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Research Trends in Parasitology

Volume - 1

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Chapter - 1

Malaria Diagnostics: Tools and Trends

Dr. Bhavinder K Arora and Dr. Sangeeta Arora

Abstract

Fast and reliable diagnosis of malaria requires confirmation of the presence of malaria parasites in the blood of patients with fever and history suggestive of malaria; hence a prompt and accurate diagnosis of malaria is the key to effective disease management. History based clinical diagnosis is widely used in areas where laboratory facilities are not available. However, it is unreliable due to non-specific nature of signs and symptoms of malaria. Confirmation of malaria infection requires the availability of a rapid, sensitive, and specific testing at an affordable cost. Conventional light microscopy of blood smear is the reference gold standard for detection of malaria parasite and well-established method of laboratory confirmation of malaria. The RDT has been said to be more sensitive for diagnosis of malarial parasite as compared to light microscopy. Rapid diagnostic tests (RDT) are also recommended by World Health Organization for quick diagnosis of malaria so that treatment can be started early and thereby preventing the complications. Both microscopy and RDT are acceptable to clinicians and patients as for both early treatment matters.

Keywords: malaria, diagnosis, diagnostic tool, microscopy, RDT, PCR

Introduction

Malaria is a parasitic disease of tropical and sub-tropical countries. Malaria is the most important of all tropical diseases in terms of morbidity and mortality with an estimated 3.4 billion people are at risk of malaria globally. Malaria is one of the leading health problems in India. About 95% of the total area of the country is endemic, with more than two third of total population in tribal areas is at risk of this infection. Malaria transmission in India is seasonal, depending mostly on altitude and rainfall. The two main seasons for transmission of malaria in India are September to November, sometimes extending to December after heavy summer rains. The malaria in human beings is caused by four species of protozoans belonging to genus

Plasmodium. These four species are *Plasmodium vivax, Plasmodium falciparum, Plasmodium ovale* and *Plasmodium malariae.* These cause the disease called malignant tertian, benign tertian, ovale tertian and quartan malaria respectively. In India, the most prevalent species is *P. vivax* in about 90% of all cases diagnosed as malaria while *P. falciparum* prevails in about 8%, *P. malariae* and *P. ovale* are the causative parasite about 2% in rest of patients. This parasite is transmitted by a vector, the female *Anopheles* mosquito. The dominant vector species are *Anopheles gambiae* and the *Anopheles funestus.* Various tools are used to diagnose malaria and start of early treatment.

1. Clinical diagnosis

In high endemic areas, the common teaching to medical and nonmedical personnel has been "fever is equal to malaria unless proven". In rural healthcare centers which are situated in faraway places from district hospitals, where parasite based diagnostic tools are not available or lack skilled technicians; the symptom based clinical diagnosis of malaria and empirical treatment is the only clinical option available. This clinical malaria diagnosis leads overdiagnosis and overtreatment with antimalarial drugs. Also, in endemic areas the malaria is traditionally accepted as the common cause of fever and is thought to be better treated by antimalarial drugs rather than missed diagnosis of malaria [1]. This quick clinical treatment of malaria has saved many lives in community-based malaria treatment programs but also led to overtreatment of masses with great financial burden on poor countries [2].

2. Light microscopy

After Laveran described the malaria parasite, Romanowsky from Russia developed a method to stain the parasites, which made the study of malaria parasite possible. Romanowsky used a mixture of eosin and methylene blue giving the nucleus purple colour and the cytoplasm blue colour. The same technique still is the basis for the presently most used staining methods of malaria parasites, i.e. Giemsa and Field stain. Microscopy allows for the identification and differentiation of malaria species, determination of parasite stages including gametocytes and the quantification of parasite density. Microscopy is still considered gold standard against which other diagnostic methods are evaluated. Malaria microscopy requires examination of both thin and thick smears from the same patient [3]. Capillary blood should be used for the preparation of blood films since various additives for venous blood such as EDTA can affect the parasite morphology making difficult to distinguish

the various species. A thick smear consists of approximately 10 μ L and a thin of 5μL blood. Optimal malaria microscopy is performed with microscopes fitted with x 10 paired eyepieces and a x 100 oil immersion objective (total magnification x 1000). 100 or 200 microscopic fields (0.2-0.5 μ L) are normally examined before a malaria infection can be excluded. In remote areas without access to electricity, microscopy can still be performed using a mirror reflecting daylight through the specimen into the eyepieces.

Giemsa staining method

Giemsa is a classical stain used for malaria microscopy [4]. It consists of commercially available Giemsa powder, glycerol and methyl alcohol (methanol). The stock solution is mostly purchased ready prepared and should be mixed with a phosphatase buffer solution of pH 7.2 prior to staining of the blood smears. Under field conditions in endemic areas often ordinary tap water is used and that works generally well, even though a pH differing from 7.2 can affect the purple-blue contrast in the specimens. Usually a concentration of 5% Giemsa for 20-30 minutes is used for both thin and thick smears. Before staining the thin smear, slide should shortly be dipped in pure methanol to fix the cells. In the thick smear on the other hand, the cells should be lysed making it possible to examine a denser layer. The sensitivity of a thick smear is 15-20 times higher than a thin film but does not allow for species determination. In the thin smear the parasites are seen within the RBC with the different characteristics of the species in terms of size, granulation and effect on the infected RBC, which generally allows for species identification if the number of parasites is not very low [5].

Field staining method

An alternative method to Giemsa is Field stain, which primarily is used for staining of thick smears. It consists of two solutions, methylene blue and eosin, and gives an excellent staining result in a few seconds if the instructions are followed carefully. Field stain also has the advantage of being very stable, allowing the same staining solutions to be used for several weeks. On the other hand, the staining can be uneven and the slide must therefore initially be scanned to find an area where both the blue and purple stains are taken up by the parasites. It is often recommended to study the colours of the leucocytes to get an idea of where to look for the parasites [6].

Parasite quantification

There are several methods for quantification of parasites in blood smears. The most common technique used in endemic areas with high parasite count is based on counting parasites in the thick smear against a standard number of

white blood cells (WBC). The number of parasites is generally counted against 500 or 200 WBC, which with an estimated 8000 WBC per µ L of blood gives a factor of 16 or 40 for calculation of parasites per µL based on the simple mathematical formula:

(Parasites counted/number of WBC counted) x 8000 = parasites per µl (p/µl).

In case of very low parasite densities the numbers are often counted in 200 microscopic fields equivalent to 0.3-0.5 µl. The numbers are then given as parasites/200 microscopic fields.

Another quantification method is to estimate the percentage of infected RBCs in a thin blood Smear [7]. For this method the parasite density is reported as % of the RBC infected.

The thick smear method has a higher sensitivity and is the first choice in endemic areas. Parasite enumeration provides useful clinical management guidance and is a useful tool for clinical trials where serial examinations of blood smears are used to determine the parasitological response to antimalarial treatment.

Sensitivity and specificity of malaria microscopy

Microscopy remains the gold standard for assessing the outcomes of drug and vaccine trials, and for serving as a reference standard in the evaluation of new tools for malaria diagnosis [8]. The sensitivity of malaria microscopy is highly dependent on the quality of the smear, the staining and not at least the skills of the microscopist. The risk of false negative results increases with decreasing parasite densities [9]. Under optimal conditions down to 5-10 p/µL (requiring more than ten minutes of thick smear examination) can be detected by an experienced microscopist, whereas under field conditions, a detection level of about 50-100 p/µL blood is more realistic. In areas with poor microscopy quality control, less skilled microscopists and poor equipment, an even higher detection limit is likely. However, overestimation, i.e. interpretation of artefacts or other dots as parasites "to be on the safe side" as well as errors in species identification are also common problems. Hence, wide ranges of malaria microscopy specificities have been reported [10]. Moreover, evaluation of the sensitivity and specificity of malaria microscopy against PCR has shown varying results [11].

Advantages and disadvantages of microscopy

Advantages

Microscopy is a cheap, well established and informative method, which allows for assessment of species, stage and quantification of malaria parasites.

Further, the finding of malaria pigment digested by neutrophils as a sign of a previous high parasitaemia as well as the effect on the parasite morphology by antimalarial drugs is of value. In the hands of an experienced technician, microscopy can also provide additional information such as anaemia, signs of bacterial infection with raised WBC and presence of other haemoparasites [12]. Blood smears are also permanent and can be used for extraction of DNA.

Disadvantages

Microscopy is a labour-intensive, time consuming method (30min-1 hour) where the quality is highly dependent on the smear preparation, the glass slides, the fixation, the staining, the microscope and the skills of the microscopist. Field microscopy often falls short of these requirements. In the era of declining malaria incidence in many areas, it is challenging to keep up the motivation for careful microscopic examination if more than 95% of the slides are negative. The examination is also prone to relatively high degree of subjectivity. All these factors may influence compliance to test results among health care providers and despite a parasite-based diagnosis, treatment decisions may still be based on clinical observations [13].

3. Fluorescent staining techniques

Acridine orange

An alternative staining of blood smears is the use of fluorescent dyes, particularly with acridine orange. The technique uses a fluorescent dye with affinity to the nucleic acid in the parasite. A common technique is thin smears fixed with methanol, stained with 0.01% acridine orange and read in a fluorescence microscope in 400-600X magnification [14].

Compared with conventional Giemsa staining, acridine orange has shown good diagnostic performance, with sensitivities of 81%-100% and specificities of 86%-100%. However, the sensitivity decreases rapidly with lower parasite densities, and species differentiation is not possible [15]. The most notable advantage of acridine orange over Giemsa staining is its promptness; results are readily available within 10 min. The simple design of an interference microscope has made direct acridine orange staining an accurate, rapid, simple and economically viable method for malaria diagnosis.[15] The microscopist, however, must learn to distinguish the stained cells of the parasite from other stained cells containing nucleic acids, such as WBCs or RBCs containing Howell Jolly bodies as well as cell debris and artefacts which could appear fluorescent [16].

Quantitative buffy coat

The quantitative buffy coat (QBC) method uses a combination of acridine orange staining and micro capillary tubes. After centrifugation the tubes are observed under the fluorescence microscope in the area just near the buffy coat region where parasites are concentrated. The sensitivity of the QBC method under field conditions is comparable with Giemsa staining but does not allow for parasite quantification or species identification [17]. The method is also, considerably more expensive which limits its usefulness for most endemic areas.

4. Immunochromatic (RDT) assay

General background

Malaria RDTs are based on immunochromatic detection of parasite antigens. The introduction of RDTs for diagnosis of malaria in the early 1990s has had a major impact on fever management in malaria endemic areas. For the first time a health worker in a remote area could rapidly and accurately distinguish between parasitaemic and non-parasitaemic febrile illness [18].

RDTs are available in two forms. One is antigen based and normally requires the use of haemolyzed RBCs while the other is antibody based and requires use of extracted serum. The antibodies are better expressed in serum otherwise could stand in place of serum for antibody-based method. The basic principles of tests is the detection of malarial parasites' protein called histidine. Where antibody type of RDT is used, it means detection of antibodies against histidine in the patients' serum and where whole is used, it means that detection of malaria parasites' histidine on RBCs [19].

Forms of immunochromatographic (RDTs) available

The RDTs come in following of formats:

a) Card
b) Dipstick
c) Hybrid cassette-dipsticks
d) Plastic cassette

The three main groups of antigens detected by commercially available RDTs are:

i) Histidine-rich protein 2 (HRP-2), specific to *P. falciparum.* It is an abundant soluble, heat stable antigen that is present in the cytoplasm and membrane of infected erythrocytes

ii) Parasite specific plasmodium lactate dehydrogenase (pLDH), currently available as P. falciparum specific, pan-specific, and P. vivax-specific. Pan-specific means that the RDT detects all the four types of plasmodia that infect humans

iii) Aldolase (pan-specific). These two antigens are conserved major enzymes in the glycolytic pathway of malaria parasites, they are abundant and are soluble in the parasite

Antigen based-RDT

RDTs have had a major impact on the accessibility to a parasite-based malaria diagnosis worldwide. The reported rate of diagnostic testing among malaria suspected cases in the African public sector has increased to 61% in 2012, mostly attributed to increased use of RDT. Wide scale distribution of RDTs free of charge to public health facilities has become a cornerstone in malaria control program recommendations and has also been increasingly advocated in a number of malaria endemic countries [20].

WHO has produced a number of guidelines evaluations and recommendations for the use of RDT and has also set up standards for the diagnostic performance of RDT with minimum requirement of sensitivities of 95% for detection of 100 p/μL (equivalent to 0.002% parasitaemia) and specificities of minimum 90% for P. falciparum compared with Microscopy [21]. WHO has also together with the Foundation for Innovative New Diagnostics (FIND), established a testing program for evaluation of the performance of commercially available RDTs. The first evaluation was published in 2008 followed by yearly reports ever since [22]. RDTs are evaluated for sensitivity and specificity in detecting P. falciparum and P. vivax at 200 and 2000 p/μL, for false positivity rate, lot variability, invalid test rate, heat stability and ease of use. The market for RDTs is enormous with more than 200 malaria RDT products currently available from more than 100 distributors worldwide.

Parasite antigens detected by RDT

Target antigens for available RDTs are:

I) Histidine Rich Protein 2 (HRP 2)

Histidine Rich Protein 2 (HRP2) is a water-soluble protein produced solely by asexual stages and young gametocytes of P. falciparum. HRP2 is a histidine and alanine-rich protein, which is localized in several cell compartments including the parasite cytoplasm and is expressed on the infected RBC membrane surface. Because of its abundance in P. falciparum,

it was the first antigen used to develop a malaria RDT [23]. The exact function of HRP2 remains incompletely understood. Studies suggest that after secretion by the parasite into the host erythrocyte cytosol, HRP2 is transported into the acidic digestive vacuole along with Hb. After Hb proteolysis, HRP2 binds the toxic haeme and mediates haemozoin (malaria pigment) formation, which is no longer toxic to the parasite [24].

HRP2 is being produced and secreted by the parasite during its growth and development and there are increasing concentrations of the protein during parasite maturation. HRP2 may be found in plasma, urine, cerebrospinal fluid and histological specimens. The fast secretion from the parasite makes HRP2 based RDTs suitable for detection also of parasites which are not circulating, i.e. mature stages of P. falciparum sequestered in the deep capillaries or placenta during infection in pregnancy [25]. Plasma concentration of HRP2 has shown to be a prognostic factor in African children with severe malaria [26]. HPR2 is a very stable protein and has even been used for immunological detection of malaria. Despite its stability there is an extensive level of sequence diversity and deletions (up to 40% in parts of South America) of the HRP2 gene [27]. This has also recently been reported from the African continent which has implications for the performance of RDTs based on detection of HRP2 [28]. HRP2 remains in the circulation up to several weeks after a cleared malaria infection [29]. A positive correlation between blood concentrations of the protein and parasite biomass has been reported and a strong correlation between the duration of positivity with the HRP2 based RDTs and initial parasite densities has been shown in several studies [30, 31]. Conversely, a wide range of HRP2 concentrations at the same parasite densities has been found [32]. Varying concentration of HRP2 is dependent on factors like duration of infection, if the blood sample is taken soon after appearance of parasites in the blood or later during infection. Other factors such as circulating parasites may not mimic the total biomass and the anti-HRP2 immune response influences the correlation between parasite density and HRP2 concentrations [33].

II) Plasmodium Lactate Dehydrogenase (pLDH)

LDH is a 33 kDa oxidoreductase. It is the last enzyme of the glycolytic pathway, essential for ATP generation and one of the most abundant enzymes expressed by the malaria parasite. The Plasmodium LDH (pLDH) isoforms can be distinguished from the human isoforms on the basis of unique epitopes within the pLDH protein as well as on its enzymatic characteristics [34]. There are no reports on antigenic variation in the pLDH gene [35]. pLDH from P. vivax, *P. malariae* and *P. ovale* exhibit 90-92% identity with pLDH from P.

falciparum and monoclonal antibodies recognizing P. falciparum and P. vivax pLDH also recognize P. knowlesi in antigen capture tests [36]. However, most pLDH based RDTs have not yet been evaluated for detection of P. knowlesi and the available results are inconsistent. Detection of pLDH has been incorporated into screening methods for the identification and quantitation of parasite growth in *in vitro* cultures. pLDH is produced only by viable parasites and is rapidly cleared from the blood stream following successful treatment [37]. The lack of antigen persistence after treatment could make the pLDH test more useful compared to HRP2 based tests in predicting treatment failure. However, pLDH is produced by all asexual and sexual stages including mature gametocytes, meaning tests can persist positive due to gametocytaemia [38].

III) Aldolase-pan malaria antigen

Plasmodium aldolase is an enzyme of the parasite glycolytic pathway expressed by the blood stages of all five human malaria species [39]. Aldolase is a highly conserved gene across all human malaria species and monoclonal antibodies against Plasmodium aldolase are pan-specific in their reaction. Aldolase is a 41 kDa protein, the presence of antibodies against p41 in the sera of human adults partially immune to malaria suggested that p41 could be involved in immune response against the parasite [40]. Low concentrations of aldolase are released from the parasites, and thus the sensitivity is dependent on the parasite density. The aldolase component in RDTs has performed poorly in several studies of the aldolase-PfHRP2 combo tests, with sensitivities of 30-50% for detection of P. vivax malaria. and for the other species, aldolase-based tests perform even worse with sensitivities below 20%. Due to the poor test performance of aldolase, this antigen RDT is not commonly available [41].

Test principle of RDTs

RDT is an immunochromatic test based on detection of malaria specific antigens (HRP2, pLDH and Aldolase). The test device is delivered in sealed envelopes to protect it from light and humidity. The envelope should be opened just before performing the test. The nitrocellulose strip is usually in a cassette test device. Capillary (or venous blood) is applied to the test well at the proximal end of the test strip, usually 5 µL. At the sample pad, dye labelled antibodies are mixed with the blood. A few drops of lysing buffer are added to the sample well or a special well next to sample to lyse the cells, release the antigen and facilitate antibody recognition. If antigen is present the antibody-antigen complex is flushed up with the blood-buffer mixture along the test strip until it reaches another target antibody bound to the strip in a thin line.

The target antibody binds to the antigen-antibody complex which now forms a visible test line. Another antibody specific for another epitope on the labelled antibody is binding excess labelled antibody to form a control line. The control band becomes positive despite no addition of blood. After passing the test band area the lysed blood is flushed into the distal end of the strip making the bands clearly visible against a bright background. The result should be interpreted after 15-20 minutes (depending on test brand) and not later than 30 minutes. RDTs are delivered as two band tests detecting only one malaria species, generally HRP2 based P. falciparum or less commonly single pLDH P. vivax or Pan-Plasmodium. Nowadays the most commonly used RDTs use a combination of HRP2 and Pan- LDH detection in a three-band format. The two options of *P. falciparum* positivity (only HRP2-band positive or both bands including the Pan band) commonly causes confusion [42]. The two-test band positivity is often misinterpreted as mixed infection, whereas the reason usually is detection of *P. falciparum* by both HRP2 and Pan-LDH antibodies, whereas a single HRP2 band positivity is usually due to a low P. falciparum parasite density since the pLDH based band may be less sensitive [43]. However, some of the more recently developed pLDH-tests based on monoclonal antibodies have shown equal sensitivity as HRP2 based tests for detection of *P. falciparum* parasite [44].

Cost effectiveness of RDT

The cost effectiveness of RDT use is dependent on the price per test as well as factors such as parasite prevalence, cost of prescribed treatment and adherence to test result. The introduction of the more expensive treatment with ACT has made treatment based on parasite-based diagnosis cost effective in low and moderate endemic areas where treatment based on clinical diagnosis has led to costly over-prescription [45]. The cost-effectiveness of RDTs mainly reflect improved treatment and health outcomes for non-malarial febrile illness, plus savings in antimalarial drug costs.

Sensitivity and specificity of RDT

In spite of more than 100 published RDT trial reports, comparative assessment is difficult because

1) Trials do not share common guidelines
2) Clinical and epidemiologic characteristics of the study populations, especially the parasitaemia levels vary
3) Reference standards are different; even among those using Giemsa microscopy, reading rules and microscopist skills vary

4) Products of different lots may differ in quality or be damaged by extreme temperature or humidity during transportation and storage

HRP 2 (Histidine Rich Protein 2)

RDTs based on HRP 2 antigen detection are estimated to have a detection limit of 50-100.

p/μL. Commercial RDTs evaluated in the WHO-FIND program show sensitivities varying.

from >50-100% in detecting 200 p/μL. In real life situations the sensitivity is highly.

Dependent on endemicity and immune status in the population tested. In low endemic areas were very low parasite densities are common among asymptomatic carriers, HRP2 based RDTs are not sensitive enough for detection of asymptomatic parasite carriers [46]. False negative result with HRP2 based RDTs are seen due to *P. falciparum* genetic diversity and deletions in some areas [47]. The prozone effect seen in samples with high density parasitaemias, due to blocking of binding sites for the antibodies by excess antigen, can also cause false negative or very faint bands [48]. The specificity of HRP2 is highly influenced by the fact that the HRP2 protein circulates in the body up to several weeks after a cleared infection, which may cause false positive results. This is especially of concern in high endemic areas were the population often are exposed to new infections after a recently cleared one, and health professionals are unable to distinguish the RDT positivity due to remaining antigens or a new infection. This may also frequently result in provision of antimalarial treatment to patients not infected [49]. RDTs based on HRP2 show higher specificity in low endemic areas where fewer individuals have recently gone through a malaria infection and unlikely carry remaining antigen [50]. Another source of false positive results with HRP2 is cross-reaction with rheumatoid factor, a common problem with diagnostic tests based on IgG antibodies [51].

pLDH (Plasmodium Lactate Dehydrogenase)

pLDH based RDTs has an overall detection limit of 100-200 p/μL for P. falciparum and 200-500 p/μL for P. vivax, and in most studies sensitivities >90% compared to microscopy. However, there is generally more performance variability among pLDH tests as compared to HRP2, and pLDH has a generally lower sensitivity at low parasite densities [52]. Since pLDH is only produced by live parasites it is not circulating in the blood after a cleared malaria episode, but can produce positive result up to 2 weeks, although less

frequently (5-10%) compared to HRP2 (>90%) after a cleared malaria episode. LDH can occasionally remain positive longer due to gametocytaemia [53]. Assessment of pLDH based tests for detection of *P. ovale* and *P. malariae* has shown poor results. With overall sensitivities ranging between 18 and 47% for *P. malariae* and between 20 and 31% for *P. ovale*, it is evident that neither test is reliable for the detection of these species [54]. However, pLDH antigen based RDTs are neither exposed to prozone effect nor genetic diversity making the main source of false negative results low parasite densities. There are reports on cross-reactions between P. vivax and P. falciparum specific LDH and pLDH can become false positive due to gametocytaemia [55]. LDH has also, been reported to be more vulnerable to high temperatures even though recent evaluations have shown high stability for several pLDH based RDTs similarly as for HRP2 based tests [56].

The usefulness of RDT

RDT is a very efficient tool for easy-of-use, rapid, stable and accurate detection of malaria infections among fever patients with P. vivax or P. falciparum infections. A disadvantage with the use of HRP2/Pan-LDH combo tests is that it is not possible to distinguish between the non-falciparum species, neither detects mixed infections. Other concerns are lot-to-lot variability and lack of internal control. However, studies on RDT in practice have shown that RDT also is a reliable tool for guidance of treatment of febrile children and even effective and safe in the hands of community health care workers [57]. However, an important factor for the effectiveness of RDT use is adherence to test result among health care workers [58].

5. Molecular methods for malaria detection

The most sensitive methods for malaria diagnosis are based on molecular detection of parasite DNA or RNA. It has been shown that when the malaria incidence in endemic areas decline in previously higher endemic areas, a large proportion of individuals carrying malaria parasites are asymptomatic with a low parasitaemia, often below the detection limit of both RDT and microscopy. These individuals, however, still constitute a risk for further transmission [59]. Also, in higher endemicity, low parasitemias are common among adults and in chronic infections. To diagnose these infections, there is a need for molecular methods with high sensitivity [60].

DNA extraction methods

Detection of malaria parasites with molecular methods is based on DNA extracted from fresh blood or blood dried on filter papers. There are several methods available for DNA extraction; Column based method is suitable for

larger sample volumes and for long DNA fragments, filter paper blood spots gives a high yield suitable for small sample volumes and for short DNA fragments, simple boil and spin methods are fast, cheap and easy but produce crude DNA sensitive to inhibition and not suitable for storage. Boil and spin methods are suitablemainly for small blood volumes.

PCR (Polymerase Chain Reaction)

PCR is the most sensitive method for detection of malaria parasites with detection limit between 0,5-10 p/µl [61]. In the past two decades, many PCR methods for parasite detection has been published [62]. Snounou *et al*., established one of the earliest nested PCR methods targeting the 18S ribosomal(r) RNA gene of the four major human Plasmodium species [63]. Later, others developed probe based real-time PCRs (qPCR) also targeting the 18S rRNA genes, and Steenkeste *et al*., published a nested PCR method targeting the Cytochrome b (Cyt b) gene in the mitochondrial DNA (Mt-DNA). PCR methods can distinguish between all human Plasmodium species, identify mixed infections and also benefit antimalarial drug efficacy monitoring, vaccine studies, and screening of vulnerable populations such as pregnant woman [64]. The possibility to apply PCR methods on extracted DNA from dried blood spots preserved on filter papers have made them applicable for screening of large series of samples collected in endemic areas [65]. PCR methods require sophistic laboratory infrastructures including PCR machines, electrophoresis and gel analyzing equipments, well trained staff, have long turn-around time and are costly [66]. These requirements are usually impossible to fulfil at point of care level in most malaria endemic areas. PCR based molecular methods are therefore, not suitable for routine diagnosis of malaria.

Loop mediated isothermal amplification

The loop mediated isothermal amplification (LAMP) method for amplification of DNA was first published by Notomi *et al*., in year 2000. They developed a method that could amplify a few copies of DNA up to 109 under isothermal conditions in less than one hour. The LAMP method uses a set of three primer pairs recognizing 4 sites of the target DNA which due to a loop formation of the amplified product acts as starting points for new primers. This autocycling strand-displacement DNA synthesis makes the amplification highly efficient and specific. The LAMP method has been evaluated for detection of malaria species in a number of studies using 18s ribosomal RNA and Mt-DNA gene targets [67]. The LAMP method has been compared with microscopy and/or PCR among symptomatic patients or on cultivated parasites generally showing sensitivities and specificities of >90%.

Usefulness of loopamp MALARIA Pan/Pf detection kit

The use of LAMP is considered a promising tool for point of care detection of asymptomatic carriers of low parasite densities, below the detection limit for both microscopy and RDT, especially in low endemic.[68] The use of LAMP kit for screening of asymptomatic individuals under field conditions needs, however, to be further evaluated. A limitation with the LAMP kit is that it like RDTs cannot distinguish between the non-falciparum species or detect mixed infections, neither quantify parasites [68].

Malaria serology

Diagnosis of malaria using serological methods is based on the detection of antibodies against asexual blood stage malaria parasites. Immunofluorescence antibody testing (IFA) or ELISA are useful tools in epidemiological surveys for assessing malaria exposure over time, especially in low endemic areas [69]. Serology is also useful for screening of potential blood donors and occasionally for providing evidence of recent infection in non-immunes. The principle of serology is that, following infection with any Plasmodium species, specific antibodies are produced within 2 weeks of initial infection, and persist for 3-6 months after parasite clearance [69]. Serology is therefore not a suitable method for point of care diagnosis of malaria.

6. Future malaria diagnostic options

Mobile phone-based microscopy

There are several studies ongoing to determine the feasibility of using mobile phones to capture microscopy images and transfer them to a central database for assessment [70]. It could have potential to be a sensitive, robust, easy to use and cost-effective system which could play a role as an alternative/complement to present diagnostic methods. However, if this is going to be a useful tool for diagnosis of malaria it has to be combined with high quality sample preparations and staining methods to prepare images suitable for computer-based analysis.

Since the World Health Organization (WHO) recognized the urgent need for new, simple, quick, accurate and cost-effective diagnostic tests for determining the presence of malaria parasites, to overcome the deficiencies of light microscopy, numerous new malaria diagnostic techniques have been developed. This has led to an increase in the use of RDTs for malaria, which are fast and easy to perform and do not require electricity or specific equipment. In clinical settings where both *Plasmodium vivax* and *Plasmodium falciparum* infection cause malaria, rapid diagnostic tests (RDTs) need to

distinguish which species is causing the patients' symptoms, as different treatments are required. The polymerase chain reaction (PCR), which is a molecular method based on DNA amplification, is the most accurate method of detecting parasites in the blood. Compared to microscopy, PCR is less prone to observer error and more sensitive at low levels of parasitaemia. PCR is currently not widely available due to logistical constraints and the need for specially trained technicians and a well-equipped laboratory. It is usually used only for research purposes. Several reports in literature have indicated that RDTs have shown a comparable level of accuracy to microscopy in clinical settings. In population-based malaria surveys, accurate diagnosis is important: microscopy provides the gold standard, whilst RDTs allow immediate findings and treatment.

In a recent study by Siwan *et al*., published in 2018, the most commonly performed diagnosis of malaria at the primary health care levels is limited to either microscopy or RDT. Whereas the gold-standard microscopic detection of malaria parasites has several limitations in proper diagnosis of malaria. Like microscopy, RDT has limitations, especially in term of differential sensitivity in different products, However, in comparison to microscopy, RDT is more sensitive in diagnosing malaria parasites in clinical settings [71].

References

1. Perkins MD, Bell DR. Working without a blindfold: the critical role of diagnostics in malaria control. Malar J. 2008; 7(1):S5-8.
2. Chandler CI, Jones C, Boniface G, Juma K, Reyburn H, Whitty CJ. Guidelines and mindlines: why do clinical over-diagnose malaria in Tanzania? A qualitative study. Malar J. 2008; 7:53.
3. WHO, Geneva. Basic Malaria Microscopy (part I and II). WHO, 1991, 1-72.
4. Barcia JJ. The Giemsa stain: its history and applications. Int J Surg. Pathol. 2007; 15(3):292-6.
5. Shillcutt S, Morel C, Goodman C, Coleman P, Bell D, Whitty CJ *et al*. Cost-effectiveness of malaria diagnostic methods in sub-Saharan Africa in an era of combination therapy. Bull World Health Organ. 2008; 86(2):101-10.
6. Uzochukwu BS, Obikeze EN, Onwujekwe OE, Onoka CA, Griffths UK. Cost-effectiveness analysis of rapid diagnostic test, microscopy and syndromic approach in the diagnosis of malaria in Nigeria: implications for scaling-up deployment of ACT. Malar J. 2009; 8:265.

7. Greenwood BM, Armstrong JR. Comparison of two simple methods for determining malaria parasite density. Trans Royal Soc Trop Med Hyg. 1991; 85(2):186-8.

8. Womgsrichanalai C, Barcus MJ, Muth S, Sutamihardja A, Wernsdorfer WH. A review of malarial diagnostic tools: microscopy and rapid diagnostic test (RDT). Am J Trop Med Hyg. 2007; 77(6):119-27.

9. McKenzie FE, Sirichaisinthop J, Miller RS, Gaser RA Jr, Wongsrichanalai C. Dependence of malaria detection and species diagnosis by microscopy on parasite density. Am J Trop Med Hyg. 2003; 69(4):372-376.

10. Durrheim DN, Becker PJ, Billinghurst K. Diagnostic disagreement-the lessons learnt from malaria diagnosis in Mpumalanga. S Afr Med J. 1997; 87(8):10-16.

11. Nicastri E, Bevilacqua N, Sané Schepisi M, Paglia MG, Meschi S, Ame SM *et al*. Accuracy of malaria diagnosis by microscopy, rapid diagnostic test, and PCR methods and evidence of antimalarial overprescription in non-severe febrile patients in two Tanzanian hospitals. Am J Trop Med Hyg. 2009; 80(5):712-717.

12. Juma E, Zurovac D. Changes in health workers' malaria diagnosis and treatment practices in Kenya. Malar J. 2011; 10:1.

13. Derua YA, Ishengoma DR, Rwegoshora RT, Tenu F, Massaga JJ, Mboera LE *et al*. Users' and health service providers' perception on quality of laboratory malaria diagnosis in Tanzania. Malar J. 2011; 10:78.

14. Kawamoto F. Rapid diagnosis of malaria by fluorescence microscopy with light microscope and interference filter. Lancet. 1991; 337(8735):200-202.

15. Keiser J, Utzinger J, Premji Z, Yamagata Y, Singer BH. Acridine Orange for malaria diagnosis: its diagnostic performance, its promotion and implementation in Tanzania, and the implications for malaria control. Ann Trop Med Parasitol. 2002; 96(7):643-54.

16. Ochola LB, Vounatsou P, Smith T, Mabaso ML, Newton CR. The reliability of diagnostic techniques in the diagnosis and management of malaria in the absence of a gold standard. Lancet Infect Dis. 2006; 6(9):582-588.

17. Lema OE, Carter JY, Nagelkerke N, Wangai MW, Kitenge P *et al*. Comparison of five methods of malaria detection in the outpatient setting. Am J Trop Med Hyg. 1999; 60(2):177-182.

18. Bell D, Peeling RW. Evaluation of rapid diagnostic tests: malaria. Nat Rev Microbiol. 2006; 4(9):S34-8.

19. Moody A. Rapid diagnostic test for malaria parasites. Clin Microbiol Rev. 2002; 15(1):66-78.

20. Masanja IM, Selemani M, Amuri B, Kajungu D, Khatib R, Kachur SP *et al*. Increased use of malaria rapid diagnostic tests improves targeting of anti-malarial treatment in rural Tanzania: implications for nationwide rollout of malaria rapid diagnostic tests. Malar J. 2012; 11:221.

21. Mouatcho JC, Goldring JP. Malaria rapid diagnostic tests: challenges and prospects. J Med Microbiol. 2013; 62(10):1491-505.

22. WHO-FIND Foundation of Innovative New Diagnostic, Malaria RDT Product Testing Reports Round 1-5 results of WHO product testing of malaria RDTs, 2008-2013.

23. Lynn A, Chandra S, Malhotra P, Chauhan VS. Heme binding and polymerization by Plasmodium falciparum histidine rich protein II: influence of pH on activity and conformation. FEBS Lett, 1999; 459(2):267-271.

24. Sullivan DJ Jr, Gluzman IY, Goldberg DE. Plasmodium hemozoin formation mediated by histidine-rich proteins. Science 1996; 271(5246):219-222.

25. Kattenberg JH, Ochodo EA, Boer KR, Schallig HD, Mens PF, Leeflang MM. Systematic review and meta-analysis: rapid diagnostic tests versus placental histology, microscopy and PCR for malaria in pregnant women. Malar J. 2011; 10:321.

26. Hendriksen IC, Mwanga-Amumpaire J, Von Seidlein L, Mtove G, White LJ, Olaosebikan R *et al*. Diagnosing severe falciparum malaria in parasitaemic African children: a prospective evaluation of plasma PfHRP2 measurement. PLoS Med. 2012; 9(8):e100-1297.

27. Baker J, Ho MF, Pelecanos A, Gatton M, Chen N, Abdullah S. Global sequence variation in the histidine-rich proteins 2 and 3 of Plasmodium falciparum: implications for the performance of malaria rapid diagnostic tests. Malar J. 2010; 9:129.

28. Gamboa D, Ho MF, Bendezu J, Torres K, Chiodini PL, Barnwell JW. A large proportion of P. falciparum isolates in the Amazon region of Peru lack pfhrp2 and pfhrp3: implications for malaria rapid diagnostic tests. PloS One. 2010; 5(1):e80-91.

29. Tjitra E, Suprianto S, McBroom J, Currie BJ, Anstey NM. Persistent ICT malaria P.f/P.v panmalarial and HRP2 antigen reactivity after treatment of Plasmodium falciparum malaria is associated with gametocytemia and results in false-positive diagnoses of Plasmodium vivax in convalescence. J Clin Microbiol. 2001; 39(3):1025-31.

30. Houzé S, Boly MD, Le Bras J, Deloron P, Faucher JF. PfHRP2 and PfLDH antigen detection for monitoring the efficacy of artemisinin-based combination therapy (ACT) in the treatment of uncomplicated falciparum malaria. Malar J. 2009; 8:211.

31. Kyabayinze DJ, Asiimwe C, Nakanjako D, Mabakooza J, Counihan H, Tibenderana JK. Use of RDTs to improve malaria diagnosis and fever case management at primary health care facilities in Uganda. Malar J. 2010; 9:200.

32. Deme AB, Park DJ, Bei AK, Sarr O, Badiane AS *et al*. Analysis of pfhrp2 genetic diversity in Senegal and implications for use of rapid diagnostic tests. Malar J. 2014; 13:34.

33. Koita OA, Doumbo OK, Ouattara A, Tall LK, Konaré A, Diakité M. False-negative rapid diagnostic tests for malaria and deletion of the histidine-rich repeat region of the hrp2 gene. Am J Trop Med Hyg. 2012; 86(2):194-198.

34. Makler MT, Piper RC, Milhous WK. Lactate dehydrogenase and the diagnosis of malaria. Parasitol Today. 1998; 14(9):376-377.

35. Talman AM, Duval L, Legrand E, Hubert V, Yen S, Bell D *et al*. Evaluation of the intra and inter-specific genetic variability of *Plasmodium* lactate dehydrogenase. Malar J. 2007; 6:140.

36. McCutchan TF, Piper RC, Makler MT. Use of malaria rapid diagnostic test to identify *Plasmodium knowlesi* infection. Emerg. Infect Dis. 2008; 14(11):1750-1752.

37. Barber BE, Williams T, Grigg MJ, Piera K, Yeo TW, Ansley NM. Evaluation of the sensitivity of a pLDH-based and an aldolase based rapid diagnostic test for diagnosis of uncomplicated and severe malaria caused by PCR-confirmed *Plasmodium knowlesi, Plasmodium falciparum, and Plasmodium vivax*. J Clin Microbiol. 2013; 51(4):1118-23.

38. Gerstl S, Dunkley S, Mukhtar A, De Smet M, Baker S, Maikere J. Assessment of two malaria rapid diagnostic tests in children under five years of age, with follow-up of false-positive pLDH test results, in a hyperendemic falciparum malaria area, Sierra Leone. Malar J. 2010; 9:28.

39. Knapp B, Hundt E, Kupper HA. Plasmodium falciparum aldolase: gene structure and localization. Mol Biochem Parasitol. 1990; 40(1):1-12.

40. Srivastava IK, Schmidt M, Certa U, Döbeli H, Perrin LH. Specificity and inhibitory activity of antibodies to Plasmodium falciparum aldolase. J Immunol. 1990; 144(4):1497-503.

41. Van Hellemond JJ, Rutten M, Koelewijn R, Zeeman Am, Verweij JJ, Wismans PJ. Human *Plasmodium knowlesi* infection detected by rapid diagnostic tests for malaria. Emerg. Infect Dis. 2009; 15(9):1478-80.

42. Harvey SA, Jennings L, Chinyama M, Masaninga F, Mulholland K, Bell DR. Improving community health worker use of malaria rapid diagnostic tests in Zambia: package instructions, job aid and job aid-plus-training. Malar J. 2008; 7:160.

43. Bell D, Wongsrichanalai C, Barnwell JW. Ensuring quality and access for malaria diagnosis: how can it be achieved? Nat Rev Microbiol. 2006; 4(9):682-695.

44. Ashley EA, Touabi M, Ahrer M, Hutagalung R, Htun K, Luchavez. Evaluation of three parasite lactate dehydrogenase-based rapid diagnostic tests for the diagnosis of falciparum and vivax malaria. Malar J. 2009; 8:241.

45. Makler MT, Piper RC. Rapid malaria tests: where do we go after 20 years? Am J Trop Med Hyg. 2009; 81(6):921-926.

46. Hopkins H, Gonzáez IJ, Polley SD, Angutoko P, Ategeka J, Asiimwe C *et al*. Highly sensitive detection of malaria parasitemia in a malaria endemic setting: performance of a new loop-mediated isothermal amplification kit in a remote clinic in Uganda. J Infect Dis. 2013; 208(4):645-52.

47. Wurtz N, Fall B, Bui K, Pascual A, Fall M, Camara C *et al*. Pfhrp2 and pfhrp3 polymorphisms in Plasmodium falciparum isolates from Dakar, Senegal: impact on rapid malaria diagnostic tests. Malar J. 2013; 12:34.

48. Gillet P, Mori M, Van Esbroeck M, Van Den Ende J, Jacobs J. Assessment of the prozone effect in malaria rapid diagnostic tests. Malar J. 2009; 8:271.

49. Olliaro P. Management of fever and malaria-policy and practice. Trop Med Int Health. 2009; 14(5):488-90.

50. Abeku TA, Kristan M, Jones C, Beard J, Mueller DH, Okia M *et al*. Determinants of the accuracy of rapid diagnostic tests in malaria case

management: evidence from low and moderate transmission settings in the East African highlands. Malar J. 2008; 7:202.

51. Laurent A, Schellenberg J, Shirima K, Ketende SC, Alonso PL, Mshinda H. Performance of HRP-2 based rapid diagnostic test for malaria and its variation with age in an area of intense malaria transmission in southern Tanzania. Malar J. 2010; 9:294.

52. Heutmekers M, Gillet P, Cnops L, Bottieau E, Van Esbroeck M, Malth J *et al*. Evaluation of the malaria rapid diagnostic test SDFK90: detection of both PfHRP2 and Pf-pLDH. Malar J. 2012; 11:359.

53. Mueller I Betuela I, Ginny M, Reeder JC, Genton B. The sensitivity of the OptiMAL rapid diagnostic test to the presence of Plasmodium falciparum gametocytes compromises its ability to monitor treatment outcomes in an area of Papua New Guinea in which malaria is endemic. J Clin Microbiol. 2007; 45(2):627-630.

54. Piper RC, Buchanan I, Choi YH, Makler MT. Opportunities for improving pLDH-based malaria diagnostic tests. Malar J. 2011; 10:213.

55. Maltha J, Gillet P, Cnops L, Van Den Ende J, Van Esbroeck M, Jacobs J. Malaria rapid diagnostic tests: Plasmodium falciparum infections with high parasite densities may generate false positive Plasmodium vivax pLDH lines. Malar J. 2010; 9:198.

56. Chiodini P. The heat stability of Plasmodium lactate dehydrogenase-based and histidine-rich protein 2-based malaria rapid diagnostic tests. Trans R Soc Trop Med Hyg. 2007; 101(4):331-337.

57. Mubi M, Janson A, Warsame M, Martensson A, Källander K, Petzold MG *et al*. Malaria rapid testing by community health workers is effective and safe for targeting malaria treatment: randomised cross-over trial in Tanzania. PloS One. 2011; 6(7):e19-753.

58. Msellem MI, Mártensson A, Rotllant G, Bhattarai A, Strömberg J, Kahigwa E *et al*. Influence of rapid malaria diagnostic tests on treatment and health outcome in fever patients, Zanzibar: a crossover validation study. PLoS Med. 2009; 6(4):e100-0070.

59. McMorrow ML, Masanja MI, Abdulla SM, Kahigwa E, Kachur SP. Challenges in routine implementation and quality control of rapid diagnostic tests for malaria-Rufiji District, Tanzania. Am J Trop Med Hyg. 2008; 79(3):385-90.

60. Moonen B, Cohen JM, Snow RW, Slutsker L, Drakeley C, Smith DL. Operational strategies to achieve and maintain malaria elimination. Lancet. 2010; 376(9752):1592-603.

61. Alemayehu S, Feghali KC, Cowden J, Komisar J, Ockenhouse CF, Kamau E. Comparative evaluation of published real-time PCR assays for the detection of malaria following MIQE guidelines. Malar J. 2013; 12:277.

62. Proux S, Suwanararusk R, Barends M, Zwang J, Price RN, Leimanis M. Considerations on the use of nucleic acid-based amplification for malaria parasite detection. Malar J. 2011; 10:323.

63. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, Do Rosario VE. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol. 1993; 61(2):315-20.

64. Steenkeste N, Incardona S, Chy S, Duval L, Ekala MT, Lim P *et al*. Towards high-throughput molecular detection of Plasmodium: new approaches and molecular markers. Malar J. 2009; 8:86.

65. Nosten F, McGready R, Mutabingwa T. Case management of malaria in pregnancy. Lancet Infect Dis. 2007; 7(2):118-25.

66. Hsiang MS, Lin M, Dokomajilar C, Kemere J, Pilcher CD, Dorsey G. PCR-based pooling of dried blood spots for detection of malaria parasites: optimization and application to a cohort of Ugandan children. J Clin Microbiol. 2010; 48(10):3539-43.

67. Han ET, Watanabe R, Sattabongkot J, Khuntirat B, Sirichaisinthop J, Iriko H. Detection of four Plasmodium species by genus- and species-specific loop-mediated isothermal amplification for clinical diagnosis. J Clin Microbiol. 2007; 45(8):2521-8.

68. Paris DH, Imwong M, Faiz AM, Hasan M, Yunus EB, Silamut K. Loop-mediated isothermal PCR (LAMP) for the diagnosis of falciparum malaria. Am J Trop Med Hyg. 2007; 77(5):972-6.

69. Bousema T, Youssef RM, Cook J, Cox J, Alegana VA, Amran J *et al*. Serologic markers for detecting malaria in areas of low endemicity, Somalia, Emerg Infect Dis. 2008, 2010;16(3):392-9.

70. Breslauer DN, Maamari RN, Switz NA, Lam WA, Flecher DA. Mobile phone based clinical microscopy for global health applications. PloS One. 2009; 4(7):e63-20.

71. Siwal N, Singh US, Dash M, Kar S, Rani S, Rawal C *et al*. Malaria diagnosis by PCR revealed differential distribution of mono and mixed species infections by *Plasmodium falciparum* and *P. vivax* in India. PLoS One. 2018; 13(3):e019-3046.

Chapter - 2
Host-Directed Therapeutic Strategies for Combating Parasitic Infections

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Chapter - 2

Host-Directed Therapeutic Strategies for Combating Parasitic Infections

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Abstract

Parasitic diseases caused by protozoans and helminths remains to the cause for significant morbidity and mortality throughout the globe. Despite of extensive research efforts in understanding host-pathogen interactions, still the chemotherapy remains to be mainstay of treatment. Conventional chemotherapeutic options have their own limitations which make them go on backfoot. Recent advances in understandings of the host immunology against parasitic diseases has opened doors for novel therapeutic strategy named host-directed therapy (HDT). HDT aims to boost host immune system to fight with infection rather than targeting the parasite with conventional anti-microbials. This chapter presents an overview of HDT and how different types of HDTs have utilized the core immunological pathways for fine tuning the immune responses to fight against the parasites.

Keywords: leishmaniasis, trypanosomiasis, helminthic infections, t-cells, macrophages, cell therapy

Introduction

Parasitic diseases threaten the global healthcare community, being the cause for significant morbidity and mortality worldwide. It affects the poor of the poorest primarily in the rural areas of developing countries, with poor sanitation and hygiene [1]. The diseases management relies on combination of active surveillance, vector control strategies, proper hygiene and patient compliance to existing chemotherapeutic regimens.

Poor patient compliance has increased the incidence of emergence of drug resistance furthermore, toxicity issues associated with current chemotherapeutics, lack of vaccine and poor disease management practices have made the parasitic infection a global threat [2]. Considering these dire limitations, reduced investment in developing new antimicrobials and World Health Organization declaration regarding the risk for entry into “post-

antibiotic era" has made it an urgent need for effective and safe intervention against parasitic diseases. Recently, there has been significant enthusiasm in targeting various host immune signaling pathways that contribute to disease pathogenesis. This approach of targeting the host factors and modifying the immune pathways for dealing with pathogens has been termed as host directed therapeutics (HDT) [3]. HDT has been known to target pathogen's virulence factors, immune cell signaling pathways to trigger protective immune machinery while rectifying the immune pathologies. This chapter provides a comprehensive overview of various HDT approaches that serves as safe, effective and adjunctive therapeutic option for parasitic diseases.

Parasitic diseases and chemotherapeutic options

Parasitic diseases pose a significant burden to global healthcare sector, with millions of deaths per year. Protozoan parasitic diseases can be divided into three categories-vector borne diseases (leishmaniasis, trypanosomiasis and malaria), food borne diseases (giardiasis, amoebiasis, coccidiosis and toxoplasmosis) and sexually transmitted diseases (trichomoniasis).

Leishmaniasis is group of diseases (visceral leishmaniasis, cutaneous leishmaniasis, mucocutaneous leishmaniasis and sequel of visceral form of disease called post-kala-azar dermal leishmaniasis) caused by protozoa *Leishmania*, transmitted by the bite of sandfly [4]. It is characterized by fever, hepatosplenomegaly, cachexia, nausea etc. there is no vaccine for the disease and treatment relies on antimonials (sodium stibogluconate), anti-fungal amphotericin B (and its liposomal formulation), miltefosine (anti-cancer drug) and paromomycin (anti-bacterial) [5].

American trypanosomiasis (Chagas disease) is caused by *Trypanosoma cruzi.* upon the bite of blood feeding triatomine bugs also called the kissing bugs. It is characterized by fever, headache, fatigue, diarrhea, heart and gastrointestinal chagomas. The chemotherapeutic options include benznidazole and nifurtimox, that helps in reducing parasitic burdens however, the treatment for chronic phase of disease remains controversial [6]. African trypanosomiasis (Human African Trypanosomiasis or sleeping sickness) is caused by protozoa *Trypanosoma* subspecies-*T. brucei, T.b. gambiense, T.b. rhodesiense.* It is transmitted by the bite of tsetse fly (*Glossina* species) and characterized by night time insomnia, seizures, chancres, if untreated for months second stage appears as confusion, poor coordination, troublesome sleep and numbness. The treatment relies mainly on use of anti-microbials as pentamidine, suramin, melarsoprol and eflornithine [7]. Malaria is another vector borne disease caused by protozoa belonging to *Plasmodium*

group, upon the bite of female *Anopheles* mosquito. The disease is characterized by fever, headache, tiredness and vomiting. The treatment options include-artemisinins in combination other antimalarial (amodiaquine, lumefantrine, sulfadoxine/pyrimethamine and mefloquine) [8]. Food-borne protozoan disease, giardiasis is an intestinal infection by *Giardia lamblia* from infected water, food and soil. It is characterized by fatigue, diarrhea, loss of appetite, vomiting, bloating, abdominal cramps etc. The treatment options include use of metronidazole, tinidazole, nitazoxanide and paromomycin [9]. Amoebiasis (amoebic dysentery) is caused by protozoa of *Entamoeba* group most commonly by *Entamoeba histolytica*. It is transmitted by the fecal-oral route and also indirectly by contact with dirty hands, contaminated food and water. The disease is characterized by abdominal pain, bloody/painful diarrhea which can complicated to cause perforation, inflammation and ulceration of colon. Treatment options include-amoebicidal nitroimidazole drugs (metronidazole and tinidazole). Cysticidal drugs include-iodoquinol, paromomycin and diloxanide [10]. Coccidiosis is caused by coccidian protozoa, infecting the intestine of animals, resulting in diarrhea which becomes bloody in severe cases. Treatment measures include administration of amprolium, that restricts the parasite growth and multiplication [11]. Toxoplasmosis is caused by parasite *Toxoplasma gondii* that results from consumption of contaminated (undercooked) meat, water, food. It is characterized by body ache, fever, fatigue, swelled lymph nodes and flu-like symptoms in some individuals. Treatment strategy includes-pyrimethamine (anti-malarial), sulfadiazine (antibiotic), clindamycin, spiramycin. Latent phase of disease is treated with antibiotics like atovaquone and clindamycin [12]. Sexually transmitted disease, trichomoniasis is caused by *Trichomonas vaginalis*. It is characterized by genital itching, pain during urination and smelly discharge from genitals. The treatment option includes the antibiotic therapy with metronidazole and tinidazole [13].

Helminthic diseases are characterized by soil transmitted helminthic infection (ascariasis, trichuriasis, strongyloidiasis), filarial nematodes (filariasis, loiasis) and platyhelminthic flukes (schistosomiasis, trematodiasis). Soil transmitted helminthic infections-ascariasis (caused by roundworm *Ascaris lumbricoides*), trichuriasis (caused by *Trichuris trichiura*) and strongyloidiasis (caused by *Strongyloides stercoralis*) manifest upon the consumption of food and/or water contaminated with parasite eggs from feces. These diseases are characterized by digestive issues as abdominal pain, diarrhea, and in some cases-shortness of breath (trichuriasis and strongyloidiasis); treatment option includes albendazole, mebendazole, levamisole, ivermectin, tribendimidine, nitazoxanide. Corticosteroids are also

used for treating infection induced inflammation [14, 15]. Filarial nematodes-roundworm (*Wuchereria bancrofti, Brugia malayi* and *Brugia timori*) and eye worm (*Loa loa, Onchocerca volvulus*) cause filariasis of lymph nodes and sub cutaneous skin layers respectively. The lymphatic form of disease is characterized by edema (elephantiasis) with thickening of skin and underlying tissues of lower extremities while subcutaneous form of disease manifest as rashes, papules and hyper-/hypo-pigmented macules. *Onchocerca* affects eyes can lead to blindness. Treatment options include the use of microfilaricides-combination therapy with albendazole and ivermectin as well as albendazole combination with diethylcarbamazine [16].

Schistosomiasis is snail fever/bilharzia caused by flatworm *Schistosoma mansoni*, that spreads upon contact with water contaminated with parasites. The disease manifests as abdominal pain, diarrhea, bloody stool and urine. Treatment option includes the use of praziquantel and oxamniquine. Praziquantel can also be used in combination with metrifonate, artesunate or mefloquine [17]. Trematodiasis, involves a number of trematodes infections, most of them spread by animals. Food borne trematodiasis has been the major form of disease that can further be divided into: Clonorchiasis, Opisthorchiasis, Fascioliasis, Paragonimiasis, Metagonimiasis, Fasciolopsiasis, Metachrosis and Dicrocoeliasis [18]. Due to advancement in technologies for understanding the parasitic diseases and the limitations associated with conventional chemotherapeutic strategies there came the demand for alternative treatment strategy.

Host immunology and parasitic diseases

Adequate understanding of the protective and/or pathological immunological responses remains the key for the development of HDT approach. Parasitic infections are characterized by immune anomalies such as tissue damage, polyclonal immune cell activation, uncontrolled inflammatory reactions, perturbed cytokine responses and apoptosis, which may be life threatening in some cases. T-cells are one of the crucial regulators of protective responses however, the intricate balance between the T_{H1} and T_{H2} determines the fate of infection. Similarly, different phenotypes of macrophages (classical M1 or alternatively activated M2 macrophages) also determines the disease fate.

Considering the immunological responses in protozoan infections, T-cells induced IFN-γ responses and monocytes/macrophage mediated nitric oxide (NO) production forms the crucial protective armory against protozoan parasites [19]. While NO possesses cytotoxicity against parasites, it also

triggers immune cell proliferation, differentiation, costimulation and cytokine production. The expression of costimulatory molecules (CD28, CD40L) induces immune responsiveness through NF-κB signaling thereby promoting the production of pro-inflammatory cytokines (IL-12 and IFN-γ). During chronic infections, impairment in immune responses can be attributed to the poor proliferative responses and immune unresponsiveness. It has been reported that T-cells induce tolerance (down regulation of CD80/CD86 on antigen presenting cells), they also undergo anergy and exhaustion, produce anti-inflammatory cytokines (IL-10 and TGF-β). Conversely, neutrophils induce neutrophil extracellular trap (NET) formation against invading pathogens. Protozoan parasites also induce strong humoral immune responses which may be detrimental or may provide limited protection [20].

Chronic helminthic infections can cause pathological consequences seen in the form of lymphedema, granuloma and fibrosis formation. In contrast to protozoan parasitic infections, the protective responses in helminthic infections are predominantly T_H2 type. The T_H2 cytokines are known to promote tissue repair and promote anti-helminthic immunity. Furthermore, these cytokines are potential activators for mast cells and increase the production of IgE that further activates mast cells, eosinophils and basophils. Eosinophils produces major basic protein, T_H2 cytokines (for example-IL-4) that mediates cytotoxic responses and tissue repair. Infiltration of mast cells is known to confer resistance toward helminths. The steep rise in the mast cells and basophilic responses can be attributed to their protective roles by diverse mechanisms. Neutrophils are also the part of host defense against certain helminths such as *Strongyloides* and *H. polygyrus* which usually work in coordination with eosinophils and macrophages to achieve optimal tissue parasite clearance [19]. Helminthic infections are also known to induce T-cells unresponsiveness and development of regulatory T-cells. Along with T_H2, Tregs, B-cells and M2 macrophages therefore, expansion of regulatory immune cell population will impair protective immune responses [21]. Therefore, deeper insight into immune regulation in parasitic infection will provide momentum to target different immune signaling pathways for fine tuning dysbalanced responses.

Host-directed therapy, types and its mechanism of targeting the immune cells

It is well known that several host immunological factors play crucial role in determining the treatment outcomes and account for conferring disease protection and/or pathology. In recent years, there has been a boom in strategies targeting the host factors rather than conventional strategy of

targeting the pathogen for treating an ailment. HDT can be defined as a targeted therapeutic approach that acts to trigger host immune responses rather than targeting the pathogen (like conventional antibiotics). It can serve to modulate the local cellular microenvironment making it hostile for pathogen sustenance thereby improving the treatment outcomes. This therapeutic approach aims to utilize innate and adaptive immune responses to effectively eliminate the diseased cells and trigger host immune responses as well as immunological memory. Thus, they are now accepted as promising adjunct to standard anti-microbial treatment.

Although a range of HDTs have been identified to date however, it can be broadly categorized into: passive and active approaches [22]. Passive approach does not rely on host immune response to fight with infection therefore, it does not require activation of immune response to pathogen. It uses the *ex vivo* generated immunogenic elements (antibodies, immune cells) thus, optimizes the immune system to specifically recognize the infected cells. It involves the routine/ frequent administration of monoclonal antibody (MAb) to target specific cell (e.g. cancer), receptor and/or surface antigen. The process of antibody treatment usually involves the following steps: MAb generation (by injecting animal with antigen, isolating the antibody-producing cells from spleen, formation of hybridoma and expansion of hybridomas to produce MAb); Immunizing the patients with antibody; antibody will bind the respective target molecule on cell of interest (immune cell/ cancer cell); trigger the effector immune responses for eliminating the pathogen. Second approach of HDT actively stimulates the immune system to recognize and destroy the infected cells. It is commonly used in cancer therapy, neurodegenerative disorders by eliciting pathogen specific or generic immune responses [23, 24]. Depending upon the type of immune of immune responses triggered it can be further sub-divided into: non-specific (it generates generic and robust immune response using cytokines, chemokines, interleukins and signaling molecules) and specific (it generates pathogen directed immune response using vaccine platform) [25].

The mechanism of action of HDTs can be attributed to their immunomodulatory potential (for example- adjuvants and pathogen associated molecular patterns as STING, C-type lectin receptors, mannose receptor, TLRs, NOD-like receptors, complement receptors, DC-SIGN etc.), enhancement of host cell functions (for example-autophagy enhancer OSU-03012) and modification of cellular microenvironment to curb disease pathology [26-28]. Several types of HDTs have approved which acts by blocking regulatory biochemical pathways or proteins, on diseased cells which are

essential for pathogen sustenance and virulence [29]. Our increasing understanding about the host-pathogen interaction has further paved the ways for development of novel strategies for HDTs are currently underway which reinvigorates pathogen antagonizing host mechanisms.

Several HDTs uses the repurposed drugs for other diseases like cancer, metabolic diseases etc. Thus, concept of HDT is not new however, it opens new avenues for dealing with drug resistant phenotypes associated with infectious diseases. HDT approach is less prone to develop treatment resistance as compared to pathogen targeted anti-microbials, used till date. Undoubtedly, the research on chemotherapeutics (targeting the pathogens) has provided impetus for treating parasitic infection but additional approaches as HDTs can interfere with mechanisms associated with pathogen persistence and enhance immunological responsiveness against the pathogen. Thus, this chapter provides an insight into different HDTs used in parasitic diseases till date.

HDT approaches for parasitic diseases

1. Immunomodulatory small molecules

Small molecules as the inhibitors of different signaling pathways are potential candidates for HDT. Imatinib (Abl/Arg tyrosine kinase inhibitor) is known to induce remodeling of cytoskeleton thereby facilitating phagocytosis of parasites and eventually reducing the severity of lesion in leishmaniasis model [30] (22665498). Similarly, AS-605240, phosphoinositide 3-kinase g (PI3Kg) (essential pathway for cytoskeleton rearrangement, phagocytosis and cell migration) inhibitor, has been proved as effective as standard drug treatment with sodium stibogluconate in treatment of *Leishmania mexicana* infection [31]. Conversely, AS101 (ammonium trichloro [1,2-ethanediolato-O,O']-tellurate), a tellurium based immunomodulator reversed T-cell anergy, promoted NO production and inhibited IL-10 signaling in *L. donovani* infection. Another PI3K inhibitor CAL-101 and IC87114 also lowered parasitic burden by improving the cytokine responses [32, 33]. Ibrutinib (small irreversible inhibitor of Bruton's tyrosine kinase) has been effective in treating leishmaniasis by boosting T_H1 polarized IFN-γ production [34]. Similarly, phospholipase A2 enzymes are known for activating NFκB in macrophages thereby boosting TNF-α and NO expression [35].

In Chagas disease, several inhibitors of GPCRs are known to prevent the entry and infection thus conferring protection [36]. Parasite derived thromboxane A2 (TXA2) has affinity for host TXA2 receptor (on dendritic cells (DCs), monocytes, cardiac myocytes, endothelial cells and platelets)

which in turn induces apoptosis, vasoconstriction, platelet aggregation and cardiomyopathy. SQ29548 (TXA2 receptor antagonist) effectively controls the *Trypanosoma cruzi* infection [37]. Leukotriene B4 has also being to possess anti-trypanocidal properties by activating leukocytes to produce NO and TNF-α-dependent pathway [38]. Similarly, platelet activating factor is also known to induce NO production [39]. Potential immunomodulatory activity has also been reported upon *ex vivo* treatment with K777, pyronaridine and furazolidone triggered CD4+ and CD8+ T-cells proliferation as well as TNF-α/IFN-γ production [40]. Further, β-adrenergic receptor blockade with carvedilol, improved cardiac functions in Chagas cardiomyopathy [41].

In Human African Trypanosomiasis (HAT), tyrosine kinase inhibitors as lapatinib and its derivatives have proven their potential in controlling the parasite (*T. brucei*) infection [42]. Lapatinib inhibits four protein kinases (TbLBPK1-4) which causes alterations in the flagellar topology and blocks parasite endocytosis [43]. Further PI3Kγ/mTOR signaling inhibitors as NVP-BEZ235 have proven their efficacy in restraining *T. brucei* infection [44]. Similarly, another small molecular inhibitor of arginase (S-(2-boronoethyl)-L-cysteine) reduces parasitic burden by diminishing the availability of growth factors released by macrophages. Inhibition of arginase-1 prevents myeloid-derived suppressor cells mediated inhibition of T-cell proliferation and IFN-γ production thereby suppressing the trypanosomes [45, 46].

Lectin based HDT for amoebiasis utilizes parasite's galactose-N-acetyl-D-galactosamine inhibitable lectin (Galectin) which triggers IL-12 production from DCs and induces T-cell proliferation and IFN-γ production [47]. Further lectin antigen from *Entamoeba histolytica* in the form of liposomal formulation with TLR-4 and TLR-7/8 agonists instigates humoral as well as cytokine (IFN-γ and IL-17) responses. In malaria, peroxisome proliferator-activator receptor gamma (PPARγ) agonist, rosiglitazone is known to enhance phagocytic clearance of parasitized erythrocytes and decreased inflammatory responses by inhibiting the parasite glycosylphosphatidylinositol-induced activation of mitogen-activated protein kinase (MAPK) and NFκB signaling. Further, rosiglitazone reduced parasitemia thus, served potent immunomodulatory agent for treating malaria [48].

In helminthic infection (Strongyloidiasis), anakinra (IL-1β receptor antagonist) treatment has shown to potentially improve innate cytokine responses (IL-33 and IL-25) leading to parasite expulsion [49] (23935505). Further, AMPK activity has therapeutic potential by regulating immunometabolism and T_H2 immune responses [50], that drives parasite expulsion from intestine. Conversely, IL-13 inhibitor has proved therapeutic

benefit by preventing tissue fibrosis due to excessive T_H2 responses [51]. Therefore, small molecular have shown potential therapeutic benefits in parasitic infection, here is just the tip of huge iceberg, research is underway to explore other molecules.

2. Cytokine (recombinant protein) supplementation and cytokine neutralization therapy

Cytokines serves as messengers for immune signaling. Drugs designed for blocking cytokine signaling have been in trials since many years however, only recently these products got approved for treating diseases. Aiming to understand myriads of cytokine signaling and their role in determining immunological responses; administration of recombinant cytokines to ameliorate disease pathogenesis has been underway over past several years. Administration of anti-cytokines (cytokine neutralization) dampens the production of cytokines, to date, interleukin-1 and tumor necrosis factor (TNF) have been amongst the most researched targets. Other chemokines, cytokines and interleukins have been under trial but no data is available in humans.

The experimental evidence for targeting cytokines for leishmaniasis therapeutics came from study of Murray *et al*. after use of anti-IL-10 receptor monoclonal antibody. The treatment effectively reduced the parasitic burden by triggering inducible nitric oxide synthase-dependent pathway [52]. Additionally, combination therapy of recombinant IFN-γ with chemotherapy has shown potent anti-parasitic activity [53]. Therefore, using immunostimulatory cytokines (IFN-γ, IL-12, GM-CSF) and/or cytokine blocking antibodies target the immune pathways and are potential targets for development of HDT.

Anti-inflammatory cytokine TGF-β has been implicated in *T. cruzi* infection induced cardiomyopathy and heart fibrosis [54]; SB-431542 (inhibitor of type I TGF-β receptor kinase) inhibits cardiomyopathy associated with Chagas disease by reducing the trypomastigotes penetration in cardiomyocytes [55, 56]. Further, treatment with recombinant IFN-γ and TNF-α has shown prompt decline in parasite burden in *T. cruzi* infection by inducing NO pathway for parasite clearance [57]. Cytokine-based therapy for amoebiasis has shown promising results by inducing protection (by upregulating IFN-γ, TNF-α and IL-17) against *E. histolytica* [58]. In giardiasis infection, blockade of TLR-2 led to improvement in IL-12 and IFN-γ production in concordance with reduction in parasitic burdens [59].

Conversely, in helminthic disease, ascariasis, MAb based blockade of IL-4 and IL-10 successfully reduced the parasite burden [60]. In trichiasis, treatment with recombinant IL-33 and IL-25 proteins prevented T_H1 polarized immune responses, while instigated protective responses thereby triggered parasite expulsion [61, 62] while MAb based blockade of IL-10 ameliorated disease pathology. IL-27 is known to block T-cell proliferation and T_H2 cytokine production thereby limits the innate as well as adaptive responses at mucosa; blockade of IL-27 receptor (WSX-1) boosted mucosal immunity [63].

MAb based IL-4 blockade therapy, in schistosomiasis, inhibited granuloma formation [64], while administration of exogenous IL-12 along with *Schistosoma* eggs inhibited granuloma and fibrosis formation [65] (7637808). Exogenous supplementation of IL-13 and IL-25 promoted intestinal functions and boosted innate lymphoid cells while blockade of IL-4 receptor induced smooth muscle hypercontraction and promoted T_H2 immunity [66-68]. Importantly, therapeutic potential of cytokine therapy opens the door for targeting other immunosuppressive factors.

3. Immune checkpoint inhibition

The development of immune checkpoint inhibitors remains the revolutionary milestone in the field of immunobiology. Parasites evolve many strategies to subvert immunosurveillance and promote disease pathogenesis by different mechanisms including the activation of immune checkpoint pathways. Immune checkpoint inhibitors reinvigorate immune responses by interrupting co-inhibitory signaling pathways.

A number of immune checkpoint molecules have been reported in case of leishmaniasis including LAG-3, CTLA-4, PD-1 etc. which negatively regulates the T-cells functionality and induce apoptosis [69-71]. Malaria infection dampens IL-2 production, MAb based inhibition of PD-1 and LAG-3 improved IFN-γ production, and abrogated T-cell inhibition [72]. Similarly, another study reported for the immune checkpoint blockade of LAG-3 and PD-L1 restored $CD4^+$ T-cells functions, increased the frequencies of follicular helper T-cells, plasma cells and germinal center B-cells. It enhanced protective antibodies production and cleared the blood stage malaria infection [73]. However, this concept has not yet been used for HAT, Chagas disease, gastrointestinal protozoan and helminthic diseases.

4. Cellular therapy

Direct transfer of immune cells has gained significant attention of researcher as HDT approach for disease management. Adoptive T-cell therapy using tumor-infiltrating lymphocytes is the best example for the clinical

success of cell therapy [74]. Similarly, direct administration of mesenchymal stromal cells and antigen specific T-cells (in tuberculosis) have raised potential hopes for further research in this field as alternative treatment strategy.

Stem cell therapy has been used since years for the treatment of many diseases but recently it has gained much attention for the treatment of parasitic diseases. Atypical progenitor cells from malaria infected mice has shown potent efficacy in fighting against the infection and transplantation of these cells helps in disease recovery [75]. Further, mesenchymal stromal cells have shown efficacy in curbing *Plasmodium berghei* infection [76]. Massive infiltration of mesenchymal stromal cells and their transplantation conferred resistance to malaria by triggering IL-12 production, suppresses IL-10 and further reduced the regulatory T-cells [77]. In Chagas disease, transplantation of bone marrow mononuclear cells reversed inflammation, fibrosis and ventricular dilation [78, 79]. Likewise, autologous transplantation of mesenchymal stem cells (MSC) and myoblasts effectively reduced ventricular dysfunctions [80]. Further, bone marrow cell transplantation proved safe and efficacious while improving the quality of life in cases with congestive heart failure due to Chagas disease [81, 82].

In toxoplasmosis, adoptive immunotherapy by transferring immune CD8+ T-cells that transiently restricted the parasite de-encystation and failed to rescue exhausted T-cells due to their short-lives [83]. Similarly, adoptive transfer strategy has been used for coccidiosis, where intraepithelial lymphocytes (IELs) and CD4+ T-cells from *Cryptosporidium parvum*-infected interferon gamma knock out mice conferred protection against infection in naïve mice [84] (19717136). Likewise, adoptive transfer of dendritic cells (DCs)-pulsed with sporozoites upon co-culture with CD4+ and CD8+ T-cells exhibited higher rates of IFN-γ production and reduced parasite burden [85].

In helminthic diseases, MSC culture supernatant inhibited macrophage activation triggered by *Schistosoma japonicum* egg antigen. Additionally, MSCs based therapy remained efficacious for *S. japonicum* induced liver injury and fibrosis.

Antibodies and alternatively activated macrophages are vital players of intestinal helminthic infections. Antibodies are known to trap the tissue migrating larvae and prevent necrosis, absence of antibodies or Fc receptors resulted in extensive tissue damage. Further antibodies mediated the attachment of macrophages to the larvae and facilitated larval immobilization,

therefore, antibodies (in the presence of helminth larvae) tuned the macrophage for expressing wound healing gene Arginase-1 [86]. In intestinal helminthic infection, IL-4 expressing memory CD4+ T-cells has been known to induce alternatively activated macrophages which serves to build protective memory responses leading to parasite elimination [87]. In Strongyloidiasis, *Nippostrongylus brasiliensis* primed macrophages hastened the parasite clearance. The reduction in parasite loads was mediated through the neutrophils that promoted alternatively activated M2 macrophage polarization that led to nematod clearance [88]. In filarial infection, CD4+ T-cells are key determinant of parasite clearance, unfortunately, T-cell acquire regulatory phenotype during infection, that bestows in hyporesponsive T-cells. Removal of these regulatory T-cells improved the antigen-specific immune responses while dampened the expression of co-inhibitory CTLA-4 [89]. Likewise, in schistosomiasis, basophil depletion strategy successfully reduced the granuloma lesions thereby ameliorated the disease pathology [90]. DCs constitute another important cell subset in driving T_H2 immune responses. Further, in schistosomal infection, *in vivo* depletion studies have established their essence in triggering the antigen specific expansion of T-cells [91]. Additionally, adoptive transfer of *Fasciola hepatica* extract induced DCs presented viable vaccination option that protected against hepatic damage by inducing T_H1 responses [92]. Similarly, transfer of *Hymenolepis diminuta* pulsed bone marrow derived DCs cells suppressed colitis by IL-4 signaling [93]. Therefore, this cell based therapeutic strategy presents potential HDT approach for helminthic infections.

5. Vitamins and Biologics

Micronutrients are important for the adequate functioning of immune responses. There are number of host factors that determines the chemotherapy outcomes including inflammatory responses, socio economic conditions, immunosuppressive drugs, micronutrients deficiency etc. There has been increasing number of evidences for the association between the parasitic infection and micronutrients deficiency. Nutritional based HDT approach using zinc supplementation lowered amastigote burdens by upregulating the immune responses and possibly attenuating the *T. cruzi* infection in fetuses [94]. Immunomodulatory therapy using zinc in combination with melatonin improved CD4+ and CD8+ T-cells frequencies, thus, affected the cytokine production in *T. cruzi* infection [95]. Further, vitamin B_{12} alone and/or in combination with vitamin C restricted parasite growth by triggering reactive oxygen species (ROS) production thus, proven its efficacy as HDT for treatment of Chagas' disease [96]. Additionally, diet supplementation with fish

oil has been shown to increase resistance against *T. cruzi* infection by modulating various host factors [97]. Vitamin C based treatment has shown to reduce the antichagasic drug induced cytotoxic effects and when used in combination with benznidazole led to dramatic reduction in parasitic burden [98]. Similarly, in gastrointestinal parasitic infections, vitamin A along with zinc supplementation reduced the *Giardia lamblia* and *Ascaris lumbricoides* burden. While, zinc supplementation alone restricted the *Entamoeba histolytica* induced diarrheal episodes [99].

Probiotics are vital players in maintaining intestinal microbiome homeostasis by limiting the pathogen growth. In line with this fact kefir (fermented milk product) administration has shown potential anti-parasitic activity by improving antibody (humoral) and T_H1 (adaptive) responses thus conferring protection against *G. intestinalis* infection [100]. Not much research on HDT for trichomoniasis has been made however, oral and/or local treatment with probiotics and pharmabiotics is known to exert homeostatic potential on natural flora [101].

Considering the helminthic infections, vitamin A supplementation along with conventional deworming strategy significantly lowered the probability of *Ascaris* reinfection amongst children [102]. The association between vitamin A deficiency and soil transmitted helminthiasis (STH) can be attributed to the fact that cure of STH significantly improved the provitamin A levels.

Further, experimental evidence from vitamin A deficient mice is known to pose serious threat to adaptive immune responses. In *Hymenolepis nana* infection, mesenteric lymph node cells (MLNCs) had dampened IL-2 production [103] therefore, provided clue for essence of micronutrients in driving immune homeostasis and can serve as potential HDT approach.

6. Drug repurposing and phytoproducts

Drug repurposing has been an inexpensive therapeutic option that bypasses the conventional drug discovery and development steps. It has provided the world with approximately 30 percent of FDA (Food and Drug Administration) approved drugs with proven safety and pharmacokinetic efficacy as compared to other conventional drugs. There remains an exhaustively long list of repurposed drugs however, only few have reached the phase of clinical trial. In context of parasitic infections, oleuropein (a biophenol, derived from *Olea europaea*) induced ROS and nitric oxide production, and also triggered T_H1 polarized immune responses to exert anti-parasitic effects [104, 105]. Berberine chloride possessed anti-leishmanial properties by inhibiting the IL-10 production and increasing the expression of

iNOS (inducible nitric oxide synthase). It induced macrophage effector responses by activating MAPK pathway [106]. Similarly, nanoliposomal formulation of berberine chloride instigated T_H1 responses and proved effective intervention option for leishmaniasis [107]. Mahanine has been another natural product that induced parasite clearance by upregulating NO/iNOS/ROS/IL-12 production thereby modulating host immune responses to serve as inexpensive HDT option [108]. Similarly, eugenol and fucoidan triggers anti-parasitic activity by promoting NO production and T_H1 responses [109, 110]. Naloxonazine (an opioid-receptor antagonist) upregulates vacuolar ATPase (vATPase, a proton pump) and actin related genes to facilitate phagolysosome formation and maturation. It enhances the acidification of parasitophorous vacuole for parasite clearance thus serves as HDT approach to circumvent parasite sustenance. The list of anti-leishmanial compounds with host-directed therapeutic potential does not end here other compounds include lupeol from *Sterculia villosa* [111], oil extract from *Nectandra* species [112], *Punica granatum* (pomegranate) [113], dehydroabietic acid from *Pinus elliottii* [114].

Statins (HMG-CoA reductase inhibitors) reduces the cholesterol which in turn weakens the parasite attachment to the macrophages thus, attenuating the parasite invasion in host cell upon lovastatin treatment [115, 116]. Terepenoids have also been known to possess anti-trypanosomal activity for example cumannin reduced parasitic loads by instigating NFκB signaling in host cell [117, 118]. Other immunomodulators- Lycopodium extracts (for Chagas disease) induced T_H1 responses [119], K777 induced pro-inflammatory responses [40], fatty acid oxidation inhibitors such as etomoxir [120], mildronate [121], trimetazidine and ranolazine [122] have been found to possess anti-parasitic activity against Chagas disease. Carvedilol (non-selective β-adrenergic blocker) has been used for treating congestive heart failure has remained safe and effective therapeutic option for improving heart functions in Chagas' disease when used in combination with renin-angiotensin inhibitors [41].

Curcumin therapy has been used for treating Chagas' cardiomyopathy by reducing the vessel inflammation, vascular permeability and IL-6/TNF-α levels to ameliorate disease pathology [123]. Curcumin has also been used for *Cryptosporidium* infection to reduce parasitic burden in immunosuppressed individuals [124]. Rottlerin, a natural polyphenol is known to restrict parasite burden during toxoplasmosis by inducing autophagy [125]. Tannic acid extract with coccidiosis vaccination emerged as potential therapeutic option [126]. Silymarin (milk thistle extract) has been known to reduce parasite load, hepatic egg load, altering granuloma size and increase the number of mast

cells in schistosomiasis. It further possessed anti-inflammatory and anti-fibrotic potential which was enhanced when it was used in combination with praziquantel [127]. Paeoniflorin is another compound with anti-fibrotic effect against schistosomiasis by inhibiting alternative activation of macrophages by dampening IL-13 production thus, blocking granuloma formation and fibrosis [128].

References

1. Lozano R *et al*. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012; 380(9859):2095-128.
2. Sundar S, Singh B. Identifying vaccine targets for anti-leishmanial vaccine development. Expert Rev Vaccines. 2014; 13(4):489-505.
3. Singh B *et al*. Host-directed therapies for parasitic diseases. Future Med Chem. 2019; 11(15):1999-2018.
4. Copeland NK, Aronson NE. Leishmaniasis: treatment updates and clinical practice guidelines review. Curr Opin Infect Dis. 2015; 28(5):426-37.
5. Sundar S, Agrawal N, Singh B. Exploiting knowledge on pharmacodynamics-pharmacokinetics for accelerated anti-leishmanial drug discovery/development. Expert Opin Drug Metab Toxicol. 2019; 15(7):595-612.
6. Ribeiro V *et al*. Current trends in the pharmacological management of Chagas disease. Int J Parasitol Drugs Drug Resist. 2019; 12:7-17.
7. in WHO interim guidelines for the treatment of gambiense human African trypanosomiasis. Geneva, 2019.
8. Nadjm B, Behrens RH. Malaria: an update for physicians. Infect Dis Clin North Am. 2012; 26(2):243-59.
9. Einarsson E, Ma'ayeh S, Svard SG. An up-date on Giardia and giardiasis. Curr Opin Microbiol. 2016; 34:47-52.
10. Shirley DT *et al*. A Review of the Global Burden, New Diagnostics, and Current Therapeutics for Amebiasis. Open Forum Infect Dis. 2018; 5(7):161.
11. Noack S. Chapman HD, Selzer PM. Anticoccidial drugs of the livestock industry. Parasitol Res. 2019; 118(7):2009-2026.

12. Torgerson PR, Mastroiacovo P. The global burden of congenital toxoplasmosis: a systematic review. Bull World Health Organ. 2013; 91(7):501-8.

13. Sutton M *et al*. The prevalence of Trichomonas vaginalis infection among reproductive-age women in the United States, 2001-2004. Clin Infect Dis. 2007; 45(10):1319-26.

14. Dold C, Holland CV. Ascaris and ascariasis. Microbes Infect. 2011; 13(7):632-7.

15. Bethony J *et al*. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet. 2006; 367(9521):1521-32.

16. Taylor MJ, Hoerauf A, Bockarie M. Lymphatic filariasis and onchocerciasis. Lancet. 2010; 376(9747):1175-85.

17. Disease GBD, Injury I, Prevalence C. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet. 2016; 388(10053):1545-1602.

18. Keiser J, Utzinger J. Emerging foodborne trematodiasis. Emerg Infect Dis. 2005; 11(10):1507-14.

19. Sturge CR, Yarovinsky F. Complex immune cell interplay in the gamma interferon response during Toxoplasma gondii infection. Infect Immun. 2014; 82(8):3090-7.

20. Arsic-Arsenijevic V *et al*. [Characteristics of the immune response in protozoan infections]. Med Pregl. 2003; 56(11-12):557-63.

21. Anthony RM *et al*. Protective immune mechanisms in helminth infection. Nat Rev Immunol. 2007; 7(12):975-87.

22. Davis ID. An overview of cancer immunotherapy. Immunol Cell Biol. 2000; 78(3):179-95.

23. Winblad B *et al*. Active immunotherapy options for Alzheimer's disease. Alzheimers Res Ther. 2014; 6(1):7.

24. Yang I, Han S, Parsa AT. Heat-shock protein vaccines as active immunotherapy against human gliomas. Expert Rev Anticancer Ther. 2009; 9(11):1577-82.

25. Baxter D. Active and passive immunization for cancer. Hum Vaccin Immunother. 2014; 10(7):2123-9.

26. Hawn TR *et al*. Host-directed therapeutics for tuberculosis: can we harness the host? Microbiol Mol Biol Rev. 2013; 77(4):608-27.

27. Chiu HC *et al*. Eradication of intracellular Francisella tularensis in THP-1 human macrophages with a novel autophagy inducing agent. J Biomed Sci. 2009; 16:110.

28. Chiu HC *et al*. Eradication of intracellular Salmonella enterica serovar Typhimurium with a small-molecule, host cell-directed agent. Antimicrob Agents Chemother. 2009; 53(12):5236-44.

29. Wykes MN, Lewin SR. Immune checkpoint blockade in infectious diseases. Nat Rev Immunol. 2018; 18(2):91-104.

30. Wetzel DM, McMahon-Pratt D, Koleske AJ. The Abl and Arg kinases mediate distinct modes of phagocytosis and are required for maximal Leishmania infection. Mol Cell Biol. 2012; 32(15):3176-86.

31. Cummings HE *et al*. Critical role for phosphoinositide 3-kinase gamma in parasite invasion and disease progression of cutaneous leishmaniasis. Proc Natl Acad Sci USA. 2012; 109(4):1251-6.

32. Khadem F *et al*. Pharmacological inhibition of p110 delta subunit of PI3K confers protection against experimental leishmaniasis. J Antimicrob Chemother. 2017; 72(2):467-477.

33. Vishwakarma P *et al*. Ammonium trichloro [1,2-ethanediolato-O,O']-tellurate cures experimental visceral leishmaniasis by redox modulation of Leishmania donovani trypanothione reductase and inhibiting host integrin linked PI3K/Akt pathway. Cell Mol Life Sci. 2018; 75(3):563-588.

34. Dubovsky JA *et al*. Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes. Blood. 2013; 122(15):2539-49.

35. De Barros NB *et al*. ASP49-phospholipase A2-loaded liposomes as experimental therapy in cutaneous leishmaniasis model. Int Immunopharmacol. 2018; 55:128-132.

36. Croxford JL *et al*. Effects of cannabinoid treatment on Chagas disease pathogenesis: balancing inhibition of parasite invasion and immunosuppression. Cell Microbiol. 2005; 7(11):1592-602.

37. Silva JF *et al*. Mechanisms of vascular dysfunction in acute phase of Trypanosoma cruzi infection in mice. Vascul Pharmacol. 2016; 82:73-81.

38. Talvani A *et al*. Leukotriene B(4) induces nitric oxide synthesis in Trypanosoma cruzi-infected murine macrophages and mediates resistance to infection. Infect Immun. 2002; 70(8):4247-53.

39. Aliberti JC *et al*. Platelet-activating factor induces nitric oxide synthesis in Trypanosoma cruzi-infected macrophages and mediates resistance to parasite infection in mice. Infect Immun. 1999; 67(6):2810-4.

40. Otta DA *et al*. Identification of Anti-Trypanosoma cruzi Lead Compounds with Putative Immunomodulatory Activity. Antimicrob Agents Chemother, 2018, 62(4).

41. Botoni FA *et al*. A randomized trial of carvedilol after renin-angiotensin system inhibition in chronic Chagas cardiomyopathy. Am Heart J. 2007; 153(4):e1-8.

42. Woodring JL *et al*. Evaluation of Aromatic 6-Substituted Thienopyrimidines as Scaffolds against Parasites That Cause Trypanosomiasis, Leishmaniasis and Malaria. Medchemcomm. 2015; 6(2):339-346.

43. Guyett PJ *et al*. Novel Effects of Lapatinib Revealed in the African Trypanosome by Using Hypothesis-Generating Proteomics and Chemical Biology Strategies. Antimicrob Agents Chemother, 2017, 61(2).

44. Diaz-Gonzalez R *et al*. The susceptibility of trypanosomatid pathogens to PI3/mTOR kinase inhibitors affords a new opportunity for drug repurposing. PLoS Negl Trop Dis. 2011; 5(8):e12-97.

45. Nzoumbou-Boko R *et al*. Trypanosoma musculi Infection in Mice Critically Relies on Mannose Receptor-Mediated Arginase Induction by a TbKHC1 Kinesin H Chain Homolog. J Immunol. 2017; 199(5):1762-1771.

46. Onyilagha C *et al*. Myeloid-Derived Suppressor Cells Contribute to Susceptibility to Trypanosoma congolense Infection by Suppressing CD4(+) T Cell Proliferation and IFN-gamma Production. J Immunol. 2018; 201(2):507-515.

47. Ivory CP, Chadee K. Activation of dendritic cells by the Gal-lectin of Entamoeba histolytica drives Th1 responses *in vitro* and *in vivo.* Eur J Immunol. 2007; 37(2):385-94.

48. Serghides L *et al*. Rosiglitazone modulates the innate immune response to Plasmodium falciparum infection and improves outcome in experimental cerebral malaria. J Infect Dis. 2009; 199(10):1536-45.

49. Zaiss MM *et al*. IL-1beta suppresses innate IL-25 and IL-33 production and maintains helminth chronicity. PLoS Pathog. 2013; 9(8):e100-3531.

50. Nieves W *et al*. Myeloid-Restricted AMPKalpha1 Promotes Host Immunity and Protects against IL-12/23p40-Dependent Lung Injury during Hookworm Infection. J Immunol. 2016; 196(11):4632-40.

51. Chiaramonte MG *et al*. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. J Clin Invest. 1999; 104(6):777-85.

52. Murray HW *et al*. Determinants of response to interleukin-10 receptor blockade immunotherapy in experimental visceral leishmaniasis. J Infect Dis. 2003; 188(3):458-64.

53. Badaro R *et al*. Treatment of visceral leishmaniasis with pentavalent antimony and interferon gamma. N Engl J Med. 1990; 322(1):16-21.

54. Silva JS, Twardzik DR, Reed SG. Regulation of Trypanosoma cruzi infections *in vitro* and *in vivo* by transforming growth factor beta (TGF-beta). J Exp Med. 1991; 174(3):539-45.

55. Waghabi MC *et al*. SB-431542, a transforming growth factor beta inhibitor, impairs Trypanosoma cruzi infection in cardiomyocytes and parasite cycle completion. Antimicrob Agents Chemother. 2007; 51(8):2905-10.

56. Waghabi MC *et al*. Pharmacological inhibition of transforming growth factor beta signaling decreases infection and prevents heart damage in acute Chagas' disease. Antimicrob Agents Chemother. 2009; 53(11):4694-701.

57. Munoz-Fernandez MA, Fernandez MA, Fresno M. Synergism between tumor necrosis factor-alpha and interferon-gamma on macrophage activation for the killing of intracellular Trypanosoma cruzi through a nitric oxide-dependent mechanism. Eur J Immunol. 1992; 22(2):301-7.

58. Guo X *et al*. CD4+ and CD8+ T cell- and IL-17-mediated protection against Entamoeba histolytica induced by a recombinant vaccine. Vaccine. 2011; 29(4):772-7.

59. Li X *et al*. TLR2(-/-) Mice Display Decreased Severity of Giardiasis via Enhanced Proinflammatory Cytokines Production Dependent on AKT Signal Pathway. Front Immunol. 2017; 8:11-86.

60. Macedo MS *et al*. Immunomodulation induced by Ascaris suum extract in mice: effect of anti-interleukin-4 and anti-interleukin-10 antibodies. Scand J Immunol. 1998; 47(1):10-8.

61. Humphreys NE *et al*. IL-33, a potent inducer of adaptive immunity to intestinal nematodes. J Immunol. 2008; 180(4):2443-9.

62. Owyang AM *et al*. Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. J Exp Med. 2006; 203(4):843-9.

63. Artis D *et al*. The IL-27 receptor (WSX-1) is an inhibitor of innate and adaptive elements of type 2 immunity. J Immunol. 2004; 173(9):5626-34.

64. Cheever AW *et al*. Anti-IL-4 treatment of Schistosoma mansoni-infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egg-induced hepatic fibrosis. J Immunol. 1994; 153(2):753-9.

65. Wynn TA *et al*. An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection. Nature. 1995; 376(6541):594-6.

66. Zhao A *et al*. Critical role of IL-25 in nematode infection-induced alterations in intestinal function. J Immunol. 2010; 185(11):6921-9.

67. Horsnell WG *et al*. IL-4Ralpha-responsive smooth muscle cells contribute to initiation of TH2 immunity and pulmonary pathology in Nippostrongylus brasiliensis infections. Mucosal Immunol. 2011; 4(1):83-92.

68. Huang Y *et al*. IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. Nat Immunol. 2015; 16(2):161-9.

69. Singh B *et al*. A molecular signature for CD8(+) T cells from visceral leishmaniasis patients. Parasite Immunol. 2019; 41(11):e12-669.

70. Esch KJ *et al*. Programmed death 1-mediated T cell exhaustion during visceral leishmaniasis impairs phagocyte function. J Immunol. 2013; 191(11):5542-50.

71. Murphy ML *et al*. B7-2 blockade enhances T cell responses to Leishmania donovani. J Immunol. 1997; 159(9):4460-6.

72. Doe HT *et al*. Expression of PD-1/LAG-3 and cytokine production by CD4(+) T cells during infection with Plasmodium parasites. Microbiol Immunol. 2016; 60(2):121-31.

73. Butler NS *et al*. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage Plasmodium infection. Nat Immunol. 2011; 13(2):188-95.

74. Rosenberg SA *et al*. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res. 2011; 17(13):4550-7.

75. Belyaev NN *et al*. Induction of an IL7-R(+)c-Kit(hi) myelolymphoid progenitor critically dependent on IFN-gamma signaling during acute malaria. Nat Immunol. 2010; 11(6):477-85.

76. Asami M *et al*. Susceptibility of multipotent haemopoietic stem cell deficient W/Wv mice to Plasmodium berghei-infection. Immunol Cell Biol. 1991; 69(Pt 5):355-60.

77. Thakur RS *et al*. Mesenchymal stem cells play an important role in host protective immune responses against malaria by modulating regulatory T cells. Eur J Immunol. 2013; 43(8):2070-7.

78. Soares MB *et al*. Transplanted bone marrow cells repair heart tissue and reduce myocarditis in chronic chagasic mice. Am J Pathol. 2004; 164(2):441-7.

79. Goldenberg RC *et al*. Bone marrow cell therapy ameliorates and reverses chagasic cardiomyopathy in a mouse model. J Infect Dis. 2008; 197(4):544-7.

80. Guarita-Souza LC *et al*. Simultaneous autologous transplantation of cocultured mesenchymal stem cells and skeletal myoblasts improves ventricular function in a murine model of Chagas disease. Circulation. 2006; 114(1):120-4.

81. Vilas-Boas F *et al*. Bone marrow cell transplantation in Chagas' disease heart failure: report of the first human experience. Arq Bras Cardiol. 2011; 96(4):325-31.

82. Vilas-Boas F *et al*. [Early results of bone marrow cell transplantation to the myocardium of patients with heart failure due to Chagas disease]. Arq Bras Cardiol. 2006; 87(2):159-66.

83. Bhadra R, Cobb DA, Khan IA. Donor CD8+ T cells prevent Toxoplasma gondii de-encystation but fail to rescue the exhausted endogenous CD8+ T cell population. Infect Immun. 2013; 81(9):3414-25.

84. Tessema TS, Dauber E, Petry F. Adoptive transfer of protective immunity from Cryptosporidium parvum-infected interferon-gamma and interleukin-12-deficient mice to naive recipients. Vaccine. 2009; 27(47):6575-81.

85. Bedi B, McNair NN, Mead JR. Dendritic cells play a role in host susceptibility to Cryptosporidium parvum infection. Immunol Lett. 2014; 158(1-2):42-51.

86. Esser-Von Bieren J *et al*. Antibodies trap tissue migrating helminth larvae and prevent tissue damage by driving IL-4Ralpha-independent alternative differentiation of macrophages. PLoS Pathog. 2013; 9(11):e100-3771.

87. Anthony RM *et al*. Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. Nat Med. 2006; 12(8):955-60.

88. Chen F *et al*. Neutrophils prime a long-lived effector macrophage phenotype that mediates accelerated helminth expulsion. Nat Immunol. 2014; 15(10):938-46.

89. Taylor MD *et al*. Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance *in vivo*. J Immunol. 2005; 174(8):4924-33.

90. Anyan WK *et al*. Basophil depletion downregulates Schistosoma mansoni egg-induced granuloma formation. Parasitol Int. 2013; 62(6):508-13.

91. Lundie RJ *et al*. A central role for hepatic conventional dendritic cells in supporting Th2 responses during helminth infection. Immunol Cell Biol. 2016; 94(4):400-10.

92. Falcon CR *et al*. Adoptive transfer of dendritic cells pulsed with Fasciola hepatica antigens and lipopolysaccharides confers protection against fasciolosis in mice. J Infect Dis. 2012; 205(3):506-14.

93. Matisz CE *et al*. Suppression of colitis by adoptive transfer of helminth antigen-treated dendritic cells requires interleukin-4 receptor-alpha signaling. Sci Rep. 2017; 7:40-631.

94. Goncalves-Neto JF *et al*. Effect of zinc supplementation in pregnant mice during experimental Trypanosoma cruzi infection. Res Vet Sci. 2011; 90(2):269-74.

95. Brazao V *et al*. Immunoregulatory actions of melatonin and zinc during chronic Trypanosoma cruzi infection. J Pineal Res. 2015; 58(2):210-8.

96. Russi EW [Therapy with beta adrenergic agents]. Schweiz Rundsch Med Prax. 1986; 75(5):94-7.

97. Lovo-Martins MI *et al*. Fish oil supplementation benefits the murine host during the acute phase of a parasitic infection from Trypanosoma cruzi. Nutr Res. 2017; 41:73-85.

98. Puente V *et al*. Anti-parasitic effect of vitamin C alone and in combination with benznidazole against Trypanosoma cruzi. PLoS Negl Trop Dis. 2018; 12(9):e000-6764.

99. Long KZ *et al*. Effect of vitamin A and zinc supplementation on gastrointestinal parasitic infections among Mexican children. Pediatrics. 2007; 120(4):e846-55.

100. Franco MC *et al*. Administration of kefir-fermented milk protects mice against Giardia intestinalis infection. J Med Microbiol. 2013; 62(Pt 12):1815-22.

101. Palmeira-De-Oliveira R, Palmeira-De-Oliveira A, Martinez-De-Oliveira J. New strategies for local treatment of vaginal infections. Adv Drug Deliv Rev. 2015; 92:105-22.

102. Payne LG *et al*. Benefit of vitamin A supplementation on ascaris reinfection is less evident in stunted children. J Nutr. 2007; 137(6):1455-9.

103. Ikeda K *et al*. Modulation of cytokine secretion by mesenteric lymph node cells from vitamin A-deficient mice during Hymenolepis nana infection. *in vivo*. 1994; 8(6):1015-7.

104. Kyriazis ID *et al*. The leishmanicidal activity of oleuropein is selectively regulated through inflammation-and oxidative stress-related genes. Parasit Vectors. 2016; 9(1):441.

105. Andreadou I *et al*. Oleuropein prevents doxorubicin-induced cardiomyopathy interfering with signaling molecules and cardiomyocyte metabolism. J Mol Cell Cardiol. 2014; 69:4-16.

106. Saha P *et al*. Berberine chloride mediates its anti-leishmanial activity via differential regulation of the mitogen activated protein kinase pathway in macrophages. PLoS One. 2011; 6(4):e18-467.

107. Want MY *et al*. Nanoliposomal artemisinin for the treatment of murine visceral leishmaniasis. Int J Nanomedicine. 2017; 12:2189-2204.

108. Bhattarai P *et al*. The effects of aging on Amyloid-beta42-induced neurodegeneration and regeneration in adult zebrafish brain. Neurogenesis (Austin). 2017; 4(1):e132-2666.

109. Charan Raja MR *et al*. Eugenol derived immunomodulatory molecules against visceral leishmaniasis. Eur J Med Chem. 2017; 139:503-518.

110. Kar S, Sharma G, Das PK. Fucoidan cures infection with both antimony-susceptible and -resistant strains of Leishmania donovani through Th1 response and macrophage-derived oxidants. J Antimicrob Chemother. 2011; 66(3):618-25.

111. Das A *et al*. Antileishmanial and immunomodulatory activities of lupeol, a triterpene compound isolated from Sterculia villosa. Int J Antimicrob Agents. 2017; 50(4):512-522.

112. Da Costa-Silva TA *et al*. Immunomodulatory and Antileishmanial Activity of Phenylpropanoid Dimers Isolated from Nectandra leucantha. J Nat Prod. 2015; 78(4):653-7.

113. Kaur G *et al*. Punica granatum (pomegranate) flower extract possesses potent antioxidant activity and abrogates Fe-NTA induced hepatotoxicity in mice. Food Chem Toxicol. 2006; 44(7):984-93.

114. Goncalves MD *et al*. Dehydroabietic acid isolated from Pinus elliottii exerts in vitro antileishmanial action by pro-oxidant effect, inducing ROS production in promastigote and downregulating Nrf2/ferritin expression in amastigote forms of Leishmania amazonensis. Fitoterapia. 2018; 128:224-232.

115. Kumar GA *et al*. Statin-induced chronic cholesterol depletion inhibits Leishmania donovani infection: Relevance of optimum host membrane cholesterol. Biochim Biophys Acta. 2016; 1858(9):2088-2096.

116. Parihar SP *et al*. Topical Simvastatin as Host-Directed Therapy against Severity of Cutaneous Leishmaniasis in Mice. Sci Rep. 2016; 6:33-458.

117. Sulsen VP *et al*. Natural terpenoids from Ambrosia species are active *in vitro* and *in vivo* against human pathogenic trypanosomatids. PLoS Negl Trop Dis. 2013; 7(10):e24-94.

118. Petersen CA *et al*. Trypanosoma cruzi infection and nuclear factor kappa B activation prevent apoptosis in cardiac cells. Infect Immun. 2006; 74(3):1580-7.

119. Brustolin Aleixo CF *et al*. Beneficial immunomodulatory and neuro digestive effect in Trypanosoma cruzi infection after Lycopodium clavatum 13c treatment. Microb Pathog. 2017; 112:1-4.

120. Paumen MB *et al*. Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis. J Biol Chem. 1997; 272(6):3324-9.

121. Liepinsh E *et al*. Mildronate, an inhibitor of carnitine biosynthesis, induces an increase in gamma-butyrobetaine contents and cardioprotection in isolated rat heart infarction. J Cardiovasc Pharmacol. 2006; 48(6):314-9.

122. Sabbah HH, Stanley WC. Partial fatty acid oxidation inhibitors: a potentially new class of drugs for heart failure. Eur J Heart Fail. 2002; 4(1):3-6.

123. Hernandez M *et al*. Curcumin exerts anti-inflammatory and vasoprotective effects through amelioration of NFAT-dependent endothelin-1 production in mice with acute Chagas cardiomyopathy. Mem Inst Oswaldo Cruz. 2018; 113(9):e180-171.

124. Asadpour M *et al*. Curcumin: A promising treatment for Cryptosporidium parvum infection in immunosuppressed BALB/c mice. Exp Parasitol. 2018; 195:59-65.

125. Ietta F *et al*. Rottlerin-mediated inhibition of Toxoplasma gondii growth in BeWo trophoblast-like cells. Sci Rep. 2017; 7(1):12-79.

126. Tonda RM *et al*. Effects of tannic acid extract on performance and intestinal health of broiler chickens following coccidiosis vaccination and/or a mixed-species Eimeria challenge. Poult Sci. 2018; 97(9):3031-3042.

127. El-Lakkany NM *et al*. Anti-inflammatory/anti-fibrotic effects of the hepatoprotective silymarin and the schistosomicide praziquantel against Schistosoma mansoni-induced liver fibrosis. Parasit Vectors. 2012; 5:9.

128. Chu D *et al*. Paeoniflorin attenuates schistosomiasis japonica-associated liver fibrosis through inhibiting alternative activation of macrophages. Parasitology. 2011; 138(10):1259-71.

Chapter - 3
Hepatic Echinococcosis

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Chapter - 3

Hepatic Echinococcosis

Dr. Namita Bhutani, Dr. Pradeep Kajal and Dr. Sachet Dawar

Abstract

Cystic echinococcosis (CE) is a widely endemic helminthic disease caused by infection with metacestodes (larval stage) of the Echinococcus granulosus tapeworm and transmitted by dogs. E. granulosus are common parasites in certain parts of the world, and are present on every continent with the exception of Antarctica. As a result, a large number of people are affected by CE. The increased emigration of populations from endemic areas where prevalence rates are as high as 5-10% and the relatively quiescent clinical course of CE pose challenges for accurate and timely diagnoses. In humans, the disease is characterized by slowly growing cyst commonly occurring in liver and lungs. Clinical features of hepatic hydatid cyst are mainly right upper quadrant pain, feeling of lump and enlarged tender liver. The cyst may be complicated by infection or rupture and may lead to anaphylactic reaction. The diagnosis depends on clinical suspicion. They appear in two ways as general (systemic) symptoms, and local symptoms based on the site and organ on which larva settles. While cysts sometimes recover spontaneously, more severe clinical presentations are observed in immunosuppressive individuals. Ultrasonography supported by serology is the main diagnostic modality. The current treatment of hydatid cyst of the liver varies from surgical intervention to minimally invasive treatments (percutaneous drainage) or medical therapies. Surgery is still the best treatment modality. Percutaneous drainage and treatment of the cyst is a good option to surgery in selected cases. Multiple, superficial single cysts are the most suitable ones for surgical treatment. Also for complicated infected cysts pressing biliary tree and vital organs, surgery should be the first choice of treatment. We believe that the laparoscopic approach should be safe to uncomplicated cysts. The success of these methods is influenced by the stage and location of hepatic cysts. However, CE can be clinically silent, and has a high risk for recurrence. It is important to consider the echinococcal parasite in the differential diagnosis of liver cystic lesions and

to perform appropriate long-term follow-ups. In this review we discuss aspects of the biology, life cycle, etiology, distribution, and transmission of the Echinococcus organisms, and the epidemiology, clinical features, treatment, and effect of improved diagnosis of the diseases they cause. New sensitive and specific diagnostic methods and effective therapeutic approaches against echinococcosis have been developed in the last 10 years. Despite some progress in the control of echinococcosis, this zoonosis continues to be a major public health problem in several countries, and in several others it constitutes an emerging and re-emerging disease.

Keywords: cystic echinococcosis, epidemiology, polycystic echinococcosis, zoonoses

Introduction

Human echinococcosis is a zoonotic infection transmitted by dogs in livestock raising areas. The causative agent of cystic echinococcosis (CE) or hydatid cyst disease of the liver is Echinococcus Granulosus (E. Granulosis) belonging to taeniidae family of cestoda class, with the height of 2-6 mm and the maximum width of 0.6 mm, consisting of 3-4 rings [1, 2]. Based on their genetic structures and biological properties, six different types of E. granulosis (sheep, cattle, horse, camel, swine and deer) have been shown, but four are of public health concern: Echinococcus granulosus (which causes cystic echinococcosis), Echinococcus multilocularis (which causes alveolar echinococcosis), and Echinococcus vogeli and Echinococcus oligoarthritis (which cause polycystic echinococcosis). Two new species have recently been identified: Echinococcus shiquicus in small mammals from the Tibetan plateau and Echinococcus felidis in African lions, but their zoonotic transmission potential is unknown. Geographically distinct strains of E. granulosus exist with different host affinities. Molecular studies using mitochondrial DNA sequences have identified 10 distinct genetic types (G1-10) within E. Granulosus [3, 4]. These include two sheep strains (G1 and G2), two bovid strains (G3 and G5), a horse strain (G4), a camelid strain (G6), a pig strain (G7), and a cervid strain (G8). A ninth genotype (G9) has been described in swine in Poland and a tenth strain (G10) in reindeer in Eurasia. The sheep strain (G1) is the most cosmopolitan form and is that most commonly associated with human infections. The other strains appear to be genetically distinct, suggesting that the taxon E. granulosus is paraphyletic and may require taxonomic revision [3]. Certain human activities (e.g., the widespread rural practice of feeding dogs the viscera of home-butchered sheep) facilitate transmission of the sheep strain and consequently raise the risk that humans will become infected [5]. There are two hosts in the life-

cycle of the parasite. The first one is the "primary host" or definitive host, and the second one is the "intermediate host" in which the illness occurs. Adult forms are present in the intestines of primary host animals including cats, dogs, wolfs and foxes, and here, they only cause intestinal parasitosis but not organ disease. Adult parasite lives approximately for 5 months in dog intestines [5, 6]. "Definitive hosts" spread millions of parasite eggs defecation. Sheep and other herbivorous animals becomes "intermediate host" for the parasite when they eat herbs contaminated with these eggs, or humans become "intermediate host" for the parasite when they eat fruits or vegetables contaminated with these eggs. Embryo (oncosphere) which comes out of the egg taken via gastrointestinal tract, adheres to intestinal wall with its hooks, then enters into circulation and reaches firstly to the liver. Thus, liver is the most common site of disease in humans accounting for 50-70% of cases, followed by the lungs (20-30%), and less frequently the spleen, kidneys, heart, bones, central nervous system, and other organs [7]. Embryo loses its scolex when it settles in an organ, and takes the cyst form consisting of cuticula (exocyst) and germinal membrane (endocyst). The cyst has sterile, clear fluid inside, and this cystic structure is wrapped with a fibrous capsule "pericyst". When alive hydatid cysts are eaten by the last host dog, the infection chain is completed, and the life cycle returns to beginning [8, 9]. Hepatic cystic echinococcosis is life-threatening disease because of their medical and economical impact and their wide geographical distribution. Despite some progress in the control of echinococcosis, this zoonosis continues to be a major public health problem in several countries, and in several others it constitutes an emerging and re-emerging disease.

In this review we discuss aspects of the biology, life cycle, etiology, distribution, and transmission of the Echinococcus organisms, and the epidemiology, clinical features, treatment, and effect of improved diagnosis of the diseases they cause.

Epidemiology

According to the World Health Organization (WHO), E. granulosus is endemic in areas of South America, Eastern Europe, Russia, the Middle East, and China, where human incidence rates are as high as 50 per 100,000 person-years. In certain areas, such as slaughter houses in South America, prevalence varies from 20% to as high as 95%. The reason of high prevalence is the abundance of sheep and goat rearing by the countries [10]. The most common intermediate hosts are farm animals, such as sheep, goats, swine, camels, horses, and cattle, as well as mule deer [11]. The incidence of surgical cases of echinococcosis reflects only a fraction of the number of

infected hosts, which, in turn, is only a fraction of the actual prevalence in endemic areas. Foci of hydatid disease also exist in India where the highest prevalence is reported in Andhra Pradesh and Tamil Nadu than in other parts of the country [12]. A recent study in Delhi showed that approximately 10% of sheep slaughtered in Delhi slaughter houses were infected with the larval forms of the parasite [13]. Tanzania, Malta, South Cyprus and New Zealand became hydatid cyst free zones with their applied public health policies [14]. Factors such as agriculture-based subsistence, low socio-economic status, regional climate, and uncontrolled and unhygienic animal slaughtering increase the incidence.

Etiology and Pathogenesis

Adult tapeworm lives in the upper small bowel of the domestic dogs, a definitive host. Other definitive hosts are wolves, jackals, domestic cats, and reindeer etc. Sheep, cattle, pigs and humans contain larval stage and are intermediate hosts. They are infected feco orally by Echinococcus eggs shed in the environment with feces of infected dogs. Upon entering the small intestine, the parasite remains firmly attached to the mucosa, and later sheds gravid proglottids that are excreted in the infected animal's feces [15]. Within each proglottid, there are hundreds of eggs. These eggs can then be ingested by intermediate hosts where they mature into cysts and daughter cysts, such as in sheep that acquire the infection by grazing upon grass contaminated with dog feces containing the eggs. Human infection does not occur by the handling or ingestion of meat or viscera from infected sheep. Rather, humans are accidental intermediate hosts that become infected either by direct contact with a dog contaminated with egg-bearing feces or by ingesting water, food, or soil contaminated with such feces. In human infection, the first stage is the asymptomatic incubation period, during which ingested eggs release oncospheres that are able to penetrate the human intestinal wall. These oncospheres enter the portal venous system, which provides access to the liver, lungs, and various other organs [16-18]. Next, the oncospheres begin cyst development. Cysts are usually unilocular, and can range anywhere from 1 cm to 15 cm in diameter. In hepatic cystic echinococcosis (CE), cyst growth ranges from 1-2 mm to 10 mm per year. They also tend to affect the right lobe more frequently than the left lobe due to the nature of portal blood flow. The cysts are composed of two derived layers of membrane: an inner, nucleated, germinal membrane, and an outer, acellular, laminated layer. The immune system responds to the cyst by forming a calcified fibrous capsule around it, which is the layer that is most often visualized on imaging studies [17]. The cyst enlarges to form a combination of protoscolices (future heads of

the adult worms) and daughter cysts. The combination of many protoscolices and cystic fluid appears grain-like on ultrasound imaging, and is thus termed "hydatid sand." Animals that consume organs infected with protoscolices will become definitive hosts, as the protoscolices attach firmly to the host's intestine, and then develop into an adult worm with a scolex (head), neck, and proglottids [17, 19]. E. granulosus infections usually present as solitary cysts, and have single-organ involvement. In 10–15% of patients, there can be involvement of two organs depending on the specific geographic region and strain of parasite [17]. (Figure 1).

Description of the pathogen

The echinococcal cyst is a fluid-filled, spherical, unilocular cyst that consists of an inner germinal layer of cells supported by a characteristic acidophilic-staining, acellular, laminated membrane of variable thickness [20]. Each cyst is surrounded by a host-produced layer of granulomatous adventitial reaction. Small vesicles called brood capsules bud internally from the germinal layer and produce multiple protoscolices by asexual division. In humans, the slowly growing hydatid cysts can attain a volume of several liters and contain many thousands of protoscolices. With time, internal septations and daughter cysts can form, disrupting the unilocular pattern typical of the young echinococcal cysts.

Clinical features

CE can go undetected for many years due to the slow growth and development of cysts and the response of the host's immune system [21, 22]. Depending on the size and location, cysts can eventually exert pressure on nearby structures, producing abdominal discomfort and pain [15, 17 18]. Cysts usually grow 1-5 mm in diameter every year, and causes symptoms a few years after the onset. Incidentally detected cysts have approximately 10-20 years of history. Symptoms develop slowly. Epigastric and/or right hypochondriac pain, nausea and vomiting are frequently observed. 85-90% of the cases have single organ involvement, and more than 70% of patients have a single cyst. Based on the organ in which the cyst settles and the environment they affect, they may show various clinical manifestations changing from cholangitis with biliary ruptures, portal hypertension, biliary obstruction and fistules and ascites to abscess formation [23]. For example, cysts in the liver can compress bile ducts, causing obstruction that can manifest as obstructive jaundice, abdominal pain, anorexia, and pruritus [24]. When in the lungs, cysts can irritate the membranes leading to chronic cough, dyspnea, pleuritic chest pain, and hemoptysis [17, 25]. Cyst rupture or leakage can cause immunologic symptoms from Elevated levels of IgE,

IgG2 and IgG4 are implicated for allergic reactions such as pruritus, hives, and anaphylactic shock [22]. Systemic findings develop as a result of complex antigenic organization via mutual down regulation of Th1 cytokines (Interferon-γ) and Th2 cytokines (IL-4, IL-5, IL-6) [26]. A major rupture can cause a life-threatening anaphylactic reaction [24]. Ruptured cysts can release viable cystic contents and protoscolices into the peritoneum, resulting in secondary hydatidosis [25]. Thus, infectious symptoms can manifest as sepsis, either due to the primary infection or to a secondary infection from leakage into the biliary tree. In one study, bacterial superinfection was found in 7.3% (37/503) of patients diagnosed with CE [27]. Four of these patients developed severe sepsis, and two patients died. Bacteria most commonly seen in the liver cyst infections included Escherichia coli, Enterococcus, and Streptococcus viridans.

Most primary infections in humans consist of a single cyst; however, 20-40% of individuals have multiple cysts or multiple organ involvement. Even though infections may be acquired in childhood, most cases of liver and lung cysts become symptomatic and are diagnosed in adult patients because of the slowly growing nature of the echinococcal cyst. Only 10-20% of cases are diagnosed in patients younger than 16 years. However, cysts located in the brain or an eye can cause clinical symptoms even when small; thus, most cases of intracerebral echinococcosis are diagnosed in children. In the lungs, ruptured cyst membranes can be evacuated entirely through the bronchi or can be retained to serve as a nidus for bacterial or fungal infection. Dissemination of protoscolices can result in multiple secondary echinococcosis disease. Larval growth in bones is atypical; when it occurs, invasion of marrow cavities and spongiosa is common and causes extensive erosion of the bone [7].

Complications

Cyst may rupture into the biliary system (leading to cholangitis with or without obstructive jaundice and marked eosinophilia), into the peritoneum (leading to anaphylaxis and/or peritoneal dissemination) or into the pleura or lung (causing pleural hydatidosis or bronchial fistula). In one series, anaphylaxis complicated 10% of all peritoneal ruptures [28]. The rupture may either be spontaneous or more usually after blunt trauma.

Rupture of echinococcal cyst can be of three types [29]

1. **Contained rupture:** only endocyst is torn and cyst contents are confined within pericyst. The size of cyst does not decrease on imaging

2. **Communicating rupture:** There is tearing of endocyst and cyst contents escape via biliary radicals or bronchioles that have been incorporated in pericyst. On imaging, cyst becomes smaller with undulating membrane
3. **Direct rupture:** Both endocyst and pericyst rupture causing spillage of contents into peritoneum or pleural space and dissemination of disease

Cysts may become infected following bacteremia or via communicating bile ducts, especially when endoscopic retrograde cholangio pancreatography (ERCP) has been performed. These patients present with high fever, sepsis syndrome and a tender liver. Pressure or mass effect on the bile ducts, portal veins, hepatic veins and inferior vena cava can cause cholestasis, portal hypertension and the Budd-Chiari Syndrome, respectively.

Diagnosis

The diagnosis is based on-

1. History and clinical examination
2. Serology
3. Imaging

Microscopic examination of the cyst content confirms the diagnosis.

Diagnosis from simple serum studies is difficult because of the low sensitivity of the tests, which is frequently due to undetectable immune responses [30]. Immune responses depend on the location, cyst wall intactness, and viability of the organisms. Serum liver enzyme tests also have low sensitivities, and are frequently unreliable in determining the underlying severity of the infection. Moreover, serum liver enzyme tests are abnormal in only 40% of CE infected patients. When present, alkaline phosphatise is commonly elevated, while aspartate/alanine transaminase ratios and bilirubin levels typically remain within the normal limits. Complete blood count tests may be helpful, as Eosinophilia may be present in 40% of patients [25].

Serum assays

In humans, infection with Echinococcus induces an antibody response, most commonly IgG (predominantly IgG1 and IgG4), followed by IgM, IgA, and IgE. However, in approximately 30-40% of patients, no antibodies of any kind are detectable, even in individuals who have circulating parasitic antigens [22]. These data suggest that the infection may be associated with an

inhibition of the host immune response, possibly at the B cell level or by a T cell-mediated mechanism. It is also possible that the impermeability of the cyst wall to the host defense system plays a role in allowing the parasite to evade immune detection and response [18]. Furthermore, the cyst may allow the organism to actively suppress the host's immune system [22]. A number of detection assays for IgG, IgM, and IgE antibodies to hydatid antigens have been described. The currently available antibody detection assays include immunoelectrophoresis, ELISA, and immunoblots, which utilize native and recombinant antibodies and a hydatid fluid fraction. Immunoblots reportedly have the highest sensitivity (80%), followed by ELISA (72%) and immunoelectrophoresis (31%) [31]. If the preliminary test with IgG ELISA is negative and there are no imaging or other signs of CE, patients do not require further work up. However, imaging results suggestive of CE in a seronegative patient necessitate repeated and extended serologic testing or consideration of cyst puncture, as well as consideration for medical and/or surgical intervention if the patient is symptomatic [32, 33]. In patients who are seropositive and have positive imaging findings, a secondary antibody test is performed, using an Arc 5 test, IgG4-ELISA, or immunoblot for antibodies against E. granulosus antigens. Secondary tests are used to rule out false-positive cross-reactivities [30]. Although antibody detection assays tend to have higher sensitivities (up to 97%) when compared to antigen assays, they do not distinguish between active and past infections [34]. Therefore, assays for antigens are preferred, as they are not only more specific, but levels have been shown to reflect improvement in surgically treated patients [35]. Enzyme linked Immuno Sorbent Assay (ELISA) is used as a screening test and immunoelectrophoresis (IE) as confirmatory test. An antibody titers >1:160 is usually considered positive. Diagnosis is established if both ELISA and IE are positive. Serology may be negative is 10-15% of cases, especially in well- encapsulated cysts. Sensitivity of serological tests for liver cysts is 80-90% and specificity is 88-96%. For pulmonary cysts sensitivity is only 50 to 60%. IgG4 response is more pronounced than that of IgG1 [30]. A disadvantage of these assays is the variability in sensitivity rates, which range from 33% to 85% [17, 35]. This variability may be due to the structure of calcified cysts, concealment of cysts by surrounding normal tissue, or the fact that antigen-antibody complexes are not easily detected by assays [36]. Traditional immunodiagnostic tests (e.g. Casoni skin test, complement fixation test) are not used today. Detection of Echinococcus antibodies has 93.5% sensitivity and 89.7% specificity [38]. In some small hydatid cysts in cases where Echinococcus antibodies could not be detected, ultrasound-guided percutaneuous biopsies may be performed. However, in order to

prevent secondary hydatid cyst formation when performing this procedure, Albendazole (oral) treatment must be initiated 4 days before the procedure, and must be continued for one month. In recent years, contrast-enhanced ultrasound (CEUS) is also used in these cases in which antibody could not be detected, to exclude malignancy [38]. In actuality, many of the serologic tests that have been developed are applicable only for research purposes, and are not broadly employable in clinical settings, especially in developing countries. Therefore, there is an emphasis on imaging modalities for diagnosing CE.

Imaging

The findings on imaging vary depending on the stage of the cyst. As several classification schemes exist for staging CE, the WHO developed a standardized classification system [10]. This system, originally developed by Gharbi and colleagues in 1981, was revised by the WHO-Informal Working Group Classification on Echinococcus (IWGE) and is currently the screening method of choice. Table 1 lists the classifying features for the development of CE [39]. Such a classification system enables a standardized approach to treatment based on diagnosed stage.

Radiology

Direct X-ray films are not diagnostic for hydatid cyst, but may lead to the suspicion of disease. In case of complicated hydatid cyst, 'air meniscus sign' due to air passage between endo-and ectocyst may be seen in chest X-ray. Other signs that may be seen in direct X ray films include 'water-lily sign', 'double-dome arch sign' and 'calcification'. Moreover, elevation in diaphragma may suggest hydatid cyst of the liver [40]. Calcification can be seen on radiographs in up to 30% of CE cases. The calcifications are usually curvilinear or ring-like, and are deposited in the pericyst. Calcification can progress throughout all stages of CE. However, it is implied that once a cyst is entirely calcified, the pathogen is inactive/dead [41].

Ultrasound

Ultrasonography is the gold standard diagnostic method [23]. This is currently the screening method of choice, due to easy availability, cost effectiveness, and ability to classify and estimation of cyst viability. Gharbi classified hepatic hydatid cysts in to 5 types depending upon the viability [39]. Ultrasound is not only helpful for diagnosis, but in post treatment monitoring. On ultrasound, the cyst wall usually has a hypoechoic layer, flanked by an echogenic line on either side [41]. A simple, unilocular cyst may not demonstrate an internal structure. However, multiple punctate

echogenic foci are often present within the CE, but are only visualized upon repositioning of the patient. These foci represent hydatid sand, a combination of fluid and protoscolices, which have recently ruptured from a vesicle within the cyst [25]. An endocyst can also detach from the pericyst. This may appear as a well-defined cystic lesion with a localized split in the wall and "floating membranes" within the cystic cavity; complete detachment observed by ultrasonography is referred to as the water lily sign [41]. Multivesicular cysts are fluid collections that often appear in a honeycomb pattern with multiple septa. The septa represent the walls of the daughter cysts, which appear as cysts within a cyst. Daughter cysts separated by the hydatid matrix can produce a "wheel-spoke pattern". The matrix is composed of detached membranes which may appear as serpentine linear structures, broken daughter vesicles, scolices and hydatid sand [33, 41]. Ultrasound has the highest sensitivity for the detection of membranes, septa, and hydatid sand within the CE. Once the matrix fills the cyst, the cyst can appear as a solid mass. In order to distinguish a cyst filled by matrix, daughter vesicles or membranes should be sought within the lesion. Calcification of cysts typically occurs within the wall, and appears hyperechoic with posterior acoustic shadowing. Severe calcification restricts ultrasound penetration, thus, only the anterior portion of the CE can then be visualized.

Gharbi classification based on the ultrasonographic appearance of the cyst was recently updated by World Health Organization-Informal Working Group on Echinococcosis (WHOIWGE) [10, 39].

Type 1: Univesicular hypodense cyst containing "hydatid sand".

Type 2: Univesicular hypodense cyst with double or undulating membrane.

Type 3: "Mother and daughter" cyst which is highly specific for hydatidosis.

Type 4: Detachment of the germinal layer which produces classical "Water Lilly" sign, echogenic content with solidifications or a pseudotumor aspect.

Type 5: Cyst wall calcification.

Type 1, 2 and 3 are viable cysts at postoperative parasitological examinations and type 4 and 5 are non-viable cysts. The contents of the cysts, the status of the adjacent organ, and vital status of cyst content can be identified. Ultrasonographic findings considered as pathognomonic for

hydatid cyst are round or oval anechoic cyst presence with snow flake sign and clearly visible cyst walls, multiseptated cyst presence with cartwheel appearance, and multiseptated cyst presence with daughter vesicles.

CT

Although ultrasound is an excellent tool for initial diagnosis, sonographic failures can occur for a multitude of reasons, including obesity, excessive bowel gas, and previous surgeries. CT is an imaging technique which can detect cysts ≥ 1 cm, has the potential of assessing each organ and is very beneficial in the differential diagnosis. CT gives valuable information regarding the size of the cyst, septation presence, the integrity of germinative membrane, status of liver parenchyma, location and the depth of the cyst and adjacency with bile ducts (Figure-3). The presence of daughter cysts and exogenous cysts can also clearly be seen on CT [42]. Pathognomonic appearances are collapsed membrane and presence of daughter vesicles. Typical eggshell-like appearance is seen in completely calcified cysts. The size of the cyst can be estimated as well. CT is valuable when we considering surgical treatment, especially the laparoscopic method is to be used. CT has a sensitivity rate approaching 94%, and plays a crucial role during the perioperative period for detection of complications, such as biliary and vascular involvement, cyst ruptures, and underlying infection [43]. CT scan is useful to visualize the relation of the hydatid cyst to the surrounding liver tissue, bile ducts, portal and hepatic veins and its segmental location.

MRI

While not often needed, MRI may provide additional information not seen on CT. MRI ensures well structural details of the hydatid cysts. Although it might be helpful for demonstrating the lesion in the liver. The signal intensity of CE resembles that of fluid on many MRI sequences. The cyst is hyperintense on T2-weighted images, and is surrounded by a low signal rim, which likely represents a collagen-rich outer layer (pericyst), and is often described as a characteristic finding of CE [33]. Daughter cysts or vesicles, if present, attach to the germinal layer and are hypointense on T1-weighted images relative to the dominant cyst. Collapsed membranes from the pericyst appear as twisted linear structures within the cyst, similar to that seen using CT. Although calcification can be clearly depicted by CT, MRI can detect early irregularities in the wall, thought to represent an impending membrane detachment [41, 44]. MRI is especially indicated in cerebral pathologies.

However, echinococcal cysts must be differentiated from benign cysts, cavitary tuberculosis, mycoses, abscesses, and benign or malignant neoplasms. A non-invasive confirmation of the diagnosis can usually be accomplished with the combined use of radiologic imaging and immunodiagnostic techniques. Radiography permits detection of echinococcal cysts in the lungs; in other sites, however, calcification is necessary for radiographic visualization. Computed tomography, magnetic resonance imaging, and ultrasonography are useful for diagnosis of deep-seated lesions in all organs and also for determination of the extent and condition of the avascular fluid-filled cysts. Antibody assays are useful to confirm presumptive radiologic diagnoses, although some patients with cystic echinococcosis do not demonstrate a detectable immune response. Hepatic cysts are more likely to elicit an immune response than pulmonary cysts. Regardless of location, the sensitivity of serologic tests is inversely related to the degree of sequestration of the echinococcal antigens inside cysts; for example, healthy, intact cysts can elicit a minimally detectable response, whereas previously ruptured or leaking cysts are associated with strong responses. The indirect hemagglutination test is sensitive but has now been replaced by the enzyme immunoassay (ELISA) for initial screening of sera. Specific confirmation of reactivity can be obtained by demonstration of specific echinococcal antigens by immunoblot assays. In seronegative individuals, a presumptive diagnosis can be confirmed by the demonstration of protoscolices or hydatid membranes in the liquid obtained by percutaneous aspiration of the cyst. Protoscolices can sometimes be demonstrated in sputum or bronchial washings; identification of hooklets is facilitated by acid-fast stains.

Treatment

The approach to management and treatment of CE depends on the extent of organ involvement, the number of cysts, presence or absence of cystic–biliary communications, and other factors, such as secondary bacterial infection and hemorrhage. It is, therefore, crucial to assess each individual case to determine the best possible outcome [25]. The ultimate goal of treatment is elimination of the germinal layer.

Currently, three treatment options are available

1) Medical
2) Surgical
3) Percutaneous

Medical treatment

Chemotherapy

Of the chemotherapeutic agents currently available for CE, the best studied are the benzimidazole carbamates, albendazole and mebendazole. Their primary mechanism of action involves interfering with parasite glucose absorption, resulting in glycogen depletion within the parasitic intracellular organelles. Studies have indicated that the effects of albendazole are superior to mebendazole [46]. From drug data reports, it appears that the principle difference between the two is in the breakdown metabolites; the metabolite of albendazole is a potent prodrug that has excellent antihelminthic properties, whereas mebendazole breaks down into multiple, poorly active metabolites [47].

Medical therapy is indicated in the following cases:

1) Inoperable cases in primary lung and liver CE with multiple cysts and peritoneal involvement
2) To reduce cyst pressure, secondary seeding, and risk of recurrence in presurgical and prepuncture cases

Contraindications include:

1) Large cysts that are likely to rupture
2) Inactive or heavily calcified cysts
3) Early pregnancy
4) Chronic hepatic conditions and bone marrow suppressive disorders where treatment results in adverse side effects [10]

The treatment dosage for a typical 70-kilogram person is 400 mg BID for 28 days. The most common toxic effect is an elevation of liver enzymes during long-term therapy, which can occur in 20% of cases. As a drug class, benzimidazoles are also known to have suppressive effects on bone marrow, which usually subside with cessation of the agent [46]. It is, therefore, crucial to monitor hepatic enzymes and complete blood counts [47, 48]. There is another broad-spectrum, anti-helminthic agent called praziquantel. However, this drug alone is not sufficient as therapy for CE, and is recommended in combination with albendazole, particularly as a preoperative regimen. Albendazole absorption is dependent on gastric pH. H2 receptor antagonist may decrease its absorption by50%. The drug is degraded by cytochrome P-450 enzymes located in the villi of small intestine.

In general there are four different goals of medical treatment.

i) Definite cure-for univesicular cyst (type 1 and 2) 3-6-month treatment has success rate of 82% and relapse rate of above 25%, most of which occurs within 2 years. Lifelong follow-up is advised.

ii) Reduction of viability and cyst size can be achieved in multivesicular cysts (type 3), however, germinal layer is rather resistant to treatment and definite cure occurs infrequently.

iii) Preoperative reduction of viability of univesicular cysts before planning elective surgery or percutaneous drainage.

iv) Perioperative or peri interventional prophylaxis-optimally should be started at least 3 days before the surgical percutaneous treatment and should be continued for 3-8 weeks post-treatment in uncomplicated cases and for 3-6 months in complicated cases.

Surgical management

Surgical management of echinococcal cysts, most commonly with partial and total cystectomy, has long been considered the definitive cure for CE [48, 49]. The aim of surgery is total removal of the cyst with avoidance of the adverse consequences of spilling the contents. Standard of care even for a surgical approach includes pre-and postprocedure adjunctive drug therapy to prevent secondary seeding of the peritoneal cavity in case of a rupture. According to WHO guidelines, treatment with albendazole or mebendazole should be started four days prior to surgery, and continued after for at least one month with albendazole and for three months with mebendazole [10].

Principles of hydatid surgery are

a) Total removal of all infective cyst parts

b) Avoidance of intra-abdominal spillage of cyst contents

There are many approaches to the surgical removal, but all must accomplish two goals: cyst removal and obliteration of the cavity. If spillage occurs, immediate washout of the peritoneum should be performed with hypertonic saline and a scolicidal agent, followed by a longer duration of postprocedure mebendazole therapy, up to six months in some cases [47, 50]. Importantly, a lack of cysto-biliary communications should be confirmed prior to use of hypertonic saline to avoid complications, such as sclerosing cholangitis and pancreatitis. This can be achieved with the use of intraoperative dyes and, if found, careful repair of such communications. Approaches vary from radical resection to simple cyst resection, but each

case varies depending on location, number of cysts, and structural complications, with the ideal approach being whole, simple resection without rupture [48]. Other approaches range from a more radical pericystectomy, all the way to a conservative approach that includes incision and drainage of cystic fluid, injection of a scolicidal agent, and aspiration of cyst contents with pericystic tissue removal. An open total pericystectomy uses protoscolicidal agents to sterilize the cyst, followed by removal of the pericystic tissue and contents. In comparison, a closed, total pericystectomy involves removal of the cyst without opening it. A newer surgical approach called subadventitial cystectomy has been developed for liver hydatid disease [51]. The pericyst is a combination of two tissue layers, namely the adventitial layer towards the liver parenchyma and the exocyst layer towards the parasitic cyst. The space between can easily be separated, therefore protecting the layer adjacent to the liver parenchyma and resulting in fewer complications from structural damage and bleeding. In accordance with many recent reports, there has been a progressive increase in surgical approaches because of fewer relapses compared to medical therapy, and also fewer postoperative complications and associated mortality [47]. In contrast, some studies have shown a higher morbidity and mortality with surgical treatment approaches, along with a relapse rate of 2-25% [10, 52]. This has shifted the focus of first-line management to less-invasive interventions, thus reserving surgery for complicated cases involving multiple cysts, rupture, bleeding, fistula formation, and compression. However, it is difficult to truly compare surgical and medical management outcomes as there are no prospective clinical trials with long-term follow-up data.

Radiofrequency thermal ablation

This is an experimental approach in which energy is applied through the needle electrodes and high temperature (up to 1000C) is achieved inside the cyst. Cyst material is aspirated after procedure. The results of this technique are preliminary and efficacy has to be proven [53].

Percutaneous aspiration injection and respiration (PAIR)

This less-invasive approach employs ultrasound or CT-guided aspiration of the cystic fluid. It plays an important role for both confirmation of diagnosis and therapeutic intervention. However, PAIR is not suitable for all cyst types. Prior determination of the number of compartments and the presence of daughter cysts is crucial for successful treatment with this strategy. 36 Indications for PAIR include WHO-IWGE classification CE1 and CE3a cysts (single compartment cysts) < 5 cm that have not responded

well to medical therapy, and in combination with medical therapy for cysts > 5cm [41, 49]. Contraindications for PAIR include percutaneously inaccessible cysts, superficial cysts due to a risk of spillage, cysts communicating with biliary structures, inactive cysts, and complex multiseptated cysts. The procedure involves aspiration, injection of scolicidal agent, and the respiration of contents. The fluid that is initially aspirated is evaluated for viable protoscolices, which confirms the diagnosis. It is also evaluated for biliary-cystic communication by testing for the presence of bilirubin in the fluid, which can also be determined prior to PAIR using cholangiography or endoscopic retrograde cholangiopancreatography. The scolicidal agent that is injected is left for approximately 15 minutes, after which there is separation of the germinal membrane from the surrounding cyst. Currently, three solutions are most commonly used: 70-95% ethanol, 15-20% hypertonic saline, or cetrimide solution [10]. The procedure also involves close monitoring for complications of anaphylaxis. Treatment with albendazole or mebendazole four hours prior to the procedure should be continued for one month postoperatively for albendazole, and for three months with mebendazole. This pre-and post-treatment reduces the risk for recurrence and secondary intraperitoneal seeding [10, 49]. Post-PAIR, serial sonographic imaging is performed to monitor the patient's response. A good response is determined by the presence of one or more of the following factors: reduction in the size of the cavity, increased wall calcification, increased areas of solidification in the cyst, and increased echogenicity of the cyst (consistent with a pseudomass appearance) [33]. Management of CE based on WHO-IWGE staging, current standards for monotherapy are for WHO-IWGE stages CE1 and CE3a (cysts with single compartment and < 5 cm in diameter). Treatment is aimed for continuous therapy from 1-3 months up to six months, depending on the clinical scenario. In multicystic liver with cysts < 5cm, peritoneal cysts, or areas where percutaneous approach is not feasible, monotherapy is also justified. PAIR, as mentioned above, is effective in smaller WHO-IWGE classification CE1 and CE3a cysts that have not responded well to medical therapy or in combination with medical therapy for larger cysts [49]. Multicompartment cyst types or cysts that contain daughter cysts (types CE2 and CE3b) require surgery in combination with medical therapy or a different type of percutaneous intervention (non-PAIR) due to the high risk for relapse after PAIR in these patients. The alternative percutaneous intervention is generally performed with a large bore catheter that is able to evacuate the entire cyst as opposed to obliterating the germinal layer with a scolicidal agent. 48 Cyst types CE4 and CE5 are inactive cysts and are managed by observation [33, 54]. Success of

PAIR is defined as detachment of endocyst, rupture of daughter cysts, and non-viable protoscoleces at microscopy of cyst fluid. Ultrasonography may show heterogeneous reflection of cyst contents at 3 months, obliteration and pseudo tumor aspect at 5 month or loss of echogenicity and disappearance of cyst at 9 months. PAIR is a safe technique with high success rate of 90-100% and low relapse rate (0-4%) in various studies. A meta-analysis comparing the clinical outcomes for 769 patients with hepatic cystic echinococcosis treated with PAIR plus albendazole or mebendazole with 952 era-matched historical control subjects undergoing surgical intervention found greater clinical and parasitological efficacy, lower rates of morbidity and mortality and disease recurrence, and shorter hospital stays than surgical treatment [43]. For multivesicular cysts with or without cystobiliary fistula and containing non-drainable material, another percutaneous technique called PEVAC has been described. In this the cyst contents are evacuated with the help of large bore Amplatz sheath (14-18f) and suction catheter without using any scolicidal agent. If cystobiliary communication is detected, endoscopic sphincterotomy and stent placement is done [55].

Follow-up period

Follow-up is recommended initially every six months for the first two years, and then once a year depending on the appropriate clinical setting. As mentioned earlier, patients undergoing chemical therapy require serial liver function tests and leukocyte counts to monitor for adverse reactions. In CE, it is difficult to assess the frequency of relapses. Therefore, monitoring with ultrasound is sometimes performed for up to ten years, a duration for which recurrences have been reported despite treatment. In the post-treatment phase, serologic studies, often with Ig levels, are difficult to interpret because they may indicate residual disease as opposed to a disease recurrence. In many cases, they remain elevated despite appropriate therapy or complete resection, which is why they are often used in combination with imaging studies during follow-up to detect cystic activity [10].

watch-and-wait method

Another treatment strategy is a relatively conservative approach to CE management. In this approach, the hypothesis is that cyst types CE4 and CE5 (Fig. 4), should be left untreated but monitored closely. The fact that some cysts are heavily calcified and remain as fairly inactive structures has been used to justify this strategy [56]. Follow-up with ultrasound in these cyst types is suggested, as opposed to serologic studies used to assess for activity. Serologic studies are not as reliable given their results vary depending on cyst stage, location, and size.

Monitoring results of treatment

The occult nature of the hydatid cyst confounds post-treatment evaluation. Objective response to treatment is best assessed with repeated evaluation of cyst size and consistency at 3-month intervals with ultrasonography, computed tomography, or magnetic resonance imaging. Since the time of the appearance of recurrence is extremely variable, such monitoring should be continued for at least 3 years. Change in titer of serologic antibody values is not reliable in itself to define the outcome of chemotherapy or PAIR.

Prevention and control

The earliest successful control program was that in Iceland initiated nearly 130 years ago, when cystic hydatid disease was recognized as affecting approximately one in every six Icelanders. An extremely effective health education campaign sensitized the entire population to the disease, and subsequent measures virtually eliminated home slaughter of sheep resulting in the gradual elimination of transmission. By the 1950s echinococcosis was considered eradicated from Iceland. Programs initiated in New Zealand (1959) and in Tasmania (1965) were primarily based upon education of rural populations and motivating them to change their practices. Strict control and prohibition of farm slaughter were key features in those programs. The initially voluntary nature of the programs was reinforced by legislative acts and strengthened efforts at enforcement as the programs progressed. This policy proved highly successful: the number of infected dogs fell steadily throughout the campaigns. Decline in canine infection preceded drops in prevalence of infection in sheep and young cattle and a reduced number of cases in humans diagnosed annually. Cystic echinococcosis has been declared provisionally eradicated in both Tasmania and New Zealand. A program in Cyprus benefited from very aggressive stray dog elimination and strict control of working dogs and those kept as pets. All used diagnostic purging of dogs with are choline as a surveillance technique for monitoring the effectiveness of the program and identifying problem farms. Tasmania quarantined infected dogs and infected sheep flocks. Regional programs in Argentina (1970), Chile (1978), and Uruguay benefited from the use of the highly effective echinococcicidal drug praziquantel. Surveillance data from all these programs documented the reduction of prevalence in dogs, animal intermediate hosts, and humans [57]. A promising advance has been the development of a recombinant vaccine (EG95), which seems to confer 96-98% protection against challenge infection. Recent trials in Australia and Argentina using EG95 have reported

that 86% of vaccinated sheep were completely free of viable hydatid cysts when examined 1 year after immunization. Vaccination reduced the number of viable cysts by 99.3% [58]. A vaccine has also been developed against the dog tapeworm stage, which conferred 97-100% protection against worm growth and egg production [59]. Mathematical modeling has revealed that the most effective intervention against echinococcosis is a combination of sheep vaccination and dog anthelmintic treatment [60]. Important advantages of this less intensive strategy would be lower cost and possibly increased compliance. It must be noted that the positive achievements of successful control programs, however significant at the local level, have not markedly changed the global distribution and public health importance of hydatid disease. In most endemic areas, effective control has not been achieved or even attempted. Much remains to be done. There is concern that echinococcosis may have become hyperendemic in areas where it was once endemic. For example, in the Peruvian central highlands, the sudden cessation of a control program at the end of the 1970s may have led to a marked increase in the prevalence of infection in intermediate and definitive hosts and in the human population [61].

Conclusion

Hepatic echinococcal cysts, although fairly uncommon, should be considered in the differential diagnosis of hepatic cysts, particularly in patients with exposure risk, such as those who have traveled to or emigrated from a region of high prevalence. Serum antibody assays generally have low sensitivities, but antigen assays may be of value. Imaging is crucial in determining cyst stage, size, and location and complications. It can also be helpful in assessing the suitability of a minimally invasive PAIR approach. Uncomplicated active cysts can be managed with chemotherapy alone or in combination with a PAIR approach. Uncomplicated, inactive cysts can be managed with the “watch-and-wait” strategy. Surgery is the first choice when there is cyst biliary fistula, significant extra-hepatic extension with high risk of perforation, complicated cysts (ruptured or infected) and when expertise to percutaneous treatment is not available. Advanced age, pregnancy, multiple cysts, comorbidity, and calcified inactive cysts are important considerations for treatment [62]. Cystic diseases can be seen in other organs should be considered in the differential diagnosis of hydatid diseases. Hydatid cyst should be considered in the differential diagnosis of the following pathologies: Simple cysts, non-organize hematoma, necrotic tumor, cystic metastatic carcinoma, hemangioma, pyogenic abscesses, Amoeba abscess, Tuberculosis, fungal infections [63, 64].

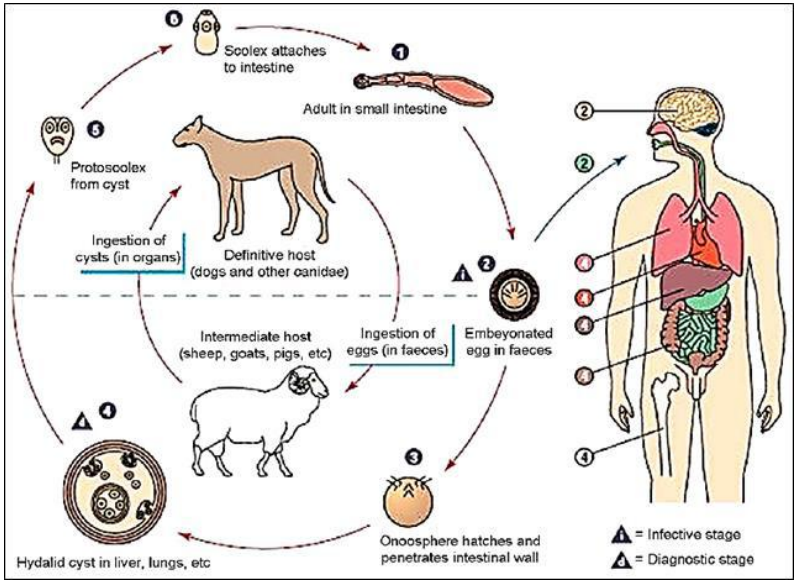

Fig 1: Life cycle of Echinococcus granulosus (Reproduced from the Centers for Disease Control and Prevention at http:// www.dpd.cdc.gov/dpdx/html/Echinococcosis.htm)

The adult Echinococcus granulosus (3-6 mm long) resides in the small bowel of the definitive hosts, dogs or other canids. Gravid proglottids release eggs that are passed in the feces. After ingestion by a suitable intermediate host (under natural conditions: sheep, goat, swine, cattle, horses, camel), the egg hatches in the small bowel and releases an oncosphere that penetrates the intestinal wall and migrates through the circulatory system into various organs, especially the liver and lungs. In these organs, the oncosphere develops into a cyst that enlarges gradually, producing protoscolices and daughter cysts that fill the cyst interior. The definitive host becomes infected by ingesting the cyst-containing organs of the infected intermediate host. After ingestion, the protoscolices evaginate, attach to the intestinal mucosa and develop into adult stages in 32 to 80 days. The same life cycle occurs with Echinococcus multilocularis (1.2-3.7 mm), with the following differences: the definitive hosts are foxes, and to a lesser extent dogs, cats, coyotes and wolves; the intermediate host are small rodents; and larval growth (in the liver) remains indefinitely in the proliferative stage, resulting in invasion of the surrounding tissues. With Echinococcus vogeli (up to 5.6 mm long), the definitive hosts are bush dogs and dogs; the intermediate hosts are rodents; and the larval stage (in the liver, lungs and other organs) develops both externally and internally, resulting in multiple vesicles. Echinococcus oligoarthritis (up to 2.9 mm long) has a life cycle that involves

wild felids as definitive hosts and rodents as intermediate hosts. Humans become infected by ingesting eggs, with resulting release of oncospheres in the intestine and the development of cysts in various organs. Image courtesy of the CDC-DPDx.

Table 1: WHO-IWGE, World Health Organization-Informal Working Group Classification on Echinococcus

Classification Type		
Gharbi Stage	**WHO-IWGE**	**Classifying features**
I	CE1	Univesicular fluid collection/simple cyst Active
III	CE2	Multivesicular fluid collection with multiple daughter cysts or septae (honeycomb) Active
II	CE3	A Fluid collection with membrane detached (water lily sign) Transitional
III	CE3	B Daughter cysts in solid matrix Transitional
IV	CE4	Cysts with heterogeneous matrix, no daughter cysts Inactive/degenerative
V	CE5	Solid cystic wall Inactive/degenerative

References

1. Eckert J, Conraths FJ, Tackmann K. Echinococcosis: an emerging or re-emerging zoonosis? Int J Parasitol. 2000; 30:1283-94.
2. Polat P, Kantarci M, Alper F, Suma S, Koruyucu MB *et al*. Hydatid disease from head to toe. Radiographics. 2003; 23:475-94.
3. McManus DP, Thompson RC. Molecular epidemiology of cystic echinococcosis. Parasitology. 2003; 127:37-51.
4. Thompson RC, McManus DP. Towards a taxonomic revision of the genus Echinococcus. Trends Parasitol. 2002; 18:452-7.
5. Moro PL, Cavero CA, Tambini M, Bricen˜o Y, Jime´nez R, Cabrera L. Identification of risk factors for cystic echinococcosis in a periurban population of Peru. Trans R Soc Trop Med Hyg. 2008; 102:75-8.
6. Yang YR, Sun T, Li Z, Zhang J, Teng J, Liu X *et al*. Community surveys and risk factor analysis of human alveolar and cystic echinococcosis in Ningxia Hui Autonomous Region, China. Bull World Health Organ. 2006; 84:714-21.
7. Kammerer WS, Schantz PM. Echinococcal disease. Infect Dis Clin North Am. 1993; 7:605-18.
8. Carrim ZI, Murchison JT. The prevalence of simple renal and hepatic cysts detected by spiral computed tomography. Clin Radiol. 2003; 58:626-9.

9. Karaman U, Mman O, Kara M, Gicik Y, Aycan OM *et al*. Hydatid cyst prevalence in the region of Kars. Turkiye. Parazitol. Derg. 2005; 29:238-40.

10. Brunetti E, Kern P, Vuitton DA. Writing Panel for the WHO-IWGE. Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. Acta Trop. 2010; 114:1-16. Doi: 10.1016/j.actatropica. 2009.11.001.

11. Otero-Abad B, Torgerson PR. A Systematic Review of the Epidemiology of Echinococcosis in Domestic and Wild Animals. Garcia. PLoS Negl. Trop Dis. 2013; 7:e22-49. Doi: 10.1371/journal.pntd.0002249.

12. Reddy CRRM. Epidemiology of hydatid disease in Kurnool. Ind J Med Res. 1968; 56:1205-20.

13. Rana UV, Sehgal S, Bhatia R, Bhardwaj M *et al*. Hydatidosis in animals in and around Delhi. J Com Dis 1986; 18:116-9.

14. Christofi G, Deplazes P, Christofi N, Tanner I, Economides P *et al*. Screening of dogs for Echinococcus granulosus coproantigen in a low endemic situation in Cyprus. Vet Parasitol. 2002; 104:299-306

15. Siracusano A, Teggi A, Ortona E. Human Cystic Echinococcosis: Old Problems and New Perspectives. Interdiscip. Perspect. Infect Dis, 2009, 474-368. doi: 10.1155/2009/474368.

16. Lewall DB. Hydatid disease: Biology, pathology, imaging and classification Clin Radiol 1998; 52:863-74. Doi: 10.1016/S0009-9260(98)80212-2.

17. Gottstein B. Hydatid Disease, Major Tropical syndromes by body system. Systemic infections, 2000, 169.

18. Siracusano A, Delunardo F, Teggi A, Ortona E. Host-Parasite Relationship in Cystic Echinococcosis: An Evolving Story. Clin Dev Immunol, 2012, 639-362. Doi: 10.1155/2012/639362.

19. Lissandrin R, Tamarozzi F, Piccoli L, Tinelli C, Silvestri A, Mariconti M *et al*. Factors Influencing the Serological Response in Hepatic Echinococcus granulosus Infection. Am J Trop Med Hyg 2016; 94:166-171. Doi: 10. 4269/ajtmh.15-0219.

20. Thompson RC. Biology and systematics of Echinococcus. In Thompson RC, Lymbery AJ. Echinococcus and hydatid disease. London: CAB International, 1995, 1-37.

21. Siracusano A, Delunardo F, Teggi A, Ortona E. Cystic echinococcosis: aspects of immune response, immunopathogenesis and immune evasion from the human host. Endocr. Metab. Immune Disord Drug Targets. 2012; 12:16-23. Doi: 10.2174/187153012799279117.

22. Zhang W, Ross A, McManus D. Mechanisms of Immunity in Hydatid Disease: Implications for Vaccine Development. J Immunol. 2000; 181:6679-6685. doi: 10.4049/jimmunol.181.10.6679.

23. Nunnari G, Pinzone MR, Gruttadauria S, Celesia BM, Madeddu G *et al*. Hepatic echinococcosis: clinical and therapeutic aspects. World J Gastroenterol. 2012; 18:1448-58.

24. Langer J, Rose D, Keystone J, Taylor B, Langer B. Diagnosis and management of hydatid disease of the liver. A 15-year North American experience. Ann Surg. 1984; 199:412-417. Doi: 10.1097/00000658-198404000-00007.

25. Symeonidis N, Pavlidis T, Baltatzis M, Ballas K, Psarras K, Marakis G *et al*. Complicated liver echinococcosis: 30 years of experience from an endemic area. Scand J Surg. 2013; 102:171-7. Doi: 10.1177/1457496913491877.

26. McManus DP, Bryant C. Biochemistry, physiology and molecular biology of Echinococcus. In: RCA Thompson, Lymbery AJ. editors. The biology of Echinococcus and hydatid disease. Wallingford: CAB International, 1995, 355-410.

27. García MB, Lledías JP, Pérez IG, Tirado VV, Pardo LF, Bellvís LM *et al*. Primary Super-Infection of Hydatid Cyst-Clinical Setting and Microbiology in 37 Cases. Am J Trop Med Hyg. 2010; 82:376-8. Doi: 10.4269/ajtmh.2010. 09-0375.

28. Ammann R, Eckert J. Cestodes. Echinococcus. Gastroenterol Clin North Am. 1996; 25:655-89.

29. Lewall DB, McCorkell SJ. Rupture of echinococcal cysts: diagnosis, classification and clinical implications. Am J Roentgenol. 1986; 146:391-4.

30. Zhang W, Wen H, Li J, Lin R, McManus D. Immunology and Immunodiagnosis of Cystic Echinococcosis: An Update. Clin Dev Immunol, 2012, 101-895. Doi: 10.1155/2012/101895.

31. Ortona E, Riganò R, Margutti P, Notargiacomo S, Ioppolo S, Vaccari S *et al.* Native and recombinant antigens in the Immunodiagnosis of human cystic echinococcosis. Parasite Immunol 2000; 22:553-9. Doi: 10.1046/j.1365- 3024.2000.00336.x.

32. Rajesh R, Dalip D, Anupam J, Jaisiram A. Effectiveness of Puncture-Aspiration-Injection-respiration in the Treatment of Hepatic Hydatid Cysts. Iran J Radiol. 2013; 10:68-73. doi: 10.5812/iranjradiol.7370.

33. Rinaldi F, Brunetti E, Neumayr A, Maestri M, Goblirsch S, Tamarozzi F. Cystic echinococcosis of the liver: A primer for hepatologists. World J Hepatol. 2014; 6:293-305. Doi: 10.4254/wjh.v6.i5.293.

34. Liance M, Janin V, Bresson-Hadni S, Vuitton DA, Houin R, Piarroux R. Immunodiagnosis of Echinococcus Infections: Confirmatory Testing and Species Differentiation by a New Commercial Western Blot. J Clin Microbiol. 2000; 38:3718-21.

35. Ravinder P, Parija S, Rao K. Evaluation of human hydatid disease before and after surgery and chemotherapy by demonstration of hydatid antigens and antibodies in serum. J Med Microbiol. 1997; 46:859-64. doi: 10.1099/ 00222615-46-10-859.

36. Sadjjadi S, Sedaghat F, Hosseini S, Sarkari B. Serum Antigen and Antibody Detection in Echinococcosis: Application in Serodiagnosis of Human Hydatidosis. Korean J Parasitol 2009; 47:153-7. Doi: 10.3347/kjp.2009.47.2.153.

37. Sbihi Y, Rmiqui A, Rodriguez-Cabezas MN, Orduña A, Rodriguez-Torres A *et al.* Comparative sensitivity of six serological tests and diagnostic value of ELISA using purified antigen in hydatidosis. J Clin Lab Anal. 2001; 15:14-8.

38. Lantinga MA, Gevers TJ, Drenth JP. Evaluation of hepatic cystic lesions. World J Gastroenterol. 2013; 19:3543-54.

39. Brunetti E, Kern P, Vuitton DA. Writing Panel for the WHO-IWGE. Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. Acta Trop. 2010; 114:1-16. doi: 10.1016/j.actatropica. 2009.11.001.

40. Von Sinner WN. Imaging of cystic echinococcosis. Acta Trop. 1997; 67:67-89.

41. Beggs I. The radiology of hydatid disease. Am J Roentgenol. 1985; 145:639-48. doi: 10.2214/ajr.145.3.639.

42. Nagarajan K, Sekar D, Babu JV, Kamath A. Hydatid cyst of the liver causing inferior vena caval obstruction. J Assoc Physicians India. 2013; 61:671-3.

43. Smego RA, Bhatti S, Khaliq AA, Beg MA. Percutaneous aspiration-injection-respiration drainage plus albendazole or mebendazole for hepatic cystic echinococcosis: A meta-analysis. Clin Infect Dis. 2003; 37:1073-83.

44. Marani SA, Canossi GC, Nicoli FA, Alberti GP, Monni SG, Casolo PM. Hydatid disease: MR imaging study. Radiology. 1990; 175:701-6. Doi: 10.1148/ radiology.175.3.2343117.

45. Zhang W, McManus DP. Recent advances in the immunology and diagnosis of echinococcosis. FEMS Immunol Med Microbiol. 2006; 47:24-41.

46. Stojkovic M, Zwahlen M, Teggi A, Vutova K, Cretu CM, Virdone R *et al*. Treatment Response of Cystic Echinococcosis to Benzimidazoles: A Systematic Review. PLoS Negl. Trop Dis. 2009; 3:e5-24. Doi: 10.1371/journal.pntd.0000524.

47. Smego R, Sebanego P. Treatment options for hepatic cystic echinococcosis. Int J Infect Dis 2005; 9:69-76. Doi: 10.1016/j.ijid.2004.08.001.

48. Tuxun T, Zhang JH, Zhao JM, Tai QW, Abudurexti M, Ma HZ *et al*. World review of laparoscopic treatment of liver cystic echinococcosis-914 patients. Int J Infect Dis. 2014; 24:43-50. doi: 10.1016/j.ijid.2014.01.012.

49. Junghanss T, Da Silva AM, Horton J, Chiodini PL, Brunetti E. Clinical management of cystic echinococcosis: state of the art, problems, and perspectives. Am J Trop Med Hyg. 2008; 79:301.

50. Paksoy Y, Odev K, Sahin M, Arslan A, Koç O. Percutaneous treatment of liver hydatid cysts: comparison of direct injection of albendazole and hypertonic saline solution. Am J Roentgenol. 2005; 185:727-34. doi: 10.2214/ajr.185. 3.01850727.

51. Peng X, Li J, Wu X, Zhang S, Niu J, Chen X *et al*. Detection of Osteopontin in the pericyst of human hepatic Echinococcus granulosus. Acta Trop. 2006; 100:163-71. doi: 10.1016/j.actatropica.2006.08.013.

52. McNanus D, Gray D, Zhang W, Yang Y. Diagnosis, treatment, and management of Echinococcosis. BMJ. 2012; 344:e38-66. doi: 10.1136/bmj.e3866.

53. Macpherson C, Milner R. Performance characteristics and quality control of community-based ultrasound surveys for cystic and alveolar echinococcosis. Acta Trop 2003; 85:203-9. doi: 10.1016/S0001-706X(02)00224-3.

54. Rinaldi F, Brunetti E, Neumayr A, Maestri M, Goblirsch S, Tamarozzi F. Cystic echinococcosis of the liver: A primer for hepatologists. World J Hepatol. 2014; 6:293-305. doi: 10.4254/wjh.v6.i5.293.

55. Schipper HG, Lameris JS, Van Delden OM, Rauws EA, Kager PA *et al*. Percutaneous evacuation (PEVAC) of multivesicular echinococcal cysts with or without cystobiliary fistulas which contain non-drainable material: first results of a modified PAIR method. Gut. 2002; 50:718-23.

56. Piccoli L, Tamarozzi F, Cattaneo F, Mariconti M, Filice C, Bruno A *et al*. Longterm Sonographic and Serological Follow-up of Inactive Echinococcal Cysts of the Liver: Hints for a "Watch-and-Wait" Approach. PLoS Negl. Trop Dis. 2014; 8:e30-57. doi: 10.1371/journal.pntd.0003057.

57. Moro P, Schantz PM. Cystic echinococcosis in the Americas. Parasitol Int 2006; 55:181-6.

58. Heath DD, Jensen O, Lightowlers MW. Progress in control of echinococcosis using vaccination-a review of formulation and delivery of the vaccine and recommendations for practical use in control programs. Acta Trop. 2003; 85:133-43.

59. Zhang W, Zhang Z, Shi B, Li J, You H, Tulson G *et al*. Vaccination of dogs against Echinococcus granulosus, the cause of cystic hydatid disease in humans. J Infect Dis. 2006; 194:966-74.

60. Torgerson PR. Mathematical models for the control of cystic echinococcosis. Parasitol. Int. 2006; 55:S253-8.

61. Moro PL, McDonald J, Gilman RH, Silva B, Verastegui M, Malqui V *et al*. Epidemiology of Echinococcus granulosus infection in the central Peruvian Andes. Bull World Health Organ. 1997; 75:553-61.

62. Gomez I, Gavara C, Lopez-Andujar R, Belda Ibanez T, Ramia Angel JM *et al*. Review of the treatment of liver hydatid cysts. World J Gastroenterol. 2015; 21:124-31.

63. Serbest S, Tiftikci U, Uludag A. Unusual Localization of a Primary Hydatid Cyst: Scaphoid Bone: A Case Report. Medicine (Baltimore). 2016; 95:e32-90.

64. Sahin B, Comoglu S, Polat B, Deger K. Hydatid cyst in unusual location: Pterygopalatine fossa-infratemporal fossa. Auris Nasus Larynx. 2016; 43:464-467.

Chapter - 4
Current Status and Drawbacks of Parasitology Research with Reference to Ethnopharmacology

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Chapter - 4

Current Status and Drawbacks of Parasitology Research with Reference to Ethnopharmacology

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Abstract

Intestinal helminth infections are a common cause in developing countries. With limited drugs and increasing resistance, anthelmintic drug research needs to be accelerated. Several workers have worked on different aspects of anthelmintic efficacy using different parasitic models. Literature also revealed that many authors have used non-parasitic models to claim anthelmintic efficacy. Most of the efficacy studies have been only confirmed by *in vitro* studies. Studies on *in vivo* anthelmintic efficacy are scanty. Also several authors have contributed by authenticating the claims of efficacy using both *in vitro* and *in vivo* studies on different parasite models. However, these studies lack toxicity assessment of the traditionally used medicinal plant. Studies that authenticate the efficacy using both *in vitro* and *in vivo* models using suitable parasite models along with toxicity assessments of the medicinal plant are necessary to access the pros and cons of its use as a medicine. These tests are the backbone of any pre-clinical testings to be carried forward for clinical tests and ultimately for human consumption. This paper is an effort to describe the studies conducted in recent years in anthelmintic research and the drawbacks of previous researches.

Keywords: anthelmintic, medicinal plants, toxicity, traditional medicine

Introduction

Helminthiasis is the most common type of infections in tropical and sub-tropical regions and affects the poor and deprived communities [1]. The highest numbers of infections occur in sub-Saharan Africa, the Americas, China and East Asia [2]. According to the WHO, approximately 1.5 billion people which comprises of 24% of the world's population are infected with soil-transmitted helminths [3]. The global target of WHO is to eliminate morbidity due to soil-transmitted helminthiases in children by 2020, which is envisaged to be obtained by regularly treating at least 75% of the children in

endemic areas [4]. Factors such as open defecation practices, lack of personal hygiene and community sanitation, lack of footwear wearing habit, poor maternal education, low literacy rate and poor socio-economic status contribute to the prevalence of STH infections [5]. Pyrantel pamoate and levamisole as well as the two benzimidazoles, albendazole and mebendazole are the four available treatments against soil-transmitted helminths [6, 7].

Actors such as limited anthelmintic drug pharmacopoeia and increasing drug resistance are now a cause of concern [8, 9, 10]. No new chemical entity or drug had been approved for these diseases during the past decade [11]. Taking these facts into account, alternate sources of medicines, which are easily available, affordable and have no or fewer side effects need to be explored. Plants possess several properties such as antioxidant, antiviral, anticancer, antimicrobial, antifungal and antiparasitic [12]. Medicinal plants are the natural raw resources needed for the development of new drugs [13, 14, 15]. Several chemical compounds in modern medicine, such as quinine, digoxin, aspirin, ephedrine, atropine, and colchicine are derived from plant sources [16, 17]. Workers have now started to venture into traditionally used medicinal plant research with the hope of finding new entities that could assist in treating helminthiasis.

***In vitro* studies**

In vitro studies is the first procedure to ascertain anthelmintic efficacy of any medicinal plant using suitable parasites. Such studies are done with commonly available parasites in the local area. From time to time, many workers have used several test parasites such as *Ascaris suum*, *Ascaridia galli*, *Raillietina echinobothrida*, *Gastrothylax crumenifer*, *Fasciolopsis buski*, *Haemonchus contortus*, *Hymenolepis diminuta*, *Syphacia obvelata* etc to test the *in vitro* anthelmintic efficacy of medicinal plants [18, 19, 20, 21, 22, 23]. *In vitro* tests are conducted using physical motility test as described by Yadav *et al*., 1992 [24]. Table 1 describes a list of a few traditionally used medicinal plants during the last decade tested for their *in vitro* anthelmintic efficacy.

Several workers have used non-parasite models such as earthworms, *C. elegans* and *Tubifex* worms to assess *in vitro* anthelmintic efficacy [25, 26, 27, 28, 29]. Such worms do not share similar features with helminth parasites in their mode of nutrition, habitat etc.

Some workers have worked on active component isolated from plants and have shown significant anthelmintic activity [30, 31]. Table 2 shows the list of a few isolated compounds tested for their *in vitro* anthelmintic efficacy.

Scanning electron microscopy (SEM) studies of the test parasites help is ascertaining the mode of action and also throws insight on the damages caused by exposure of the parasite to the plant extract. Several authors have processed the worms for SEM studies and evaluated the effects of the extract [22, 32].

In vivo studies

In vitro studies alone do not validate the anthelmintic efficacy of plants [33]. *In vivo* studies usually supplement the *in vitro* results and are performed using various parasite-animal models [34]. Several authors have only established *in vitro* anthelmintic efficacy alone [35, 36]. A few authors have established both *in vitro* and *in vivo* anthelmintic efficacy of traditionally used medicinal plants using various models such as *Syphacia obvelata*-mice model [37], *Hymenolepis diminuta*-rat model [34], *Haemonchus contortus*-goat model [38]. Other *in vivo* models used include *Dactylogyrus intermedius*-goldfish [39] and *Trichinella spiralis*-mice [40]. Table 3 shows a list of plants tested for their *in vivo* anthelmintic efficacy and table 4 shows a list of few plants tested for their *in vitro* and *in vivo* anthelmintic efficacy in the recent years.

Table 1: *In vitro* studies

Sl. No	Scientific name	Family	Extract	Model parasite	Workers
1.	*Dicerocaryum eriocarpum* *Pappea capensis* *Aloe ferox* *Helichrysum mill* *Senecio congestus* *Senecio barbertonicus* *Gardenia* sp.	Pedaliaceae Sapindaceae Asphodelaceae Asteraceae Asteraceae Asteraceae Rubiaceae	Aqueous	*Haemonchus contortus*	Chitura *et al*., 2019 [36]
2.	*Hymenodictyon pachyanta*	Rubiaceae	Methanol	*Haemonchus contortus*	Olayemi *et al*., 2019 [41]
3.	*Ficus hispida* *Hemigraphis alternata* *Senna sophera*	Moraceae Acanthaceae Fabaceae	Methanol	Earthworms *Pheretima posthuma*	Rahman *et al*., 2019 [29]
4.	*Caesalpinia coriaria*	Caesalpiniaceae	Methanol	*Haemonchus contortus*	Jesús-Martínez *et al*., 2018 [35]
5.	*Achyranthes aspera*	Amaranthaceae	Ethyl acetate Ethanol Aqueous	Earthworm *Pheretima posthuma*	Esther *et al*., 2018 [28]
6.	*Piper sylvaticum*	Piperaceae	Methanol	Aquarium worm *Tubifex tubifex*	Paul *et al*., 2018 [27]
7.	*Amaranthus dubius* *Basella alba* *Cleome gynandra*	Amaranthaceae Basellaceae Cleomaceae	Aqueous	Earthworm *Eisenia fetida*	George *et al*., 2018 [42]
8.	*Acanthus ilicifolius*	Acanthaceae	Ethanol Aqueous n-hexane	*Ascaridia galli* *Pheretima posthuma*	Husori *et al*., 2018 [23]
9.	*Cissus quadrangularis* *Schinus molle*	Vitaceae Anacardiaceae	Methanol	*Haemonchus contortus*	Zenebe *et al*., 2017 [43]

10.	*Chrysopogon aciculatus* *Cyperus brevifolius* *Ruellia tuberosa* *Saccharum spontaneum*	Poaceae Cyperaceae Acanthaceae Poaceae	Hexane Chloroform	Earthworm *Eudrilus eugeniae*	Pueblos *et al*., 2017 [26]
11.	*Lophira lanceolata*	Ochnaceae	Ethanol, methanolic-methylene chloride	*Onchocerca ochengi* *Caenorhabditis elegans*	Kalmobé *et al*., 2017 [25]
12.	*Plumeria rubra*	Apocynaceae	Methanol	Earthworm *Pheretima posthuma*	Srivastava *et al*., 2017 [44]
13.	*Acacia nilotica* *Acacia raddiana*	Fabaceae Fabaceae	Aqueous Acetone	*Haemonchus contortus* *Caenorhabditis elegans*	Zabré *et al*., 2017 [45]
14.	*Azadirachta indica* *Allium sativum* *Ocimum tenuiflorum*	Meliaceae Amaryllidaceae Lamiaceae	Ethanol	*Haemonchus contortus*	Kumar *et al*., 2017 [46]
15.	*Acacia arabica* *Cadaba farinosa* *Capparis tomentosa* *Dichrostachys cinerea* *Dodonaea angustifolia* *Euclea racemosa* *Maerua angolensis* *Maytenus senegalensis* *Rhus natalensis* *Senna singueana*	Fabaceae Capparaceae Capparaceae Fabaceae Sapindaceae Ebenaceae Capparaceae Celastraceae Anacardiaceae Fabaceae	Acetone water (70:30)	*Haemonchus contortus*	Mengistu *et al*., 2017 [21]
16.	*Marattia fraxinea*	Marattiaceae	Ethanol	*Gastrothylax crumenifer*	Rajesh *et al*., 2017 [22]
17.	*Azadirachta indica* *Nicotiana tabacum* *Momordica charantia*	Meliaceae Solanaceae Cucurbitaceae	Methanol	*Haemonchus contortus*	Akther *et al*., 2015 [47]

	Curcuma domestica *Alstonia scholaris* *Cuscuta* spp. *Mimosa pudica*	Zingiberaceae Apocynaceae Convolvulaceae Fabaceae			
18.	*Ocimum sanctum* *Murraya koenigii* *Mallotus philippensis*	Lamiaceae Rutaceae Euphorbiaceae	Aqueous Methanol	*Haemonchus contortus*	Sujith *et al*., 2015 [48]
19.	*Senna occidentalis*	Leguminosae	Ethanol	*Hymenolepis diminuta*	Kundu *et al*., 2015 [49]
20.	*Alpinia nigra*	Zingiberaceae	Ethanol	*Fasciolopsis buski*	Swargiary and Roy, 2015 [19]
21.	*Fumaria indica*	Papaveraceae	Methanol	*Haemonchus contortus*	Khan *et al*., 2014 [50]
22.	*Alpinia nigra*	Apocynaceae	Aqueous Chloroform	Earth Worm *Terrestris lumbricoides*	James *et al*., 2014 [51]
23.	*Cassia alata* *Cassia angustifolia* *Cassia occidentalis*	Fabaceae	Ethanol	*Heterakis gallinarum*, *Raillietina tetragona* *Calotropis* sp.	Kundu *et al*., 2014 [52]
24.	*Vernonia amygdalina* *Secamone africana*	Asteraceae Apocynaceae	Ethanol Aqueous	*Ascaris suum*	Nalule *et al*., 2013 [53]
25.	*Luffa cylindrica*	Cucurbitaceae	Aqueous Methanol	Earthworm *Pheretima posthuma*	Partap *et al*., 2012 [54]

Table 2: *In vitro* studies on compounds isolated from plants

Sl. No.	Compound	Test parasite	Workers
1.	Mangiferin Rutin Quercetin β-sitosterol	*Haemonchus* *Trichostrongylus* *Chabertia* *Teladorsagia/Ostertagia*	Giovanelli *et al*., 2018 [30]
2.	Ursolic acid Betulinic acid	*Syphacia obvelata*	Vijaya *et al*., 2018 [31]
3.	Artesunate	*Raillietina echinobothrida*	Lalchhandama, 2015 [18]
4.	Astragalin	*Fasciolopsis buski*	Swargiary and Roy, 2015 [19]
5.	Resveratrol	*Hymenolepis diminuta*	Giri *et al*., 2015 [55]

Table 3: *In vivo* studies

Sl. No	Scientific name	Family	Extract	Model	Workers
	Acorus calamus	Acoraceae	Methanol	*Hymenolepis diminuta*-rat	Nath and Yadav, 2016 [56]
1.	*Caesalpinia sappan* *Lysimachia christinae* *Cuscuta chinensis* *Artemisia argyi* *Eupatorium fortunei*	Caesalpiniaceae Primulaceae Convolvulaceae Asteraceae Asteraceae	Aqueous	*Dactylogyrus intermedius*-goldfish	Huang *et al*., 2013 [39]
2.	*Clerodendrum colebrookianum*	Lamiaceae	Methanol	*Hymenolepis diminuta*-rat	Yadav and Temjenmongla, 2012 [57]
3.	*Solanum myriacanthum*	Solanaceae	Methanol	*Hymenolepis diminuta*-rat	Yadav and Tangpu, 2012 [58]
4.	*Lasia spinosa*	Araceae	Methanol	*Trichinella spiralis*-mice	Yadav and Temjemnongla, 2012 [40]

Table 4: *In vitro* and *in vivo* studies

Sl. No	Scientific name	Family	Extract	Model	Workers
1.	*Caesalpinia bonducella*	Caesalpiniaceae	Methanol	*Hymenolepis diminuta* *Syphacia obvelata*	Gogoi and Yadav, 2017 [37]
2.	*Artemisia sieversiana* *Artemisia parviflora*	Asteraceae Asteraceae	Methanol	*Haemonchus contortus*	Irum *et al*., 2017 [38]

3.	*Prunella vulgaris*	Lamiaceae	Aqueous. Methanol	*Haemonchus contortus*	Lone *et al*., 2017 [59]
4.	*Croton joufra*	Euphorbiaceae	Methanol	*Hymenolepis diminuta*- rat	Gogoi and Yadav, 2016 [20]
5.	*Hibiscus rosa-sinensis*	Malvaceae	Methanol	*Hymenolepis diminuta*	Nath and Yadav, 2016 [60]
6.	*Oroxylum indicum*	Bignoniaceae	Methanol	*Hymenolepis diminuta*	Deori and Yadav, 2016 [34]
7.	*Gynura angulosa*	Compositae	Methanol	*Hymenolepis diminuta*	Yadav *et al*., 2014 [61]

Toxicity studies

Parallel to the fact that some plants have been found to be efficacious against helminths, there is also some misconception among many people that since medicinal plants are natural in origin, they do not cause any adverse effects to their users. Therefore, the therapeutic products of these plants are usually considered to be safe for use as medicines [62]. However, the safety of such traditionally used medicinal plants remains doubtful unless it is testified by scientific researches [63]. Although herbal medicines are supposed to be safe [64], many toxic effects have been reported by several authors [65, 66]. Medicinal plants contain bioactive principles with potential to cause adverse effects [67]. In view of such serious risks, a much greater attention seems necessary to not only authenticate the bioefficacies of traditionally used medicinal plants, but to also evaluate their safety profiles and to ensure their beneficial role in the treatment and control of helminth parasite infections [62]. Table 5 displays a list of traditionally used medicinal plants tested for their toxic effects in the recent years. Several medicinal plants await evaluation of their toxicological profile. Toxicity tests include acute oral toxicity studies, sub-acute oral toxicity studies, haematology, biochemical studies, histopathology, transmission electron microscopy (TEM) studies of vital organs, genotoxicity, cytotoxicity, elemental analysis etc. Acute oral toxicity studies is conducted following OECD 425, 2008 [68] whereas sub-acute oral toxicity studies comprising repeated dose 28-day oral toxicity study is conducted following OECD 407, 2008 [69].

Evaluations are made by assessing fluctuations from normal values of various parameters such as food and water consumption, relative organ weights, body weights, enzyme assays and other blood parameters. Damages in the chromosomal level can be assessed by genotoxicity study in the cells of the animal after dosing for a certain period. Presence of toxic elements in the medicinal plant could also contribute to the toxicity of the plant. Hence elemental analysis of the studied medicinal plant is necessary.

Table 5: Toxicity studies on medicinal plants

Sl. No	Scientific name	Family	Uses	Extract	Toxicity	Workers
1.	*Brugmansia*	Solanaceae	Spasmolytic, Anti-asthmatic, Anticholinergic, Narcotic and Anesthetic	Plant	Neurotoxic	Parez *et al*., 2012 [70]
2.	*Scutellaria baicalensis*	Lamiaceae	Anti-inflammatory, Arthritis	Plant	Hepatotoxic	Yang *et al*., 2012 [71]
3.	*Grewia crenata*	Malvaceae	Treatment of Fractured Bones, Wound Healing and Anti-inflammatory	Methanolic	No toxicity	Ukwuani *et al*., 2012 [72]
4.	*Sida acuta Sida cordifolia*	Malvaceae	Malaria, Fever, Pain, Variola, Antibacterial, Anti-Inflammatory, Analgesic, Hepatoprotective	Aqueous acetone	No toxicity	Konaté *et al*., 2012 [73]
5.	*Eucalyptus camaldulensis*	Myrtaceae	Anaesthetic, Astringent, Antiseptic, Cough, Diarrhoea, Sore Throat, Cold, Dysentery, Hemorrhage, Wound Healing, Antibacterial	Aqueous	Inflammatory, anaemic, hepatotoxic	Musa *et al*. 2011 [74]
6.	*Parkia biglobosa*	Fabaceae	Malaria, Diarrhoea, Pain	Aqueous Methanolic	Hypercholesterolemic, Hyperglycemic Hypocholesterolemic, Hypoglycemic	Builders *et al*., 2012 [75]
7.	*Ananas comosus*	Bromeliaceae	Antimicrobial, Vermicide, Purgative, Emmenagogue, Abortifacient, Anti-oedema, Anti-inflammatory, Wound Healing, Antimicrobial	Aqueous	No toxicity	Dutta and Bhattacharyya, 2013 [76]
8.	*Carica papaya*	Caricaceae	Anemia, Diabetes mellitus, Intestinal helminthiasis, Malaria, Diarrhoea, Jaundice, Dysentery, Algesic, Emmenagogue, Febrifuge, Laxative	Aqueous	Uric acid and triglycerides accumulation	Tarkang *et al*., 2012 [77]
9.	*Cassia fistula*	Caesalpinioideae	Skin diseases, Fever, Abdominal pain and Leprosy, Astringent, Tonic, Febrifugal and Purgative, Cardiac	Methanolic	No toxicity	Jothy *et al*., 2011 [78]

			Disorders, Biliousness, Rheumatic Condition, Haemorrhages, Wounds, Ulcers and Boils, Tubercular Glands and Various Skin Diseases			
10.	*Acorus calamus*	Acoraceae	Emetic, Expectorant, Aphrodisiac, Antimicrobial, Laxative, Diuretic, Antispasmodic, Carminative, Anthelmintic	Methanolic	Genotoxic	Nath *et al*., 2017 [79]
11.	*Lysimachia ramosa*	Primulaceae	Intestinal helminthiasis	Aqueous	Cytotoxic	Roy and Dey, 2016 [80]
12.	*Aconitum*	Ranunculaceae	Pain	Plant	Cardiotoxic, neurotoxic	Sheth *et al*., 2015 [81]
13.	*Psidium guajava*		Gastrointestinal infections, Malaria, Respiratory Infections, Oral/Dental Infections, Skin infections diabetes, Hypertension, Cancer, Malnutrition, Pain, Fever, Liver and Kidney Problems	Ethanol	No toxicity	Atik *et al*., 2019 [82]

Discussion

With developing resistance, the need for alternative medicines or drugs for the treatment of helminthiasis is the need of the hour. Systematic clinical assays with proper scientific analysis needs to be carried out. *In vitro* studies alone do not validate the anthelmintic efficacy of the plant. A study by Bogh *et al*. (1996) [83], on anthelmintic efficacy of fruits of *Embelia schimperi*, the plant extract showed significant *in vitro* against *Hymenolepis microstoma*, *Echinostoma caproni* and *Heligmosomoides polygyrus*, but it failed to show *in vivo* effects. *In vitro* studies need to be supplemented with *in vivo* studies. Several authors have only shown *in vitro* effects of the plant extracts and hence such studies needs to be further studied for their *in vivo* efficacy using suitable models [21, 24]. The parasite model used should be a suitable model. Non-parasitic models such as tubifex worms, *C. elegans*, earthworms are not parasitic and their mode of action of the extracts could be different. These worms differ from parasitic worms in the tegument structure, habitat and feeding habits. Hence, data from sources using such models cannot be used for pre-clinical or clinical testings.

The body surface of the parasite is the interface with the hostile environment, and it also serves as the principal route by which the plant extract could damage the parasite through its action on body surface [84]. Hence studies on surface alterations of the parasite assists in validating the mode of action or damages caused by the plant extract. Several authors have used SEM as a tool to identify the damage caused to the parasite on exposure to drugs or plant extracts [22, 85]. Since optical microscopy does not reveal the body surface changes in detail, SEM is employed to observe alterations on body surface of test parasites [86].

Many people have been using plants as a medicine without scientific knowledge and proper guidance for thousands of years. Studies have shown that not all plants are safe and the presence of toxic compounds in them cannot be denied [87]. Medicinal plants can be easily contaminated during growth, processing and collection. Also, adulteration and heavy metal contamination are the two major problems in Herbal medicines [88]. Hence, the strong dependence on traditional plants as medicines, demands a comprehensive research of their efficacy and safety issues [89].

Several plants have only been tested for their claims of efficacy and still await toxicological assessment. The general perception that herbal medicines are safe and devoid of any adverse effects is not only untrue, but also misleading. Traditionally used medicinal plants have been shown to be

capable of causing several undesirable or adverse reactions some of which are capable of causing serious life-threatening conditions and even death [90, 91, 92]. In view of such serious risks involved with the use of herbal medicines, the need to not only authenticate the bioefficacies of traditionally used medicinal plants, but to also evaluate their safety profiles and to ensure their beneficial role in the treatment and control of helminth parasite infections seems necessary [62]. Following these tests, the active compound present in medicinal plants can be isolated and tested further for their efficacy and toxicity. On completion of these assessments, the compound can be characterised and the dosage can be estimated for human consumption. Most findings have been done without approval for use of animals by Institutional Animal Ethical Committee (IAEC) [93, 94]. For any study on animals, proper approval from an IAEC, declaring to carry out the work in an ethical manner by complying with the rules should be sought [95].

Conclusion

There is a lack of literature comprising of the complete efficacy of an anthelmintic medicinal plant using suitable parasitic models both *in vitro* and *in vivo*, toxicity studies and active component isolation. Every traditionally used medicinal plant must undergo these tests to be authenticated safe for human consumption. Medicinal plants lacking in any one of these tests must be subjected to such studies. Such systematic studies could result in finding new drugs, or alternatives or supplements to the already existing pharmacopeia.

References

1. Mitra AK, Mawson AR. Neglected Tropical Diseases: Epidemiology and Global Burden. Tropical Medicine and Infectious Disease, 2017, 2(3). https://doi.org/10.3390/tropicalmed2030036.
2. Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D *et al*. Soil-transmitted helminth infections: ascariasis, trichuriasis and hookworm. Lancet. 2006; 367(9521):1521-1532.
3. WHO. Soil transmitted helminth infections, Fact Sheet. https://www.who.int/news-room/fact-sheets/detail/soil-transmitted-helminth-infections. 8 June, 2020.
4. WHO. https://www.who.int/news-room/fact-sheets/detail/soil-transmitted-helminth-infections. 5 October, 2019.
5. Salam N, Azam S. Prevalence and distribution of soil-transmitted helminth infections in India. BMC Public Health, 2017, 17(201). https://doi.org/10.1186/s12889-017-4113-2.

6. Keiser J, Utzinger J. The drugs we have and the drugs we need against major helminth infections. Advanced Parasitology. 2010; 73(1):197-230.
7. WHO. WHO model list on essential medicines, 2019, 6.
8. Kopp SR, Kotze AC, McCarthy JS, Coleman GT. High-level pyrantel resistance in the hookworm *Ancylostoma caninum*. Veterinary Parasitology. 2007; 143(3-4):299-304.
9. Schougaard H, Nielsen MK. Apparent ivermectin resistance of *Parascaris equorum* in foals in Denmark. Veterinary Record. 2007; 60(13):439-440.
10. Lindgren K, Ljungvall O, Nilsson O, Ljungstrom BL, Lindahl C, Hoglund J. *Parascaris equorum* in foals and in their environment on a Swedish stud farm, with notes on treatment failure of ivermectin. Veterinary Parasitology. 2008; 151(2-4):337-343.
11. Pedrique B, Strub-Wourgaft N, Some C, Olliaro P, Trouiller P, Ford N *et al*. The drug and vaccine landscape for neglected diseases (2000-11): a systematic assessment. The Lancet Global Health. 2013; 1(6):e371-e379.
12. Chopra A, Doiphode V. Ayurvedic medicine: Core concept, therapeutic principles and current relevance. Medical Clinics of North America. 2002; 86(1):75-89.
13. Heinrich M. Ethnobotany and its role in drug development. Phytotherapy Research. 2000; 14(7):479-488.
14. WHO. World Health Organisation Media Centre, Traditional Medicine, 2008, 20.
15. Shakya AK, Sharma N, Saxena M, Shrivastava S, Shukla S. Evaluation of the antioxidant and hepatoprotective effect of Majoon-e-Dabeed-ul-ward against carbon tetrachloride induced liver injury. Experimental Toxicology and Pathology. 2012; 64(7-8):767-773.
16. Moteriya P, Satasiya R, Chanda S. Screening of phytochemical constituents in some ornamental flowers of Saurashtra region. Journal of Pharmacognosy and Phytochemistry. 2015; 3(5):112-120.
17. Ram J, Moteriya P, Chanda S. Phytochemical screening and reported biological activities of some medicinal plants of Gujarat region. Journal of Pharmacognosy and Phytochemistry. 2015; 4(2):192-198.

18. Lalchhandama K. *In vitro* anthelmintic activity of artesunate against the cestode *Raillietina echinobothrida*. International Research Journal of Pharmacy. 2015; 6(4):234-238.

19. Swargiary A, Roy B. *In vitro* anthelmintic efficacy of *Alpinia nigra* and its bioactive compound, astragalin against *Fasciolopsis buski*. International Journal of Pharmacy and Pharmaceutical Sciences. 2015; 7(10):30-35.

20. Gogoi S, Yadav AK. Therapeutic efficacy of the leaf extract of *Croton joufra* Roxb. against experimental cestodiasis in rats. Journal of Parasitic Diseases. 2016; 41(2):417-422.

21. Mengistu G, Hoste H, Karonen M, Salminen JP, Hendriks WH, Pellikaan WF. The *in vitro* anthelmintic properties of browse plant species against *Haemonchus contortus* is determined by the polyphenol content and composition. Veterinary Parasitology. 2017; 15(237):110-116.

22. Rajesh KD, Subramani V, Annamalai P, Rajesh NV, Narayanaperumal J, Ponraj P *et al*. *Gastrothylax crumenifer*: Ultrastructure and histopathology study of *in vitro* nematicidal action of *Marattia fraxinea* (Sm.). Clinical Phytoscience, 2017, 3(3). https://doi.org/10.1186/s40816-016-0039-y.

23. Husori DI, Sumardi, Tarigan H, Gemasih S, Ningsih SR. *In vitro* anthelmintic activity of *Acanthus ilicifolius* leaves extracts on *Ascaridia galli* and *Pheretima posthuma*. Journal of Applied Pharmaceutical Science. 2018; 8(2):164-167.

24. Yadav AK, Tandon V, Rao HSP. *In vitro* anthelmintic activity of root-tuber's extract of *Flemingia vestita* against *Ascaris suum*. Fitoterapia. 1992; LXIII:395-398.

25. Kalmobé J, Ndjonka D, Boursou D, Vildina JD, Liebau E. Phytochemical analysis and *in vitro* anthelmintic activity of *Lophira lanceolata* (Ochnaceae) on the bovine parasite *Onchocerca ochengi* and on drug resistant strains of the free-living nematode *Caenorhabditis elegans*. BMC Complementary and Alternative Medicine, 2017, 17(404). https://doi.org/10.1186/s12906-017-1904-z.

26. Pueblos KRS, Bajalla M, Pacheco D, Ganot S, Paig D, Tapales R *et al*. Comparative anthelmintic activity investigation of selected ethno-medicinal weeds AIP Conference Proceedings, 2017, 1803(020027). https://doi.org/10.1063/1.4973154.

27. Paul A, Adnan M, Majumder M, Kar N, Meem M, Rahman MS *et al*. Anthelmintic activity of *Piper sylvaticum* Roxb. (family: Piperaceae): *In vitro* and in silico studies. Clinical Phytoscience, 2018, 4(17). https://doi.org/10.1186/s40816-018-0077-8.

28. Esther J, Sangeetha N, Balabhaskar R, Gunalan G. *In vitro* anthelmintic activity of *Achyranthes aspera* Linn. (whole plant) against *Pheretima posthuma*. Journal of Research in Siddha Medicine. 2018; 1(1):68-71.

29. Rahman SMM, Islam M, Haque MR, Hossain MI, Atikullah M, Ahammed K *et al*. Cytotoxic and anthelmintic activity of three selected medicinal plants used in Bangladesh. International Journal of Pharmaceutical Sciences Review and Research. 2019; 55(1):114-119.

30. Giovanelli F, Mattellini M, Fichi G, Flamini G, Perrucci S. *In vitro* anthelmintic activity of four plant-derived compounds against sheep gastrointestinal nematodes. Veterinary Sciences. 2018; 5(3):78. https://doi.org/10.3390/vetsci5030078.

31. Vijaya, Yadav AK, Gogoi S. *In vitro* and *in vivo* anthelmintic efficacy of two pentacyclic triterpenoids, ursolic acid and betulinic acid against mice pinworm, *Syphacia obvelata*. Journal of Parasitic Diseases. 2018; 42(1):144-149.

32. Swarnakar G, Kumawat A. *In vitro* anthelmintic effect of *Citrullus colocynthis* on tegument of amphistome *Orthocoelium scoliocoelium* (Trematoda: Digenea). International Journal of Current Microbiology and Applied Science. 2014; 3(6):571-582.

33. Athanasiadou S, Githiori J, Kyriazakis I. Medicinal plants for helminth parasite control: facts and fiction. Animal. 2007; 1(9):1392-1400.

34. Deori K, Yadav AK. Anthelmintic effects of *Oroxylum indicum* stem bark extract on juvenile and adult stages of *Hymenolepis diminuta* (Cestoda), an *in vitro* and *in vivo* study. Parasitology Research. 2016; 115(3):1275-1285.

35. De Jesús-Martínez X, Olmedo-Juárez A, Olivares-Pérez J, Zamilpa A, De Gives PM, López-Arellano ME *et al*. *In vitro* anthelmintic activity of methanolic extract from *Caesalpinia coriaria* J. Willd fruits against *Haemonchus contortus* eggs and infective larvae. BioMed Research International, 2018. https://doi.org/10.1155/2018/7375693.

36. Chitura T, Shiba MR, Afful DB, Shai K, Muvhali PT, Tsotetsi-Khambule AM. *In vitro* anthelmintic activity of seven medicinal plants used to control livestock internal parasites in chief Albert Luthuli

municipality, South Africa. Livestock Research for Rural Development, 2019, 31(2).

37. Gogoi S, Yadav AK. *In vitro* and *in vivo* anthelmintic effects of *Caesalpinia bonducella* (L.) Roxb. leaf extract on *Hymenolepis diminuta* (Cestoda) and *Syphacia obvelata* (Nematoda). Journal of Intercultural Ethnopharmacology. 2017; 5(4):427-433.

38. Irum S, Ahmed H, Mirza B, Donskow-Łysoniewska K, Muhammad A, Qayyum M *et al*. *In vitro* and *in vivo* anthelmintic activity of extracts from *Artemisia parviflora* and *A. Sieversiana*. Helminthologia. 2017; 54(3):218-224.

39. Huang AG, Yi YL, Ling F, Lu L, Zhang QZ, Wang GX. Screening of plant extracts for anthelmintic activity against *Dactylogyrus intermedius* (Monogenea) in goldfish (*Carassius auratus*). Parasitology Research. 2013; 112(12):4065-4072.

40. Yadav AK, Temjenmongla. Efficacy of *Lasia spinosa* leaf extract in treating mice infected with *Trichinella spiralis*. Parasitology Research. 2012; 110(1):493-498.

41. Olayemi DO, Onakpa MM, Jegede OC. Anthelmintic activity of *Hymenodictyon pachyanta* stem bark extracts against *Haemonchus contortus*. Folia Veterinaria. 2019; 63(2):30-36.

42. George B, Kousalya P. *In vitro* anthelmintic efficacy of *Amaranthus dubius*, *Basella alba* and *Cleome gynandra*. Der Pharma Chemica. 2018; 10(6):142-148.

43. Zenebe S, Feyera T, Assefa S. *In vitro* anthelmintic activity of crude extracts of aerial parts of *Cissus quadrangularis* L. and leaves of *Schinus mole* L. against *Haemonchus contortus*. Bio Med Research International, 2017. https://doi.org/10.1155/2017/1905987.

44. Srivastava A, Gupta AK, Rajendiran A. Phytochemical screening and *in vitro* anthelmintic activity of methanolic extract from the stem bark of *Plumeria rubra* Linn. International Journal of Pharmaceutical Sciences and Research. 2017; 8(12):5336-5341.

45. Zabré G, Kaboré A, Bayala B, Katiki LM, Costa-Júnior LM, Tamboura HH *et al*. Comparison of the *in vitro* anthelmintic effects of *Acacia nilotica* and *Acacia raddiana*. Parasite, 2017, 24(44). https://doi.org/10.1051/parasite/2017044.

46. Kumar A, Samantaray S, Das AK. *In vitro* and *in vivo* anthelmintic effect of some medicinal plant parts against *Haemonchus contortus* in goats. Journal of Bio Innovation. 2017; 6(6):931-937.

47. Akther S, Dey AR, Hossain S, Dey TR, Begum N. *In vitro* anthelmintic effect of some medicinal plants against *Haemonchus contortus*. Journal of Animal Science Advances. 2015; 5(1):1162-1170.

48. Sujith S, Sreedevi R, Priya MN, Deepa CK, Darsana U, Sreeshitha SG *et al*. Anthelmintic activity of three Indian medicinal plants. International Journal of Pharmacognosy and Phytochemical Research. 2015; 7(2):361-364.

49. Kundu S, Roy S, Nandi S, Ukil B, Lyndem LM. *In vitro* anthelmintic effects of *Senna occidentalis* (l.) Link (Leguminosae) on rat tapeworm *Hymenolepis diminuta*. International Journal of Pharmacy and Pharmaceutical Sciences. 2015; 7(6):286-371.

50. Khan A, Tak H, Nazir R, Lone BA, Parray JA. *In vitro* anthelmintic and antimicrobial activities of methanolic extracts of *Fumaria indica*. Clinical Microbiology, 2014, 3(161). https://doi.org/10.4172/2327-5073.1000161.

51. James O, God UOT. *In vitro* anthelmintic activity of *Saba florida* (Benth) extracts against Nigerian adult earth worm (*Terrestris lumbricoides*). American Journal of Phytomedicine and Clinical Therapeutics. 2014; 2(6):758-766.

52. Kundu S, Roy S, Lyndem LM. Broad spectrum anthelmintic potential of *Cassia* plants. Asian Pacific Journal of Tropical Biomedicine. 2014; 4(1): S436-S441.

53. Nalule AS, Mbaria JM, Kimenju JW. *In vitro* anthelmintic potential of *Vernonia amygdalina* and *Secamone africana* on gastrointestinal nematodes. Agriculture and Biology Journal of North America, 2013. https://doi.org/10.5251/abjna.2013.4.1.54.66.

54. Partap S, Kumar S, Kumar A, Sharma NK, Jha KK. *In vitro* anthelmintic activity of *Luffa cylindrica* leaves in Indian adult earthworm. Journal of Pharmacognosy and Phytochemistry. 2012; 1(2):27-30.

55. Giri BR, Bharti RR, Roy B. *In vivo* anthelmintic activity of *Carex baccans* and its active principle resveratrol against *Hymenolepis diminuta*. Parasitology Research. 2015; 114(2):785-788.

56. Nath P, Yadav AK. Anthelmintic activity of a standardized extract from the rhizomes of *Acorus calamus* Linn. (Acoraceae) against experimentally induced cestodiasis in rats. Journal of Intercultural Ethnopharmacology. 2016; 5(4):390-395.

57. Yadav AK, Temjenmongla. *In vivo* anthelmintic activity of *Clerodendrum colebrookianum* Walp. A traditionally used tanacidal plant in Northeast India. Parasitology Research. 2012; 111(4):1841-1846.

58. Yadav AK, Tangpu V. Anthelminthic activity of ripe fruit extract of *Solanum myriacanthum* Dunal (Solanaceae) against experimentally induced *Hymenolepis diminuta* (Cestoda) infections in rats. Parasitology Research. 2012; 110(2):1047-1053.

59. Lone BA, Chishti MZ, Bhat FA, Tak H, Bandh SA. Anthelmintic activities of aqueous and methanol extracts of *Prunella vulgaris* L. Natural Products Chemistry and Research. 2017; 5(4):269. https://doi.org/10.4172/2329-6836.1000269.

60. Nath P, Yadav AK. Anticestodal properties of *Hibiscus rosa-sinensis* L. (Malvaceae): an *in vitro* and *in vivo* study against *Hymenolepis diminuta* (Rudolphi, 1819), a zoonotic tapeworm. Journal of Parasitic Diseases. 2016; 40(4):1261-1265.

61. Yadav AK, Temjenmongla, Deori K. *In-vitro* and *in-vivo* anthelmintic effects of *Gynura angulosa* (Compositae) leaf extract on *Hymenolepis diminuta*, a zoonotic tapeworm. Journal of Biologically Active Products from Nature. 2014; 4(3):171-178.

62. WHO. WHO country cooperation strategy, 2006-2011, Supplements on traditional medicine, WHO Country Office for India, New Delhi, 2002, 1-13.

63. Zhu M, Lew KT, Leung P. Protective effect of plant formula on ethanol induced gastric lesions in rats. Phytotherapy Research. 2002; 16(3):276-280.

64. Gesler WM. Therapeutic landscapes: medicinal issues in light of the new cultural geography. Social Science and Medicine. 1992; 34(7):735-746.

65. Unuofin JO, Otunola GA, Afolayan AJ. Toxicity evaluation of *Vernonia mespilifolia* Less (A South Africa medicinal plant) using brine shrimp. Journal of Pharmacology and Toxicology. 2017; 12(2):103-110.

66. Akindele AJ, Oladimeji-Salami JA, Oyetola RA, Osiagwu DD. Sub-chronic toxicity of the hydroethanolic leaf extract of *Telfairia occidentalis* Hook. f. (Cucurbitaceae) in male rats. Medicines (Basel). 2018; 5(1):4. https://doi.org/10.3390/medicines5010004.

67. Bent S, Ko R. Commonly used herbal medicines in the United States: A review. The American Journal of Medicine. 2004; 116(7):478-485.

68. OECD. OECD guidelines for the testing of chemicals. Acute oral toxicity-up-and-down procedure (UDP), 425, Adopted, 2008, 27.

69. OECD. OECD guidelines for the testing of chemicals, repeated dose 28-day oral toxicity study in rodents, 407, Adopted, 2008, 8.

70. Parez BE, Rodriquez OR, Sanchez VMC. Toxic plants: *Brugmansia* (Floripondio) neurotoxicity. Archivos de Medicina de Urgencia de México. 2012; 4(3):119-124.

71. Yang L, Aronsohn A, Hart J, Jensen D. Herbal hepatoxicity from Chinese skullcap: a case report. World Journal of Hepatology. 2012; 4(7):231-233.

72. Ukwuani-Kwaja AN, Abubakar MG, Hassan SW, Agaie BM. Toxicological studies of hydromethanolic leaves extract of *Grewia crenata*. International Journal of Pharmaceutical Sciences and Drug Research. 2012; 4(4):245-249.

73. Konaté K, Bassolé IH, Hilou A, Aworet-Samseny RR, Souza A, Barro N *et al*. Toxicity assessment and analgesic activity investigation of aqueous acetone extracts of *Sida acuta* Burn f. and *Sida cordifolia* L. (Malvaceae), medicinal plants of Burkina Faso. BMC Complementary and Alternative Medicine, 2012, 12(120). https://doi.org/10.1186/1472-6882-12-120.

74. Musa DA, Nwodo FOC, Ojogbane E. Phytochemical, antibacterial and toxicity studies of the aqueous extract of *Eucalyptus camaldulensis* Dehnh. Asian Journal of Plant Science and Research. 2011; 1(3):1-10.

75. Builders MI, Isichie CO, Aguiy JC. Toxicity studies of the extracts of *Parkia biglobosa* stem bark in rats. British Journal of Pharmaceutical Research. 2012; 2(1):1-16.

76. Dutta S, Bhattacharyya D. Enzymatic, antimicrobial and toxicity studies of the aqueous extract of *Ananas comosus* (pineapple) crown leaf. Journal of Ethnopharmacology. 2013; 150(2):451-457.

77. Tarkang PA, Agbor GA, Armelle TD, Yamthe TLR, David K, Ngadena YSM. Acute and chronic toxicity studies of the aqueous and ethanol leaf extracts of *Carica papaya* Linn in Wistar rats. Journal of Natural Product and Plant Resources. 2012; 2(5):617-627.

78. Jothy SL, Zakaria Z, Chen Y, Lau YL, Latha LY, Sasidharan S. Acute oral toxicity of methanolic seed extract of *Cassia fistula* in mice. Molecules. 2011; 16(6):5268-5282.

79. Nath P, Yadav AK, Soren AD. Sub-acute toxicity and genotoxicity assessment of the rhizome extract of *Acorus calamus* L., a medicinal plant of India. European Journal of Pharmaceutical and Medical Research. 2017; 4(8):392-399.

80. Roy B, Dey P. Cytotoxic effects of *Lysimachia ramosa* Wall. Ex Duby (Primulaceae) on Swiss albino mice. Journal of Pharmacognosy and Natural Products, 2016, 2(126). https://doi.org/10.4172/2472-0992.1000126.

81. Sheth S, Tan EC, Tan HH, Tay L. Herb-induced cardiotoxicity from accidental aconitine overdose. Singapore Medical Journal. 2015; 56(7):116-119.

82. Atik N, Muda I, Rahmadi AR, Achadiyani Djunaedy DD. Phytochemical screening and histology appearance of acute oral toxicity study on ethanol extract of *Psidium guajava* Linn. fruit in mice. Asian Journal of Pharmaceutical and Clinical Research. 2019; 12(1):351-355.

83. Bogh HO, Andreassen J, Lemmich J. Anthelmintic usage of extracts of *Embelia schimperi* from Tanzania. Journal of Ethnopharmacology. 1996; 50(1):35-42.

84. Anuracpreeda P, Chawengkirttikul R, Ngamniyom A, Panyarachun B, Puttarak P, Koedrith P *et al*. The *in vitro* anthelmintic activity of the ethanol leaf extracts of *Terminalia catappa* L. on *Fasciola gigantica*. Parasitology. 2017; 143(4):1931-1942.

85. Devi RK, Vasantha S, Panneerselvam A, Rajesh NV, Jeyathilakan N, Venkataramanan R. *Gastrothylax crumenifer*: ultrastructure and histopathology study of *in vitro* trematodicidal effect of *Microlepia speluncae* (L.) Moore. Journal of Applied Animal Research. 2017; 46(1):427-434.

86. De Oliveira RN, Rehder VLG, Oliveira ASS, Montanari Jr I, De Carvalho JE, De Ruiz ALTG *et al*. *Schistosoma mansoni*: *in vitro* schistosomicidal activity of essential oil of *Baccharis trimera* (Less) DC. Experimental Parasitology. 2012; 132(2):135-143.

87. Wink M. Introduction: Biochemistry, Physiology and Ecological Functions of Secondary Metabolites, Annual Plant Reviews. Edn. 2, Blackwell Publishing Limited, 2010, 40.

88. Dash B, Sharma BK. Charak Samhita, Edn 7, Chaukhamba Sanskrit Series, Varanasi (India), 2001.

89. Farnsworth NR. Ethnopharmacology and drug development. Ciba Foundation Symposium. 1994; 185(1):42-59.

90. Ernst E. Toxic heavy metals and undeclared drugs in Asian herbal medicines. Trends in Pharmacological Sciences. 2002; 23(3):136-139.

91. Hoenerhoff MJ, Pandiri AR, Snyder SA, Hong HH, Ton TV, Peddada S *et al*. Hepatocellular carcinomas in B6C3F1 mice treated with *Ginkgo biloba* extract for two years differ from spontaneous liver tumors in cancer gene mutations and genomic pathways. Toxicologic Pathology. 2013; 41(6):826-841.

92. Alonso-Castro AJ, Domínguez F, Ruiz-Padilla AJ, Campos-Xolalpa N, Zapata-Morales JR, Carranza-Alvarez C *et al*. Medicinal plants from North and Central America and the Caribbean considered toxic for humans: The other side of the coin. Evidence Based Complementary and Alternative Medicine, 2017. https://doi.org/10.1155/2017/9439868.

93. Alferah MAZ. Toxicity induced histological changes in selected organs of male (wistar) rats by *Lawsonia inermis* leaf extract. European Journal of Medicinal Plants. 2012; 2(2):151-158.

94. Ughachukwu P, Okonkwo P, Nwozor C, Okafor J, Nwafia I. Effect of aqueous leaf extract of *Ocimum gratissimum* on antiretroviral drug-induced hepatotoxicity in rats. European Journal of Medicinal Plants. 2016; 13(1):1-11.

95. Parasuraman S. Toxicological Screening. Journal of Pharmacology and Pharmacotherapeutics. 2016; 2(2):74-79.

Chapter - 5
Life Cycle, Pathogenesis and Laboratory Diagnosis of Malaria Parasite

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Chapter - 5

Life Cycle, Pathogenesis and Laboratory Diagnosis of Malaria Parasite

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Abstract

Malaria is an infectious vector-borne parasitic disease of the genus plasmodium and transmitted by the bite of infected Anopheles mosquitoes. There are four recognized and distinct species are Plasmodium vivax, Plasmodium falciparum, Plasmodium ovale, Plasmodium malaria. Out of the four species Plasmodium vivax and Plasmodium falciparum are most prevalent in India that infect the human. Plasmodium. vivax malaria is commonly called "benign tertian malaria" and Plasmodium. Falciparum is called malignant tertian. However, a fifth species, Plasmodium knowlesi, has been identified as a cause of human malaria in almost all countries in Southeast Asia and extending to the Nicobar and Andaman Islands in India.

Keywords: malaria, plasmodium vivax, plasmodium falciparum, etc.

Introduction

Malaria is one of the most important infectious diseases in the world and its history extends into antiquity [1]. Malaria or a disease resembling malaria has been noted for more than 4,000 years. From the Italian for "bad air," mal' aria has probably influenced to a great extent of human populations and human history [2]. It was centuries before the true causes of malaria were understood. Previously, it was thought that miasma. (Bad air or gas from swamps-mal-air-ia) caused the disease [3]. The characteristic periodic fever of malaria are recorded from every civilized society from China in 2700 BC through the writings of Greek, Romania, Assyrian, Indian, Arabic and European physicians up to the 19th century following the establishment of the germ theory and the birth of microbiology [1].

Charles Louis Alphonse Laveran, first discovered the parasite on the 6th of November 1880 and was awarded the Nobel Prize in 1907 for his discovery. Italian neurophysiologist named Camillo Golgi, described in detail two species (P. vivax and P. falciparum) of human malaria parasites in 1880 and

got a Nobel Prize in 1906. Then in 1890 P. vivax and P. falciparum were named by Giovanni Batista Grassi and Raimondo Filetti. In 1897 William H. Welch named the P. falciparum. John William and Watson Stephens described the fourth human malaria parasite P. ovale in 1922 [2]. Romanowsky developed a staining method for demonstrating plasmodia in blood smear in 1891. The mosquito transmits malaria parasites were first demonstrated by Ronald Ross in 1897 and got Nobel Prize in 1902 for his discovery [4].

Then in 1898 Italian investigator Grassi, Bignonia and Bastianelli described the mosquito cycle of human malaria parasites. In 1900 Patrick Manson confirmed the theory of mosquito transmission [4].

Shortt, Granham, Covell and Shute described pre-erythrocytic forms of P. vivax in 1948 and pre-erythrocytic forms of P. falciparum in the human liver in 1949. The pre-erythrocytic stage of P. ovale was discovered in 1954 by Granham [5].

However, a fifth species, Plasmodium knowlesi, has been identified as a cause of human malaria in almost all countries in Southeast Asia [6] and extending to the Nicobar and Andaman Islands in India [7]. There are about 380 species of Anopheles mosquito, but only 60 or more can transmit the parasite Malaria is a life-threatening blood disease caused by parasites transmitted to humans through the bite of the Anopheles mosquito. Once an infected mosquito bites a human and transmits the parasites, those parasites multiply in the host's liver before infecting and destroying red blood cells [8].

Epidemiology of malaria

Geographical distribution

Of the four species of plasmodium that cause human malaria: P. falciparum and P. vivax are Widespread and P. malariae and P. ovale are less widespread [9]. Plasmodium falciparum is found mainly in the hotter and more humid regions: tropical and sub-tropical parts of Africa and parts of Central America and South America, Bangladesh, Pakistan, Afghanistan, Nepal, Sri Lanka, South East Asia, Indonesia, Islands of Melanesia, parts of India, Middle East and the eastern Mediterranean. Plasmodium vivax is mainly found in South America, Mexico, Middle East, Northern Africa, India, Pakistan, Sri Lanka, Papua New Guinea, Solomon Islands and is also found in parts of South East Asia, Indonesia, Philippines, Madagascar, tropical and subtropical Africa, Korea and China. Plasmodium malariae is found in tropical and subtropical regions: Guyana, India, Sri Lanka, and Malaysia. Plasmodium ovale has a restricted distribution and low prevalence. It is mainly in West Africa, Philippines, Indonesia, China and parts of the Far East, South East Asia, and South America [10].

Environmental factors: Several environmental elements such as temperature, humidity, rainfall, altitude influence malaria epidemiology.

Temperature: The ideal temperature for the development of the malaria parasite is between 20 to 300C with a relative humidity of 60% [11].

Rainfall and Altitude: Rain increases atmospheric humidity and breeding places for mosquitoes. However, heavy rain may hurt flushing out the breeding places. Anopheles mosquitoes are not found at altitudes above 2000-2500 meters [12].

Vector: For transmission of malaria female Anopheles mosquito acts as a vector. There are about 380 species of Anopheles mosquitoes. Only 60 or more species act as the vector [3]. Anopheles mosquitoes are found worldwide except Antarctica. The success of the development of the malaria parasite in the mosquito depends on several factors. The most important is ambient temperature and humidity [2].

The vector in India

Of the 58 Anopheles species in India, only six are epidemiologically important for malaria transmission with regional distribution, though other species may be key local vectors. Each species exhibits specific behavior and preferred habitat. Multiple vector species may be present across any region, but no vector species is found throughout all of India.

Anopheles culicifacies is the main malaria vector in rural, peri-urban areas and the plains, and is Responsible for an estimated 65% of malaria in India. This species is mostly zoophagic and breeds in the plain-land ecosystem.

Anopheles stephensi is the key vector for malaria in urban areas, primarily zoophagic, but prefers human hosts in the absence of cattle. Curing waters in construction sites are a primary breeding area for this vector.

Anopheles dirus is an efficient vector in the forest areas of north-eastern regions. It is exophagic and exophilic and breeds in temporary water collections.

Anopheles minimus is also a vector in the forest areas of north-eastern regions, exhibiting zoophilic and exophilic behavior, breeding in slow-flowing streams.

Anopheles fluviatilis is associated with hill and foothill areas, contributing 15% of malaria transmission in India.

Anopheles sundaicus breeds in brackish water and is the main malaria vector in Andaman and Nicobar Islands [13].

The life cycle of the malarial parasite

Malaria parasite passes its life cycle in 2 hosts.

Definitive host: Female Anopheles mosquito.

Intermediate host: Man.

The life cycle of the malarial parasite comprises 2 stages-an asexual phase occurring in humans and a sexual phase occurring in the mosquito, which serves as a definitive host for the parasite.

Asexual phase: In this stage, the malaria parasite multiplies by division or splitting a process known as Schizogony (from schizo: to split, and gone: generation). Because this asexual phase occurs in man, it is also called the vertebrate, intrinsic, or endogenous phase. In humans, schizogony occurs in two locations-in the red blood cell (known as erythrocytic schizogony) and in the liver cells (known as exoerythrocytic schizogony or the tissue phase). Because schizogony in the liver is an essential step before the parasites can invade erythrocytes, it is called pre-erythrocytic schizogony. The products of schizogony, whether erythrocytic or exoerythrocytic, are called merozoites (meros: apart, zoon: animal).

Sexual phase: The sexual phase takes place in the female Anopheles mosquito, even though the sexual forms of the parasite (known as gametocytes) originate in human red blood cells. Maturation and fertilization take place in the mosquito, giving rise to a large number of sporozoites (from sporos: seed). Hence this phase of sexual multiplication is called sporogony. It is also called the invertebrate, extrinsic, or exogenous phase.

Human cycle (Schizogony)

Human infection takes place by the inoculation of sporozoites through the bite of the infective female Anopheles mosquito to man (Figure. 1.1). The sporozoites are infective forms of the parasite which are present in the salivary gland of the infected mosquito. They are injected into blood capillaries when the mosquito feeds on blood after piercing the skin. Usually, 10-15 sporozoites are injected at a time, but occasionally, many hundreds may be introduced. Then sporozoites pass into the bloodstream, where many are destroyed by the phagocytes, but some reach the liver and enter the parenchymal cells (hepatocytes).

Pre-erythrocytic cycle

During the bite of an infected female mosquito, sporozoites are released into the circulating blood of the host. Within 30 to 45 minutes, sporozoites

enter hepatocytes where growth and division in the liver for the human malaria parasites take approximately 6 to 15 days depending on the species: approximately 6, 10, and 15 days for P. falciparum, P. vivax, and P. ovale and P. malariae, respectively. In P. vivax and P. ovale, some of the sporozoites appear to develop for about 24 hours before becoming dormant as a hypnozoite stage; this form can remain as such for months and even years until reactivated to complete the liver cycle, releasing merozoites into the blood to precipitate a relapse infection [14].

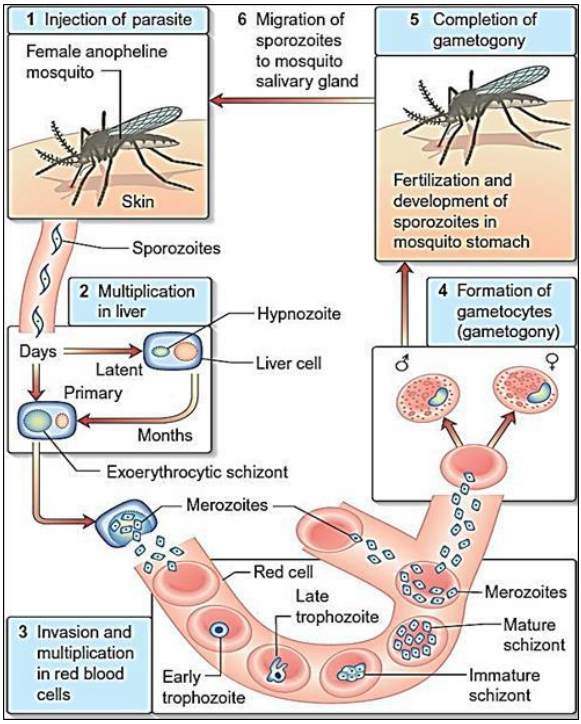

Fig 1.1: A schematic life cycle of Plasmodium vivax

Source: Kumar & Clark's Clinical Medicine, 7th Edition

Erythrocytic cycle

At the end of the pre-erythrocytic cycle, thousands of merozoites are released into the blood and within 15 to 20 seconds, attach or invade erythrocytes through a receptor, glycophorin, which is a major glycoprotein present on the red blood cells. The differences in the glycophorins of red cells of different species may account for the species specificity of malaria parasites. The asexual erythrocytic cycle produces more merozoites that are released with the destruction of the red blood cell after 48 or 72 hours for the

human malaria parasites, depending on the species, and which then immediately invade additional erythrocytes. In the erythrocyte, the merozoite loses its internal organelles and appears as a rounded body having a vacuole in the center with the cytoplasm pushed to the periphery and the nucleus at one pole. These young parasites are, therefore called the ring forms or young trophozoites. The parasite feeds on the hemoglobin of the erythrocyte. It does not metabolize hemoglobin completely and therefore, leaves behind a hematin-globin pigment called the malaria pigment or hemozoin pigment, as residue. As the ring form develops, it enlarges in size becoming irregular in shape and shows amoeboid motility. This is called the amoeboid form or late trophozoite form. When the amoeboid form reaches a certain stage of development, its nucleus starts dividing by mitosis followed by a division of the cytoplasm to become mature schizonts or meronts. A mature schizont contains 8-32 merozoites and hemozoin. The mature schizont bursts releasing the merozoites into the circulation. The merozoites invade fresh erythrocytes within which they go through the same process of development. This cycle of erythrocytic Schizogony or merogony is repeated sequentially. The asexual cycle usually continues until controlled by the immune response or chemotherapy or until the patient dies (in the case of P. falciparum). After invading red blood cells, eventually, some merozoites differentiate into sexual forms called gametocytes [14]. The rupture of the mature schizont releases large quantities of pyrogens. This is responsible for the febrile paroxysms characterizing malaria. The interval between the entry of sporozoites into the host and the earliest manifestation of clinical illness is the incubation period. This is different from the prepatent period, which is the time taken from the entry of the sporozoites to the first appearance of the malaria parasite in peripheral blood. In P. falciparum, erythrocytic schizogony always takes place inside the capillaries and vascular beds of internal organs. Therefore, in P. falciparum infections, schizonts, and merozoites are usually not seen in the peripheral blood. The erythrocytic stages of all the 4 species of Plasmodium are shown in Figure. 1.2.

Gametogony

After a few erythrocytic cycles, some of the merozoites that infect RBCs do not proceed to become trophozoites or schizonts but instead, develop into sexually differentiated forms, the gametocytes. They grow in size until they almost fill the RBC, but the nucleus remains undivided. Development of gametocytes generally takes place within the internal organs and only the mature forms appear in circulation. The mature gametocytes are round in shape, except in P. falciparum, in which they are crescent-shaped.

In all species, the female gametocyte is larger (macro-gametocyte) and has cytoplasm staining dark blue with a compact nucleus staining deep red. In the smaller male gametocyte (microgametocyte), the cytoplasm stains pale blue or pink and the nucleus is larger, pail stained, and diffuse. Pigment granules are prominent. Female gametocytes are generally more numerous than the male. Gametocyte appears in circulation 4-5 days after the first appearance of asexual form in the case of P. vivax and 10-12 days in P. falciparum. A person with gametocytes in the blood is a carrier or reservoir. The gametocytes do not cause any clinical illness in the host but are essential for the transmission of the infection. A gametocyte concentration of 12 or more per cumm of blood in the human host is necessary for mosquitoes to become infected.

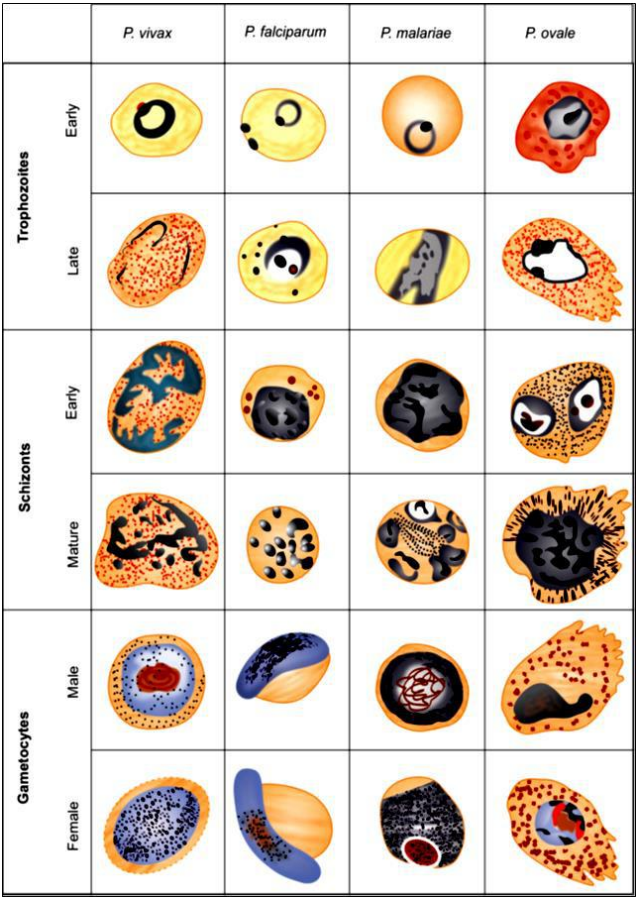

Fig 1.2: Malaria parasites-Erythrocytic stages of the four species

Source: Paniker's Textbook of Medical Parasitology

Mosquito cycle

After ingestion by another female mosquito, gametocytes will mature to male and female gametes. After fertilization, the resulting zygote matures within 24 hours to the motile ookinete, which burrows through the midgut wall to encyst on the basal lamina, the extracellular matrix layer separating the hemocoel from the midgut. Within the developing oocysts, many mitotic divisions are resulting in oocysts full of sporozoites. Rupture of the oocysts releases the sporozoites, which migrate through the hemocoel to the salivary glands to complete the cycle approximately 7 to 18 days after gametocyte ingestion, depending on host-parasite combination and external environmental conditions [14]. All stages in the life cycle are haploid, apart from the diploid zygote, which immediately after fertilization undergoes a two-step meiotic division, the resulting cell containing a nucleus with four haploid genomes. The sexual process and meiotic division following fertilization allow genetic recombination, which is reflected in the genetic makeup of the sporozoites and together with mutations provides the raw material upon which selective pressures such as antimalarial drugs can work [14].

Transmission: Malaria parasites are usually transmitted to humans by the bite of female Anopheles mosquito [3]. The parasite can also be transmitted during a blood transfusion, organ or tissue transplants or needle stick injuries, [15-16].

Prepatent period: This is the interval between the bite of an infected mosquito and parasitemia. Prepatent period for species are as follows: P. falciparum, 9-10 days; P. vivax, 8-13 days; P. ovale, 9-14 days; and P. malariae, 15-16 days [16].

Incubation period: The interval between infection and the first symptom appears is termed as the incubation period. During the incubation period, patients are asymptomatic. The usual incubation periods for different species are as follows: P. falciparum, 7-14 days; P. vivax, 12-17 days; P. ovale, 15-18 days; and P. malariae, 18-40 days [17]. The incubation period is shorter when infection results from a blood transfusion, organ or tissue transplants, or needle stick injuries because no preliminary hepatic cycle occurs before infection of erythrocytes [16].

Vivax malaria: P. vivax has the widest geographical distribution, extending through the tropics, subtropics and temperate regions. This type of malaria is less severe than P. falciparum malaria. It may cause severe anemia. Despite its uncomplicated course, it requires special attention for two reasons. First, diagnosis often is complicated by the late onset of symptoms. Second,

case management is complicated by the fact that parasites can remain dormant in the liver as hypnozoites. Thus, even if blood stages of the parasite are cleared, reactivation of these life forms may cause relapses within a few months [18]. It may cause death as a result of the ruptured spleen or in association with reticulocytosis and high parasitemia after anemia [24]. All stages of erythrocytic schizogony can be seen in peripheral smears (Fig. 1.3).

Falciparum malaria: The name falciparum comes from the characteristic sickle shape of the gametocytes of this species (falx: sickle, parere: to bring forth). This is the most severe form of malaria and is associated with more intense parasitemia hence, the name malignant tertian or pernicious malaria. The fatality rate is up to 25% in non-immune adults and 30% in non-immune infants may occur if appropriate therapy is not instituted promptly [19]. Infection with P. falciparum is not limited to RBCs of a particular age and hence represents the highest level of parasitemia. The infected erythrocytes are of normal size. The early ring form in the erythrocyte is very delicate and tiny, measuring only a sixth (1/6) of the red cell diameter. Rings are often seen attached along the margin of the red cell, the so-called form appliqué, or accole. Binucleate rings (double chromatin) are common. Several rings may be seen within a single erythrocyte. In course of time, the rings become larger, about a third of the size of the red cell, and may have 1 or 2 grains of pigment in its cytoplasm. The subsequent stages of the asexual cycle-late trophozoite, early and mature schizonts-are not ordinarily seen in peripheral blood, except in very severe or pernicious malaria. The erythrocytic schizogony takes about 48 hours or less so that the periodicity of febrile paroxysms is 36-48 hours. They show a few (6-12) coarse brick-red dots which are called Maurer's clefts. Some red cells show basophilic stippling. The mature gametocytes, which are seen in peripheral smears are curved oblong structures, described as crescentic, sickle, sausage, or banana-shaped. They are usually referred to as crescents. The male gametocytes are broad and sausage-shaped or kidney-shaped, with bluntly rounded ends in comparison to the female gametocytes, which are thinner and more typically crescentic, with sharply rounded or pointed ends. The cytoplasm in the male it is pale blue or pink while in the female, gametocyte is deep blue. The nucleus is pink, large and diffuse, with the pigment granules scattered in the cytoplasm in male gametocyte, while it is deep red and compact with the pigment granules closely aggregated around it. Falciparum crescents can survive in circulation for up to 60 days, much longer than in other species. Gametocytes are most numerous in the blood of young children (9 months to 2 years old). This species also causes vascular obstruction due to its ability to adhere to endothelial cell walls. This property leads to most complications of P.

falciparum infection. Severe falciparum malaria is associated with cerebral malaria, blackwater fever, severe anemia, hypoglycemia, acidosis, renal failure, pulmonary edema, hypotension, hemostatic normalities, jaundice and hepatic dysfunction, and cardiovascular collapse shock [10, 16, 20].

Plasmodium malariae: Malariae malaria was formerly known as quartan malaria. In Africa, it accounts for up to 25% of Plasmodium infections and often accompanies infection with P. falciparum. There is no dormant hypnozoites stage in P. malariae and thus no relapses [21]. This is the mildest and most chronic of all malaria. It causes quartan malaria, in which febrile paroxysms occur every fourth day, with 72 hours' interval. Recrudescence may be provoked by splenectomy or immunosuppression. P. malariae preferentially infects older erythrocytes and the degree of parasitization is low. The ring forms resemble those of P. vivax, although thicker and more intensely-stained. The old trophozoites are sometimes seen stretched across the erythrocyte as broadband. These band forms are a unique feature of P. malariae. The gametocytes develop in the internal organs and appear in the peripheral circulation. The male gametocyte has pale blue cytoplasm with a large diffuse nucleus, while the female gametocyte has deep blue cytoplasm and a small compact nucleus.

Ovale malaria: Malaria caused by P. ovale is referred to as ovale malaria and was previously known as ovale tertian malaria because of the presence of an oval shape with a fimbriated margin. This oval appearance of the infected erythrocyte is the reason for the name ovale given to this species. Like P. vivax it is also a relapsing species [21]. The schizonts resemble those of P. malariae, except that the pigment is darker and the erythrocyte is usually oval, with prominent Schuffner's dots. Of all types of malaria, this is the least common. This is commonly found in conjunction with P. falciparum infection [19].

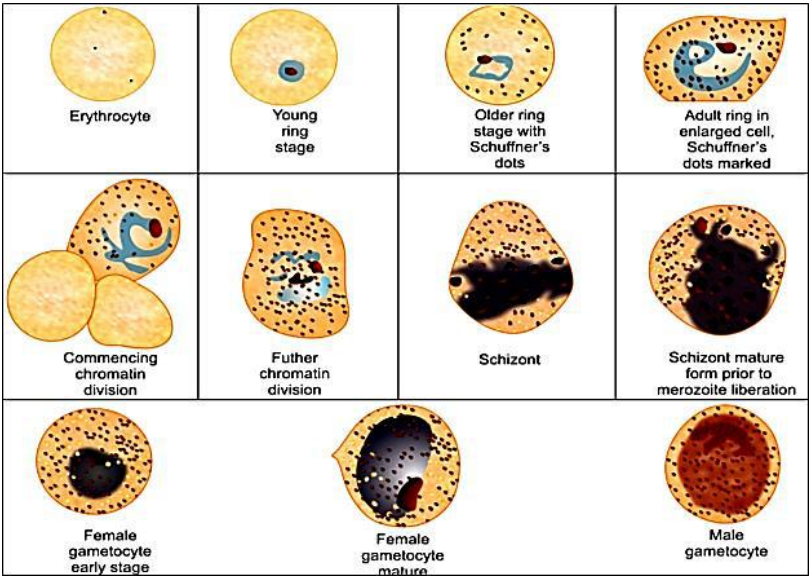

Fig 1.3: Plasmodium vivax (Morphological forms present in Blood)

Source: Paniker's Textbook of Medical Parasitology.

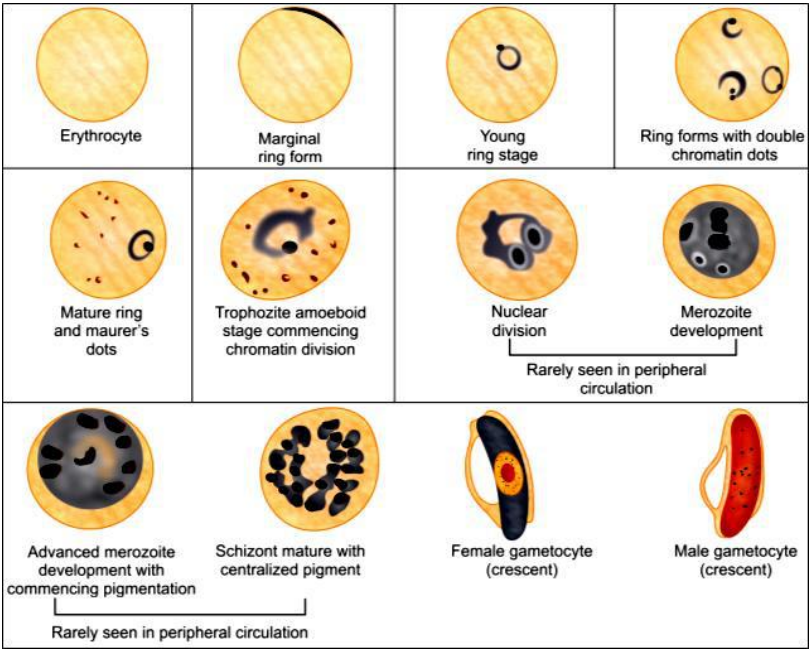

Fig 1.4: Morphological forms of Plasmodium falciparum

Source: Paniker's Textbook of Medical Parasitology

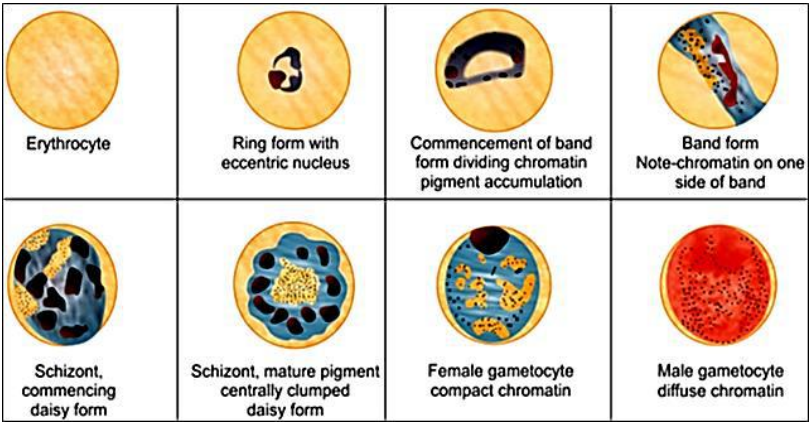

Fig 1.5: *Plasmodium malariae* stages of erythrocytic schizogony

Source: Paniker's Textbook of Medical Parasitology

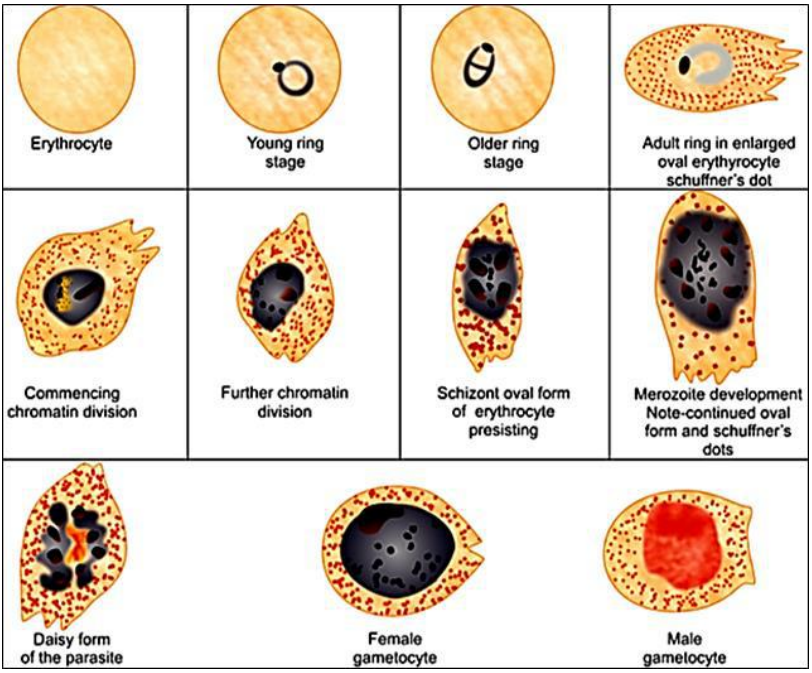

Fig 1.6: *Plasmodium ovale* stages of erythrocytic schizogony

Source: Paniker's Textbook of Medical Parasitology

Pathogenesis of malaria

Virulence factors: Plasmodium falciparum is one of the world's most devastating pathogens. It has an astonishing array of sequences and genes that

play key roles in pathogenesis and immune evasion. Several factors such as PfEMP1, RIFIN protein, stevor antigen, hemozoin, Glycosyl phosphatidylinositol act as virulence factors in the pathogenesis of malaria.

P. falciparum erythrocyte membrane protein 1 (PfEMP1)

Virulence of plasmodium falciparum is associated with the expression of variant surface antigens designated PfEMP1 that are encoded by a family of var genes [22]. These PfEMP1 proteins are high molecular weight proteins that are transported to the surface of the infected red cell, where they have been demonstrated via CD36 and to uninfected red cells via complement receptor1 and heparan sulfates. Several vascular receptors have been identified of which intracellular adhesion molecule (ICAM-1) is probably most important in the brain, chondroitin sulfate B in the placenta and CD36 in most other organs. Indirect evidence also suggests that PfEMP1 is the ligand that binds to intercellular adhesion molecule [23].

RIFIN protein: In the genome sequence of Plasmodium falciparum, other unique multi-copy gene families have been identified. The largest of which belongs to the Rif (repetitive interspersed family) gene family [24]. This gene family encodes clonally variant proteins (RIFINS) that are expressed on the infected red cell.

Stevor antigen: The third family of variant proteins comprises stevor antigens. Although stevor genes are located in the tandem with Rif and var genes, they seem to be much more conserved among strains than the Rif and var genes. The stevor genes (30 to 40 copies per haploid genome) have a two-exon structure similar to that of Rif genes and code for 30 to 40 kDa proteins with a rather short intracellular domain that are expressed over a brief period by mature trophozoites and possibly by sporozoites and gametocytes as well [25].

Others: The hemozoin (malaria pigment) has been implicated in the modulation of immune responses during malaria infection [26]. Glycosyl phosphatidylinositols are the anchor molecules of some membrane proteins of plasmodium species that have also been implicated in the induction of TNFá and IL-1 during malaria infection [27-28].

Virulence events

Cytoadherence: It is an ability of parasites to adhere to the vascular endothelium, was recognized as early as 1892 by Marchiafava and Bignami. Mature forms of parasites (asexual stage and gametocytes) can adhere to the vascular endothelium of several organs (lung, heart, brain, lung, liver, and

kidney), the subcutaneous adipose tissues and the placenta. This feature of the disease *in vivo* has been related exclusively to P. falciparum [29].

Sequestration: It occurs principally during the second half of the intra-erythrocytic asexual growth phase of the parasite, following adherence [30]. Sequestration is the characteristic feature of infection with P. falciparum. This is the process involving the accumulation of large numbers of parasitized erythrocytes in various organs. It is mediated by adhesive interactions between parasite ligands on the surface of the infected erythrocytes (IEs) and host molecules present on microvascular endothelium [31]. Sequestration is facilitated by the expression of knob-like productions under the surface of the infected RBC membrane [32].

Rosetting: Erythrocytes containing mature parasites, in addition to adhering to the endothelial cells and syncytiotrophoblast, mature-stage parasitized RBCs (pRBCs) can also adhere to the uninfected RBCs. This process leads to the formation of rosettes when suspensions of parasitized erythrocytes are viewed under the microscope [33].

Deformability/RBC changes: As the parasite matures inside the erythrocyte, the normally flexible biconcave disc becomes progressively more spherical and rigid. The deformability results from reduced membrane fluidity, increasing sphericity, and the enlarging and relatively rigid intra-erythrocytic parasite [33]. The membrane fluidity of the pRBCs is much lower than that of uninfected cells, and this renders pRBCs less flexible, more liable to damage in the circulation, and more susceptible to splenic clearance. However, uninfected RBCs also show alterations in their rheological characteristics during malaria which may also render them more susceptible to damage or splenic clearance [16]. The differences between parasite-infected and uninfected RBCs are great such as the mechanical properties of pRBCs are changed. The membrane is less flexible, which makes it difficult for the cell to pass through the microvasculature, parasite nutrients: carbohydrates, amino acids, and purine bases are transported into the cells. Furthermore, some pRBCs membrane components are digested or modified. Most interestingly several parasite-derived polypeptides, including PfMP1 and rosettins/RIFINs are inserted into and then protrude from the membrane. All these parasite-derived polypeptides significantly change the nature of the RBC membrane [34].

Immunity to malaria: Both innate and acquired immunity plays an important role in malaria. Initially, the host responds to Plasmodium infection by activating the non-specific defense mechanism [35].

Innate immunity: Innate immunity is thought to play a crucial role in clearing parasite from an infected host. Most of the elimination occurs in the spleen under normal circumstances. Although the liver has been shown to function as an alternative clearing site. Within the spleen, this task is assumed by macrophages of the red pulp [28]. Several inherited alterations in red blood cells give partial immunity to malaria in some populations. It seems to clear that Sickle cell disease, G6PD deficiency, thalassemia, and other hemoglobinopathies provide some protection against lethal levels of falciparum infection. People who are heterozygous for the sickle cell trait (HbS), they are less likely to die from P. falciparum infection. HbS trait causes the parasites to grow poorly or die at low oxygen concentrations, perhaps because of low potassium levels caused by potassium efflux from red blood cells on hemoglobin sickling. HbC protects against severe malaria by reducing parasite proliferation. Individuals with the HLA B-53 are resistant to Plasmodium falciparum because HLA B-53 presents liver stage-specific antigens to cytotoxic T cells [36]. Another red cell deformity is ovalocytosis, which confers a reduced risk of infection with P. falciparum and P. vivax malaria. Due to the absence of the Duffy antigen (Fy Fy) people become resistant to P. vivax infection, because this antigen acts as a receptor for P. vivax malaria [37].

Acquired immunity: Both cellular and humoral immunity is involved in the process of acquired immunity [3]. Individuals who are repeatedly exposed to malaria develop antibodies against sporozoites, liver stage, blood-stage, and sexual stage of parasite antigens. It is thought these antibodies are responsible for the decreased susceptibility to malaria infection and disease [38]. Passive transferred IgG from mother to fetus Contributes to the relative protection of infants from severe malaria in the first months of life. Another type of acquired immunity has been observed that appears to depend upon the presence of low-level parasitemia that somehow inhibits new infections on maintains the infection at a non-symptomatic level. This is called premonition. This type of immunity is soon lost after the parasites disappear from the blood [20].

Immunity to the pre-erythrocytic stage: Following the bite of the female mosquito, sporozoites circulate in the bloodstream for a very brief period. Some of the parasites invade hepatocytes, others being filtered out by a variety of non-specific mechanisms. At this stage, the parasite would be susceptible to an antibody-mediated attack directed to components on the surface of the sporozoites. Such antibody could potentially exert its protective effect by any one of a variety of mechanisms, including opsonization, complement-mediated lysis, or neutralization. Once the invasion of

hepatocytes has taken place HLA B-53 molecules present processed antigen to the cytotoxic T cell. Then the parasites are killed by either direct lysis or by the range of soluble mediators [39].

Erythrocytic stage: Once inside the red cells, the parasites appear well-positioned to avoid host responses, but there are several gaps in its defenses. When parasites mature inside RBC it induces a series of morphological, functional antigenic changes in the host red cell membrane. Some changes are a result of an alteration of host constituents, but others result from the parasite inserting its molecules into the host cell membrane. The host`s immune system acts against these neoantigens. Cytokines produced by immune cells play an important role in this stage [39].

Natural killer cells: Natural killer (NK) cells derived from pluripotent hematopoietic stem cells are important cells of the immune system that have two main functions: a cytolytic activity and a cytokine-producing capacity. New insights into NK cell biology have suggested their major roles in the control of infections, particularly in P. falciparum infection. It was also observed that during pregnancy cell function might be altered due to the production of hormones and other pregnancy regulatory factors. A causal relationship between high cortisol levels and depressed NK cell cytotoxicity against P. falciparum parasitized erythrocytes and susceptibility to malaria has been demonstrated [40].

T cell: Both cellular and humoral arms of the adaptive immune system are pivotal elements in the eradication of plasmodium from the body, and both are critically dependent on α/β CD4+ lymphocytes. Both the Th1 and Th2 subsets of CD4+ T cells have regulatory functions in human malaria [28]. CD8+ T cells have been implicated as critical effects or cells in protection against pre-erythrocytic stage malaria. It is established that role CD8+ T cells is dependent not only on IFN-ã and NO but also on IL-12 and in part on NK cells [41].

Clinical features

Symptoms and signs: Common symptoms and signs of malaria are: fever, chill, sweating, headache, nausea, vomiting, body aches, generalized malaise, elevated temperature, perspiration, weakness, and an enlarged spleen. In P. falciparum malaria additional findings may include mild jaundice, hepatomegaly, tachypnoea [2]. Classical malaria fever starts with a sudden inappropriate feeling of cold. Mild shivering quickly turns into violent teeth chattering and shaking of the whole body. The skin is cold, dry, pale, cyanosed, and goose-pimpled. The pulse is rapid and low volume. Rigor lasts

for 15-60 minutes. After which shivering ceases, the patient feels some waves of warmth and the hot stage ensues. In this stage, the patients become unbearably hot. Other features are: throbbing headache, palpitation, tachypnoea, prostration, epigastric discomfort, nausea, vomiting and thirst develop as the temperature reaches its peak of 40-410C or more. During this stage, the patient may become confused or delirious. This stage lasts for 2 to 6 hours. In the sweating stage, the patient breaks out into a profuse, drenching sweat. The fever declines over the next 2-4 hours.

Complications of malaria

Cerebral malaria: Cerebral malaria is an acute febrile and mainly diffuse encephalopathy, occurring in a patient infected with P. falciparum. The pathogenesis of cerebral malaria is heterogeneous and neurological complications are often part of a multisystem dysfunction. The clinical presentation and pathophysiology differ between adults and children [42]. Cerebral malaria is the most severe and common complication affecting up to 7% of all P. falciparum malaria cases and with mortality rates up to 50%. Initially, patients often complain of nonspecific symptoms, even days before the onset of the paroxysm: malaise, headache, myalgia, and fatigue are easily mistaken for beginning viral illness. Children usually experience an abrupt onset, frequently with hyperpyrexia, headache, restlessness, and vomiting [43]. Within hours but sometimes much slower features of CNS involvement set in. In malignant falciparum malaria, however, an asynchronous cycle of parasite multiplication leads to continuous, remittent, or irregular. Unarousable coma may be preceded by severe headache, confusion, drowsiness, and in many instances convulsions. Meningeal irritation is rare as extrapyramidal and cerebellar signs, retinal hemorrhage, and exudates are infrequent. Hyper pyrexia, splenomegaly is frequently physical findings. Hepatomegaly is less often observed. In advanced disease, severe anemia, icterus, renal failure, acute pulmonary edema, heart failure, bleeding tendencies, spontaneous hemorrhages and hypoglycemia complicate the course of P. falciparum infection.

Lactic acidosis: Commonly co-exists with hypoglycemia in malaria patients. It is an important contributor to death from severe malaria. The prognosis of lactic acidosis is poor [20]. The most common clinical sign, deep (Kussmaul) breathing, occurs in nearly two-thirds of patients with acidosis. Acidaemia and hyperlactatemia may also be present [44].

Renal impairment: It is more common among adults than children with severe falciparum malaria. It may be due to erythrocyte sequestration interfering with renal microcirculatory flow and metabolism [16].

Hematologic abnormalities: Common hematologic abnormalities are thrombocytopenia (platelet count<150x109/L) occurs in up to 70% of patients and anemia in 25% patient. The leukocyte count is normal or low. Leukocytosis is seen in less than 5% of cases and is a poor prognostic factor [45].

Liver dysfunction: Jaundice is more common among adults than children and results from hemolysis, hepatocyte injury, and cholestasis. Liver dysfunction carries a poor prognosis [20].

Hyper reactive malarial splenomegaly: Hyper-reactive malarial splenomegaly (HMS) represents one of the leading causes of massive splenomegaly in malaria-endemic countries. HMS is caused by an aberrant immune response to a chronic antigenic stimulation in subjects long exposed to malaria parasites [46].

Blackwater fever: Is a manifestation of falciparum malaria occurring in previously infected subjects and is characterized by sudden intravascular hemolysis followed by fever and hemoglobinuria. It is associated with infection by Plasmodium falciparum, most commonly observed among the non-immune (nonindigenous) individuals who have resided in malaria-endemic countries previously for 6 months to 1 year and have had inadequate doses of quinine for both suppressive prophylaxis and treatment of repeated clinical attacks. In these cases, quinine often acts as a precipitating factor. Other factors that have been known to precipitate an attack of blackwater fever are cold, exposure to the sun, fatigue, trauma, pregnancy, and parturition and X-ray treatment of the spleen [5].

Malaria in pregnancy: During pregnancy, malaria is an important cause of maternal and infant morbidity and mortality. The impact of malaria during pregnancy depends on levels of pre-existing malarial immunity, the intensity, and stability of malaria transmission and parity [31]. Erythrocytes infected with P. falciparum congregate in the maternal placental vascular space where the sinusoidal and low-pressure blood flow, and possibly parasite adherence to endothelial cells, allow parasites to sequester and replicate. An active immune response involving antibody production, cytokine release, and cellular response is frequently observed in malaria-infected placentas. The infection and possibly, aspects of the immune response contribute to poor pregnancy outcomes of prematurity and intrauterine growth retardation [47].

Malaria in children: Malaria is still a major cause of severe disease, which is responsible for millions of deaths, mostly in children under 5 years old in tropical countries, especially sub- Saharan Africa [48]. Children with

malaria often have special clinical features that differ from those of adults. The age-related differences in specific clinical manifestations are likely due to multiple factors, including differential parasite organ. Sequestration, low levels of complement regulatory proteins leading to increased red cell destruction, inadequate reticulocyte production, and possibly the need for exposure to specific strains in cerebral malaria [49]. Symptoms of malaria in children >2 months of age that are non-immune vary widely from low-grade fever to >1040F. Apart from fever, other features include headache, drowsiness, hepatosplenomegaly, anemia, anorexia, nausea, vomiting, diarrhea, thrombocytopenia, and a normal or low white blood cell count. Recurrent infection in children in the endemic area may result in malnutrition and subsequent growth retardation. Fever with convulsion is an important clinical presentation of acute malaria as well as cerebral malaria in non-immune and semi-immune children below two years of age.

Differential diagnosis of malaria

The clinical manifestations of malaria are nonspecific and may be present in a variety of other febrile illnesses. Common infections such as influenza, viral gastroenteritis, viral hepatitis, encephalitis, meningitis may present similarly. Other conditions such as typhoid fever, relapsing fever, tuberculosis, pyelonephritis, yellow fever, brucellosis, amoebic liver abscess, etc. may also be included in the differential diagnosis. Non-infectious causes of fever similar to malaria are Hodgkin disease and drug-induced fever with hemolysis [35-36].

Laboratory diagnosis of malaria

Several approaches are available for the diagnosis of Malaria, which includes:

- Clinical diagnosis
- Microscopic examination of thin and thick blood film
- Microscopic examination of Centrifuged buffy coat smear
- Fluorescent microscopy (Quantitative Buffy Coat examination)
- Polymerase chain reaction (PCR)
- Malarial antigen and antibody-based rapid diagnostic tests (RDT)

The latest automated hematology analyzers can flag suspicious samples by detecting malarial pigment in the white blood cells but still requires confirmation by another method [50]. Diagnosis of malaria based on clinical grounds alone is unreliable and should be confirmed by laboratory tests. The

accurate diagnosis of malaria depends on the demonstration of parasites in stained blood smears. The microscopic examination of blood film is regarded as the gold standard method.

Microscopic examination of thin and thick blood film

Leishman, Giemsa, or Romanowsky's stained peripheral blood smear examination remains the gold standard for the diagnosis of malaria in malaria-endemic countries. For best results, the smear should be stained with a 3% Giemsa solution (pH of 7.2) for 30-45 minutes. The parasite detection threshold for thin blood film is 100 parasites/μL. For the thick film, the threshold is lower about 5-20 parasites/μL. It is sensitive, informative, and relatively inexpensive, provides a permanent record, and can be shared with other disease control programs [51]. Blood obtained by pricking a finger or earlobe is the ideal sample because the density of developed trophozoites or schizonts is grater in blood from this capillary-rich area. Blood obtained by venepuncture collected in heparin or sequestrate (EDTA) anticoagulant coated tubes is acceptable if used shortly after being drawn to prevent alteration in the morphology of white blood cells (WBC) and malarial parasites [52]. The parasites are more easily detected in the film when the blood has been taken several hours after the height of the paroxysm has been reached. Schizogony of P. vivax, P. malariae and P. ovale occurs in the peripheral blood, hence the parasites can be readily be demonstrated both during the febrile and afebrile periods. In the case of P. falciparum parasite disappears from the peripheral blood during the afebrile period, hence the best time for a demonstration of P. falciparum is a few hours after the febrile paroxysm reach its peak [5].

Microscopic examination of centrifuged buffy coat smear

Buffy coat smear was prepared by a hematocrit tube. A tube filled with blood up to mark and centrifuged it for 30 minutes at 1500-2000 rotation per minute. Once the process is done there a layer of blood will appear at the top plasma layer at bottom red blood cells and in middle, there is another layer that is formed by the deposition of white blood cells known as the buffy coat layer. Discard the plasma layer by picking out by lumbar puncture needle without disturbing the buffy coat layer. After discarding the plasma layer pick the buffy coat layer and prepare smear [53].

Fluorescence microscopy with the light microscope: Fluorochrome staining to detect malarial parasites in blood film is more sensitive, easier to do, and less time consuming but it requires standard epi-illuminated, mercury vapor and fluorescence microscope. This method is expensive, especially for tropical countries where malaria is endemic. Fluorescence microscopy with a

standard light microscope and a new interference filter specially designed for the fluorochrome stain, acridine orange (A.O) is used to detect malarial parasites in thick and thin blood films. In this system, two fluorescence colors, green (nuclei) and red (cytoplasm), are emitted from stained parasites [54].

Quantitative Buffy Coat technique (QBC): The Quantitative Buffy coat technique is a method of diagnosing hemiparasites based on micro centrifugation, fluorescence, and density gradient of infected red cells. This technique is an attractive alternative in the diagnosis of malaria. This technique is an attractive alternative in the diagnosis of malaria. A precisely constructed capillary tube is internally coated with EDTA and acridine orange, filled with capillary or venous blood to a predetermined level (60 µl) and capped. Staining of the nuclear DNA of the parasites by acridine orange makes the determination of specimen immediately possible [55].

Post-mortem diagnosis: The diagnosis of cerebral malaria post-mortem can be confirmed from a brain smear. A needle aspirate or biopsy is obtained through the superior orbital foramen or the foramen magnum. A smear of grey matter is examined after staining the slide in the same way as for a thin blood film. Capillaries and venules are identified microscopically under low power and examined under high power. If the patient died in the acute stage of cerebral malaria the vessels are packed with erythrocytes containing mature parasites and large amounts of pigment [33].

Rapid diagnostic tests

Detection of antigens: One of the cornerstones in the control of malaria is early diagnosis and treatment. For these reasons, rapid diagnostic strips have been developed. They are based on the capture of Plasmodium antigens [56]. These tests are based on the detection of antigens derived from malarial parasites in lysed blood, using immunochromatographic methods. These tests can be performed in 15-20 minutes [50]. Currently targeted antigens are histidine-rich protein-2 (HRP-2), plasmodium lactate dehydrogenase (pLDH) and plasmodium aldolase [57].

Detection of HRP-II antigen: histidine-rich protein-II (HRP-II) is a water-soluble protein produced by trophozoites and young gametocytes of P. falciparum [57]. It is expressed on the RBC membrane surface. HRP II antigen detection kits are rapid, do not require expertise, and can detect P. falciparum infection when the parasites are sequestered. Following antimalarial therapy, HRP II has been shown to persist in the blood for 7-14 days and up to 28 days. The positivity depends upon the number of parasites per microlitre of blood [58].

Detection of plasmodium aldolase: The energy metabolism of the blood stages of human malaria lacks a functional citric acid cycle, and the generation of ATP depends fully on the glycolytic cycle. Aldolase is a key enzyme in this pathway. This enzyme within the glycolytic pathway recognized and considered as targets for rapid malaria diagnostic tests [59]. Many first-generation rapid diagnostic products relied on the detection of the histidine-rich protein II (HRP II) antigen of P. falciparum and therefore could not detect other plasmodium species.

Detection of plasmodium lactate dehydrogenase (pLDH): It is (pLDH) a metabolic enzyme actively produced by all human malaria parasites during their growth in red cells. This enzyme does not persist in the blood but clears at about the same time as the parasites following successful treatment. The test is therefore of use for monitoring responses to drug therapy and for detecting drug-resistant malaria because pLDH reflects the presence of viable malaria parasites in the blood [60].

Serological methods for antibody detection: Serological methods are based on the detection of antibodies against malaria parasites. These methods have been used since the early 1960s when indirect fluorescent antibody tests (IFAT) and indirect hemagglutination assay (IHA) was described [60]. The main use of serological tests is for: retrospective confirmation of empirically treated non-immunes, tracing asymptomatic infections in blood donors, investigating congenital malaria especially when mother's blood smear is negative and epidemiological studies to define malaria transmission areas and monitor the effectiveness of preventive intervention strategies [61].

Indirect fluorescent antibody test (IFAT): Malaria antibody detection can be performed by using the indirect fluorescent antibody test (IFAT). The species-specific test is available for the four-human species: P. falciparum, P. vivax, P. malariae, and P. ovale. Blood stage Plasmodium species schizonts are used as antigens. The patient's serum is exposed to the organisms; if a homologous antibody is present, it attaches to the antigen and forms an antigen-antibody (Ag-Ab) complex. The fluorescent-labeled anti-human antibody is then added, which attaches to the patient's malaria-specific antibodies. When examined with a fluorescence microscope, a positive reaction is when the parasites fluoresce an apple green color. The disadvantages of IFAT are the requirement for a fluorescence microscope, the subjectivity of the reading, and the fact that the method is relatively labor-intensive [60].

Enzyme-linked immunosorbent assay (ELISA) method: Malaria specific antibody and antigen can be detected by using commercially available enzyme-linked immunosorbent assay (ELISA). The kits can detect antigen and antibody reliably and quite specific [62]. In ELISA the antibody or antigen is fixed to a surface, such as a well of a microlitre plate or a plastic bead. The test sample is applied and bound material is detected by a secondary enzymatically labeled antibody. They require highly purified reagents; uses of mAbs and recombinant antigens have greatly facilitated the widespread use of ELISA [63]. Immuno-reactivity of the ELISA method is dependent on antigen and antibody binding on the plastic surface of plates [64]. An enzyme-linked immunosorbent assay (ELISA), based on the monoclonal and polyclonal antibody was developed for the detection of P. falciparum antigens. The monoclonal antibodies were originated from Thai strains of P. falciparum in mice models and polyclonal antibodies originated against Nepali strains of P. falciparum in the rabbit model [65].

Molecular diagnosis

Molecular diagnosis of malaria was created as a more sensitive and specific methodology to detect parasites [66].

DNA probe: The presence of parasites in the blood means the presence of parasite DNA or RNA. Various methods have been developed based on the principle of nucleic acid hybridization to detect this. In this method, a known sequence of nucleic acid (Oligonucleotide) is synthesized and labeled with either radioactive 32p or a non-radioactive colorimetric reagent and this probe is used to detect parasite nucleic acid taking advantage of the fact that complementary sequences will hybridize. The simplest version of this technique is the use of DNA probes to detect parasites directly in a drop of patient's blood immobilized on a filter paper [60]. Hybridization using labeled probes was described first in 1984. In comparison with thick blood smear, the test displayed a sensitivity ranging from 65% to 81% and specificity close to 100% [67]. In 1989, the use of four species-specific oligonucleotides for the small subunit ribosomal RNA (SSR RNA) genes were reported. They were used in hybridization studies as probes for the detection of all Plasmodium species in humans. However, this methodology has disadvantages, such as presenting low sensitivity and using radioactive material for which special laboratory conditions are necessary [66].

Polymerase chain reaction (PCR): This method is based on the molecular biology technique to identify parasite genetic material. The amplification principle behind the test allows us to pick up a negligible amount

of parasite DNA sequence and multiply it million times for easy detection [61]. Several PCR assays for malaria diagnosis have also been developed, most often based on the genus or species-specific sequences of the parasites 18S subunit rRNA gene and single-stranded rRNA. Real-time PCR, a new methodology that employs fluorescent labels to enable the continuous monitoring of amplicon (PCR product) formation thought the reaction has recently been adapted to detect all four human malaria parasites indiscriminately and screen large numbers of samples [68]. To improve the sensitivity of this methodology, a nested PCR was developed. This technique utilizes two genus-specific primers in a first amplification reaction and a species-specific primer in a second reaction [66]. It has been estimated that PCR can detect malaria infections with parasitemia as low as 5 parasites/μL (0.0001% parasitemia). This procedure also can establish a species-specific diagnosis and recognize mixed infections; this makes PCR a very attractive tool [69]. However, molecular techniques are a costly procedure, including the cost of labor and access to reagents, compared to the examination of blood smears. This represents a true impairment for its implementation in reference laboratories located in poor regions of the world, where malaria is endemic [70].

Strategy for malaria control: The World Health Organization suggests that there are three essential elements of malaria control. First, is the selective vector control by reduction of the numbers of mosquitoes. Second, is early diagnosis, effective and prompt treatment of the cases. The third element is early detection or forecasting of epidemics and rapid application of control measures [14].

Prevention of malaria

Following measures can prevent malaria such as:

- Personal protection by mosquito nets, repellents, protecting clothing
- Anti-mosquito measures
- Prophylactic drugs
- Vaccination against malaria

Personal protection

Personal protection can be achieved by the following measures: by using a mosquito net, insecticide-treated bed net, repellent creams and spray, and wearing long sleeves and trousers [71].

Antimosquito measures: These may be directed toward adult mosquitoes and their larvae. Destruction of adult mosquitoes can be carried out by spraying with insecticides, such as DDT or Gammexane. Anti-larval measures consist of the elimination of breeding places of the mosquitoes and use larvicide. (oil, Paris green, DDT dissolved in oil) and culture of Guppy fish, Tilapia fish, etc in small ponds [71].

Prophylactic drugs: All travelers, unless eradication is complete, should take regular prophylactic drugs. Several, most of which are used for the treatment of malaria, can be taken for prophylaxis. For prophylaxis commonly used are drugs; mefloquine, doxycycline, and Proguanil. The choice of drug is usually driven based on the drug resistance status of the area [72].

Vaccination against malaria

Three main types of vaccine are currently under development:

- Pre-erythrocyte stage vaccine
- Transmission-blocking vaccine
- Asexual blood-stage vaccine [14]

Pre-erythrocyte stage vaccine: This is designed to prevent infection. Several pre-erythrocytic stages P. falciparum vaccine has been developed and others are in progress. These are CSP recombinant vaccines, synthetic multiple antigen peptide vaccines, and carboxy-terminal synthetic peptide from the PfCSP. Clinical trials with these vaccines show poor efficacy [72].

Transmission-blocking vaccines: This is designed to arrest the development of the parasite in the mosquito thereby reducing or eliminating transmission of the disease [3]. The three target antigens of transmission-blocking vaccine are pre-fertilization antigens expressed on gametocytes, postfertilization antigens expressed on zygotes or ookinetes, and late midget-stage antigens such as parasite-produced chitinase required for the ookinete to penetrate through the peritrophic membrane [14].

Erythrocytic vaccine: Among the several pre-erythrocytic vaccines the most promising is the SPf66 vaccine. Most of the work has been done with this vaccine. Initial trials showed 30% or greater immunity. But the recent trial of SPf66 is disappointing [71]. Several other experimental vaccines are purified recombinant PfMSP-1 vaccine, purified recombinant proteins vaccine based on three (MSP-1, MSP-2, and a portion of RESA) blood-stage P. falciparum proteins, recombinant P. falciparum apical membrane-1 (PfAMA-1) vaccine and MSP-3 synthetic peptide vaccine [72].

Antimalarial drug resistance: Drug resistance is a major problem in the treatment and prophylaxis of malaria. Malaria parasites have been developed resistance against many different types of drugs, such as the aminoquinolines as well as the antifolates. Drug resistance has been confirmed in 2 of the 4 human malaria parasite species, P. falciparum and P. vivax [2].

Drug-resistant P. falciparum: Chloroquine-resistant P. falciparum (CRPF) was first developed in Southeast Asia, Oceania, and South America in the late 1950s and early 1960s. Since then, chloroquine resistance has spread to nearly all areas of the world where falciparum malaria is transmitted. P. falciparum has also developed resistance to nearly all of the other currently available antimalarial drugs, such as sulfadoxine/ pyrimethamine, mefloquine, halofantrine, and quinine [2].

Drug-resistant P. vivax Chloroquine-resistant P. vivax (CRPV) malaria was first identified in 1989 among Australians living in or traveling to Papua New Guinea. CRPV has also now been identified in Southeast Asia, in the Indian subcontinent, and South America. Vivax malaria, particularly from Oceania, also exhibits decreased susceptibility to primaquine [2].

Mechanism of resistance

Chloroquine: Defect in pigment formation: Many chloroquine-resistant plasmodium strains are incapable of pigment formation. The absence of pigment would protect the parasite against the toxic action of chloroquine [3]. Amplification of the pf MDR gene: In many chloroquine-resistant isolates multiple copies of a gene homologous to the mammalian MDR gene are found. The MDR gene is often amplified and present in multiple copies in tumor cells that have become resistant to many different anti-cancer drugs. Interestingly an inhibitor of the MDR protein called verapamil is capable of reversing chloroquine resistance in plasmodium when administered at the same time as the anti-malarial drugs [3].

Quinine, mefloquine and halofantrine: Amplification of the pf mdr1 gene has been associated with resistance to mefloquine, halofantrine, and quinine in P. falciparum [16].

Antifolate drugs: Resistance to antifolates (Dihydrofolate reductase [DHFR] inhibitors) developed more quickly after their introduction. Resistance occurs due to point mutations in the substrate-binding site of the target enzymes. At present, there are seven identified point mutations occurring in the DHFR gene which are associated with the reduced binding capacity in resistant DHFR strains of P. falciparum [14].

References

1. Cox: History of human parasitology: Journal of clinical microbiology. 2002; 15(2):595-612.
2. Centre for Disease Control and Prevention. Malaria disease, 2015. Available at www.cdc.gov/malaria
3. World health organization. World malaria report, 2015. Available from www.who.int/malaria/worldreport2015
4. Gilles HM. Historical outline. In: Warrell DA, Gilles HM, editors. Essential malariology. 4th edn Arnold; London, 2002, 1-7.
5. Chaterjee KD. Parasitology and Helminthology 20th edn. Chaterjee Medical Publishers. 4 Amrita Banerjee Road, Calcutta, 2006, 216.
6. Singh B, Daneshvar C. Human infections and detection of Plasmodium knowlesi. 2013; 26:165-184.
7. Tyagi RK, Das MK, Singh SS, Sharma YD. Discordance in drug resistance associated mutation patterns in marker genes of Plasmodium falciparum and Plasmodium knowlesi during coinfections. J Antimicrob. Chemother. 2013; 68:1081-1088.
8. Pedro. Alonso. Malaria: developing a candidate vaccine (RTS, s/ASO2A) for an old source of humankind. International Journal of microbiology. 2006; 9:83-93.
9. National vector born disease control program, report, India 2015-2016. Available from: www.nvbdcp.gov.in
10. Cheesbrough M. District Laboratory practice. In: Tropical Countries, Part-1, Low price edn. Bath press and bounded in at the University press, Cambridge, 1999, 239-258.
11. Srinivasulu N, Gujju Gandhi B *et al*. Influence of climate change on malaria incidence in Mahabubnagar district of Andhra Pradesh, India. Int. J Curr. Microbiol. 2013; 2(5):256-266.
12. Moses Tesi. Global warming and health: The issue of malaria in Eastern Africa`s highlands. Discussion paper series no, 2011.
13. Anupkumar R. Anvikar, Naman Shah *et al*. Epidemiology of *Plasmodium vivax* Malaria in India. Copyright by the American Society of Tropical Medicine and Hygiene, 2016.
14. Phillips RS. Current Status of Malaria and Potential for Control. Clinical Microbiology Reviews. 2001; 14(1):208-226.

15. Filler S, Causer ML, Newman DR, Barber MA, Roberts MJ, MacArthur J *et al.* CDC. Malaria Surveillance-United States. 2001, 2003; 52:1-4.

16. Warrell DA. Clinical features of malaria. In: Warrel Da, Gilles HM. editors. Essential Malariology 4th edn. Arnold, a member of the Hodder Headline Group, London, 2002, 191-205.

17. Bradley DJ, Newbold CI, Warrel DA. Malaria. In Concise Oxford Textbook of Medicine. Weatherall DJ, Ledingham JGG, Warrel DA. 3rd edn. Oxford Medical Publication, Oxford, 2000, 1735-1750.

18. Mahlberger N, Jelinek T, Gascon J, Probst M, Zoller T, Schunk M *et al.* Epidemiology and clinical features of vivax malaria imported to Europe: Sentinel surveillance data from Trop Net Europ. Malaria Journal. 2004; 3:3-5.

19. Krause PJ. Malaria (Plasmodium). In: Behrman RE, Kliegroan RM, Jeson HB editors. Nelson Textbook of Pediatrics. 17th edn. Philadelphia. Saunders, 2000, 1049-1043.

20. White NJ, Breman JG. In: Kasper DL, Fauci AS, Longo D, Braunwald E, Hauser S, Jameson JL. editors. Harrison. Principles of Internal Medicine 16th edn. McGrow Hill, New York, 2005, 1203-1210.

21. Swash M. Parasites in the blood and faeces. In: Hutchison. Clinical Methods. 20th edn. W. B. Saunders Company Ltd. London, 1996, 427-433.

22. Lavstsen T, Magistrado P, Hermsen CC, Salanti A, Jensen ATR, Sauerwein R *et al.*, Expression of *Plasmodium falciparum* erythrocyte membrane protein1 in experimentally infected humans. Malaria Journal. 2005; 4:4-21.

23. Kyes SA, Rowe JA, Kriek N, Newbold CI. RIFINS: A second family of clonally variant proteins expressed on the surface of red cells infected with *Plasmodium falciparum.* Proceeding of the National Academy of Sciences of the United States of America. 1999; 96(16):9333-9338.

24. Abdel Latif MS, Khattab A, Lindenthal C, Kremsner PG, Klinkert M. Recognition of variant RIFIN Antigens by Human Antibodies Induced during Natural *Plasmodium falciparum* Infections. Infection and Immunity. 2002; 70 (12):7013-7021.

25. Ferreira MU, Nunes MS, Wunderlich G. Antigenic Diversity and Immune Evasion by Malaria Parasites. Clinical and Diagnostic Laboratory Immunology. 2004; 11(6):987-995.

26. Coban C, Ishii KJ, Sullivan DJ, Kumar N. Purified malaria pigment (hemozoin) enhances dendritic cell maturation and modulates the isotype of antibodies induced by a DNA vaccine. Infection and Immunity. 2002; 70(7):3939-3943.

27. Souza JB, Todd J, Krishegowda G, Gowda DC, Kwiatkowski D, Riley EM. Prevalence and boosting of antibodies to *Plasmodium falciparum* glycosyl phosphatidyl inositols and evaluation of their association with protection from mild and severe clinical malaria. Infection and Immunity. 2002; 70(9):5045-5051.

28. Angulo I, Fresno M. Cytokines in the Pathogenesis of and Protection against Malaria. Clinical and Diagnostic Laboratory Immunology. 2002; 9(6):1145-1152.

29. Beatrice Aitino *et al*. Pathogenesis of Malaria in Tissue and Blood. Mediterr J Hematol infection Dis, 2012, 4(11).

30. Mackintosh CL, Beeson JG, Marsh K. Clinical features and pathogenesis of severe malaria. Trends in Parasitology. 2004; 20(12):597-602.

31. Beeson JG, Brown GV, Molyneux ME, Mhango C, Dzinjalamala F, Stephen J *et al*., *Plasmodium falciparum* Isolates from Infected Pregnant Women and Children Are Associated with Distinct Adhesive and Antigenic Properties. The Journal of Infectious Diseases. 1999; 180:464-472.

32. Hayward RE, Tiwari B, Piper KP, Baruch DI, Day KP. Proceeding of the National Academy of Sciences of the United States of America. 1999; 96(8):4563-4568.

33. White NJ. Malaria. In: Gordon C Cook, Zumla A. Editors. Manson s Tropical Diseases, 21st edn. Saunders Elsevier Science, Health Science Division, London, 2003, 1205-1295.

34. Chen Q, Schlichtherle M, Wahlgren M. Molecular Aspects of Severe Malaria. Clinical Microbiology Reviews. 2000; 13(3):439-450.

35. Shulman C, Dorman E. Clinical features of malaria in pregnancy. In: Warrel DA, Gilles HM. Essential malariology 4th edn. Arnold, a member of Hodder Headline group, 338 Euston Road, London, 2002, 219-235.

36. McAdam AJ, Sharpe AH. Infectious Diseases. In: Kumar V, Abbas AK, Fausto N. Editors. Robins and Cotran Pathologic Basis of Disease. 7th edn. Saunders an Imprint of Elsevier. Philadelphia, Pennsylvania, 2004, 401-403.

37. Carter R, Mendis KN. Evolutionary and Historical Aspects of the Burden of Malaria. Clinical Microbiology Reviews. 2002; 15(4):564-594.

38. Krause PJ. Malaria (Plasmodium). In: Behrman RE, Kliegroan RM, Jeson HB. Editors. Nelson Textbook of Pediatrics. 17th edn. Philadelphia. Saunders, 2000, 1049-1043.

39. Marsh K. Immunology of malaria. In: Warrel DA, Gilles HM editors. Essential Malariology 4th edn. Arnold, a member of the Hodder Headline Group, 338 Euston Road, London, 2002, 252 257.

40. Mavoungou E. Interactions between Natural Killer Cells, Cortisol and Prolactin in Malaria during Pregnancy. Clinical Medicine & Research. 2005; 4(1):33-41.

41. Doolan DL, Hoffman SL. IL-12 and NK Cells Are Required for Antigen-specific Adaptive Immunity against Malaria Initiated by CD8+ T cells in the *Plasmodium yoelii* Model. The Journal of Immunology. 1999; 163:884-892.

42. Newton CRJC, Hien TT, White N. Neurological Aspects of Tropical Disease. Journal of Neuropsychiatry. 2000; 69:433-441.

43. Schmutzhard E. Cerebral Malaria: Clinical features and neurological sequelae. The African Journal of neurological sciences. 1998; 17(1):512-517.

44. Taylor TE, Molyneux ME. Clinical features of malaria in children. In: Warrell DA, Gilles HM editors. Essential malariology 4th edn. Arnold, a member of Hodder Headline group, 338 Euston Road, London, 2002, 206-217.

45. Suh KN, Kain KC, Keystone JS. Malaria. Canadian Medical Association Journal. 2004; 170(11):1693-1703.

46. Bedu-Addo G, Bates I. Causes of massive tropical splenomegaly in Ghana. Lancet. 2002; 360:449-454.

47. Steketee RW. Pregnancy, Nutrition and Parasitic Diseases. Journal of Nutrition. 2003; 133:1661-1667.

48. Malaguarnera L, Musumeci S. The immune response to *Plasmodium falciparum* malaria. Lancet Infectious Disease. 2002; 2(8):472-478.

49. Idro R, Bitarakwate E, Tumwesigre S, John CC. Clinical Manifestations of Severe Malaria in The Highlands of Southwestern Uganda. American Journal of Tropical Medicine and Hygiene. 2005; 72(5):561-567.

50. Khan SA, Anwar M, Hussain S, Qureshi AH, Ahmad M, Afzal S. Comparison of Optimal Malarial Test with Light Microscopy for the diagnosis of Malaria Journal of Pakistan Medical Association. 2004; 54:404-408.

51. Mendiratta DK, Bhutada K, Narang R, Narang P. Evaluation of different methods for diagnosis of P. falciparum malaria. Indian Journal of Medical Microbiology. 2006; 24(1):49-51.

52. Moody A. Rapid Diagnostic Tests for Malaria parasites. Clinical Microbiology Reviews. 2002; 15(1):66-78.

53. Prajapati *et al*. Comparative study of three diagnostic techniques for diagnosis of malaria at a tertiary care hospital in Kanpur. Journal of Medical Science & Clinical Research. 2018; 6(10):437-442.

54. Kawamoto F. Rapid diagnosis of malaria by fluorescence microscopy with light microscope and interference filter. Lancet 1991; 337:200-202.

55. Estacio RH, Edwin ER. Dy Cresswell S, Coronel RF, Alora AT. The Quantitative Buffy Coat Technique (QBC) in Early Diagnosis of Malaria: The Santo Tomas University Hospital Experience. Philippines Journal of Microbiology and Infectious Disease. 1993; 22(2):56-59.

56. Tarazona AS, Zerpa LS, Requena DM, Cuentas AL, Magill A. Evaluation of the rapid diagnostic test optimal for diagnosis of malaria due to *Plasmodium vivax.* Brazilian Journal of Infectious Disease, 2004, 8(2).

57. Tierney LM, McPhee SJ, Papadakis MA. Malaria. In: Current Medical diagnosis and Treatment 49thedn, 2005, 1417-1426.

58. Mendiratta DK, Bhutada K, Narang R, Narang P. Evaluation of different methods for diagnosis of P. falciparum malaria. Indian Journal of Medical Microbiology. 2006; 24(1):49-51.

59. Moody A. Rapid Diagnostic Tests for Malaria parasites. Clinical Microbiology Reviews. 2002; 15(1):66-78.

60. Hommel M. Diagnostic methods in malaria. In: Warrel DA, Gilles HM editors. Essential Malariology 4th edn. Arnold, a member of the Hodder Headline Group, 338 Euston Road, London, 2002, 36-58.

61. Malaria Roll Back. Malaria Diagnostics, 2002. WWW.malaria.am.

62. Iqbal J, Khalid N, Hira PR. Comparison of Two Commercial Assays with Expert Microscopy for confirmation of symptomatically Diagnosed Malaria. Journal of Clinical Microbiology. 2002; 40(12):4675-4678.

63. Lowell C. Clinical Laboratory Methods for Detection of Antigens and Antibodies. In: Parslow TG, Sites DP, Terr Al, Imboden JB editors. Medical Immunology 10th edn. The McGrow-Hill Companies. 1997, 215-233.

64. Mya MM, Saxena RK, Roy A, Rao DN. Determination of Immunoreactivity of *P. falciparum* Antigens, Serum Dilutions and Biomaterials. Indian Journal of Clinical Biochemistry. 2004; 19(1):88-92.

65. Ndao M, Bandyayera E, Kokoskin E, Gyorkos TW, Maclean JD, Ward BJ. Comparison of Blood Smear, Antigen Detection, and Nested-PCR Methods for Screening Refugees from Regions Where Malaria is Endemic after a Malaria Outbreak in Quebec, Canada. Journal of Clinical Microbiology. 2004; 42(6):2694-2700.

66. Disanti SM, Kirchgatter K, Brunialti KCSA, Oliveira AM, Ferreira SRS, Boulos M. PCR-Based diagnosis to evaluate the performance of malaria reference centers. Reveiw. Instituto de Medicina tropical de Sao Paulo. 2004; 46(4):183-187.

67. Berry A, Fabre R, Benoit VF, Cassaing S, Magnaval JF. Contribution of PCR based methods to diagnose and management of imported malaria. Tropical Medicine. 2005; 65(2):176-183.

68. Perandin F, Manca N, Calderaro A, Piccolo G, Galati L, Ricci L *et al.* Development of a Real-Time PCR Assay for detection of *plasmodium falciparum*, *Plasmodium vivax*, and *plasmodium ovale* for Routine clinical diagnosis. Journal of Clinical Microbiology. 2004; 42(3):1214-1219.

69. Ndao M, Bandyayera E, Kokoskin E, Gyorkos TW, Maclean JD, Ward BJ. Comparison of Blood Smear, Antigen Detection, and Nested-PCR Methods for Screening Refugees from Regions Where Malaria Is Endemic after a Malaria Outbreak in Quebec, Canada. Journal of Clinical Microbiology. 2004; 42(6):2694-2700.

70. Johnston SP, Pieniazek NJ, Xayavong MV, Slemenda SB, Wilkins PP, Silva AJ. PCR as a Confirmatory Technique for Laboratory Diagnosis of Malaria. Journal of Clinical Microbiology. 2006; 44(3):1087-1089.

71. Beales PF, Gilles HN. Rationale and technique of malaria control. In: Essential Malariology, Eds. David A Warrell, Herbert M Gilles. 4th Edition, Arnold, 2002, 107-190.

72. Hoffman *et al*. Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. J Infect Dis. 2002; 185:1155-1164.

Chapter - 6
In vitro Antiplasmodial Effect of Plants Extracts *Caesalpinia bonducella* and *Tinospora cordifolia* of Assam

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Chapter - 6

In vitro Antiplasmodial Effect of Plants Extracts *Caesalpinia bonducella* and *Tinospora cordifolia* of Assam

Shyamalima Gogoi and Abdul Mabood Khan

Abstract

Malaria is an infectious disease caused by parasites of the genus Plasmodium, of which *Plasmodium vivax* and *Plasmodium falciparum* are the major species that cause the disease in humans. Comparatively there are very few alternatives for malaria treatment, therefore it is necessary to search for new therapeutic options. The North East India possesses a great diversity of medicinal plants, which are potential sources of new compounds as therapeutics against different diseases including malaria. Thus, in this study the antiplasmodial effect of extracts from two species of plants *Caesalpinia bonducella* (Caesalpiniaceae) and *Tinospora cordifolia* (Menispermaceae) was evaluated *in vitro*. These plants were chosen because of their potent use in traditional medicine system of Assam.

Keywords: antiplasmodial, therapeutics, *in vitro*, medicinal plant, malaria

1. Introduction

Malaria is still one of the major tropical diseases leading to high morbidity and mortality in tropical and sub-tropical countries of the world. India is endemic for malaria and some states viz. Jharkhand, Orissa and Chhattisgarh are highly endemic. The contribution of India in malaria cases and deaths is 4% to total malaria burden of world [1]. While, North East India alone contributes to 12% of India's *Plasmodium falciparum* (*Pf*) cases [2]. In spite of persistent efforts by the Government of India to control/eliminate malaria, it is still major public health concern due to development of drug resistance against most of the existing antimalarial.

According to the World Malaria Report no significant success in reducing malarial infection have been made during the period of 2015-2017 [1]. Globally, after unprecedented period of time, the progress has been stopped regarding the successful control of malaria. Despite of full logistics support and increased centrally funded schemes, the emergence of multiple drug

resistant strains of *Pf* has been found in many endemic areas including Assam [3]. Therefore, it is highly indispensable to search for new anti-malarial agents that could be used in drug resistance cases having excellent anti-malarial properties.

2. Commercial drugs and its limitations

The antimalarial drugs currently available fall into three broad categories-

i) Aryl aminoalcohol compounds (quinine, quinidine, chloroquine, amodiaquine, mefloquine, halofantrine, lumefantrine, piperaquine, tafenoquine)

ii) Antifolate compounds (pyrimethamine, proguanil, chlorproguanil, trimethoprim)

iii) Artemisinin compounds (artemisinin, dihydroartemisinin, artemether, artesunate)

But the limitations of these drugs are widely known. The available commercial drugs are no longer effective against *Pf* in most of the endemic regions. Chloroquine resistance in *Pf* was first reported from Assam, India in 1973 and gradually it has spread to other parts of India [4]. Apart from chloroquine, resistance against sulfadoxine pyrimethamine has rapidly developed in most malaria affected areas. The emergence of multi-drug resistant malaria has become a challenging task for treatment and prophylaxis in most malaria endemic regions of the world. Increase in the rapid use of these drugs in large scale raises the potential risk of development of drug resistance and their constant efficacy. Therefore, there is an urgent need of development of newer and effective antimalarial drugs that would fight against resistant malaria with new mode of actions. To develop a new antimalarial drug for uncomplicated *Pf* it must be efficacious against the resistant strains, safe, suitable and affordable.

3. Plants as alternative source

Medicinal plants have been used as primary source of treatments against various diseases and ailments; in fact, there is a consensus among the scientific community that natural products have a dominant presence in discovering new leads for the development of drug treatment for human diseases [5]. Herbal medicines based on the traditional beliefs of different cultures for medicinal purposes has been used long before recorded history. It is estimated that about 70-80% of the population of developing countries, like Africa and Asia still depends on traditional medicine to meet their demands of primary health care. In fact, medicinal plants and/or plant-based products are being used from time

immemorial and are believed to be the most reliable and alternative source. The first antimalarial drug the alkaloid quinine extracted from the bark of the Cinchona (Rubiaceae) species in 1820, still largely used. Afterwards it was isolated and characterized [6] and recognized as the most important antimalarial drug to be used till date.

The indigenous tribes of North East India have been using traditionally a variety of medicinal plants from years as anti-malarial and found them quite efficacious. Despite vast resources of medicinal plants available in North East India, very less have been documented and validated scientifically to justify their efficacy.

4. Methods

4.1 Collection of data and plant materials

For the collection of data, a well-prepared semi-structured questionnaire was used. Primary information was collected on the traditionally used plants and its usage to treat malarial fever and its preparations as traditional formulations.

- Traditional healers (male and female) of different age groups were interviewed
- Questions were asked about the frequently used plants, plant parts used and method of preparation, frequency, dosages and their efficacy and the respective experiments were conducted with standard methods

A pilot study was conducted during June, 2018 to December, 2018 in some of the districts of upper Assam viz. Lakhimpur, Dhemaji and Dibrugarh, in order to collect the information of traditional knowledge, belief and practices held by the people of the study areas, about the use of medicinal plants for treating malarial fever. This information was collected using a semi-structured questionnaire and about 20 respondents including both male and female were interviewed, using a random sampling technique.

4.2 Extraction and fractionation collected from field

Plant parts were collected from the field; the leaves of the plant were separated and washed with water and shade dried field were washed and the dried plant materials were then grounded into fine powder in a laboratory blender and were subjected to organic solvents for extraction.

Plant crude extracts were prepared by Soxhlet extraction in solvent diethyl ether and then methanol for 3-4 days at 40-60 °C for about 4-5 h and

the process was repeated thrice with fresh solvents. The ratios of samples to solvents were 1:10 (m/v). After, each plant extract was subsequently filtered; the methanol filtrates were combined and evaporated in rotary evaporator to dryness under reduced pressure.

The final crude extracts were stored in glass vials at +4°C until use for farther experiments.

The percentage yields of various plant extracts were determined using the following formula:

$$\% \text{ yield of extract} = \frac{\text{Weight of the crude extract}}{\text{Weight of fresh plant material}} \times 100$$

4.3 *In vitro* studies

In vitro study is essential as the screening of plants for their perpetuate efficacy is needed to carry out the *in vivo* study further. In vitro antimalarial efficacy of methanolic crude extracts of *Caesalpinia bonducella* (leaves) and *Tinospora cordifolia* (leaves/stem). Experiments were carried out in Parasitology Division, CDRI, Lucknow. These plant extracts have been studied for chloroquine sensitive and resistant parasite *Plasmodium falciparum* 3D7 and K1 respectively at different doses (a total of seven concentrations) taking initial concentration 50µg/ml diluted in the ratio 1:2 in phosphate buffered saline (PBS, pH 7.4). IC50 was calculated using a templet as compared with positive controls.

4.4 Cytotoxic activity using VERO cell line

The cytotoxic effects of extracts on host cells were assessed by functional assay using VERO cell line. The cytotoxicity test of the two plant extracts was carried out taking concentrations from 500 µg/ml to 0.68 µg/ml using 1:3 dilutions.

5. Results

About 20 plant species were found to be used as antimalarial in the areas surveyed. Plant species viz., *Caesalpinia bonducella* (Fig.1) and *Tinospora cordifolia* (Fig. 2) emerged out as the most frequently used and therefore selected for the study.

Percentage yield of plant: The crude extracts of *C. bonducella* and *T. cordifolia* gives a yield of 3.61% and 0.77% respectively.

Fig 1: *Caesalpinia bonducella*

Fig 2: *Tinospora cordifolia*

In vitro study

C. bonducella crude extract showed promising activity against *Pf* K1 (chloroquine resistant strain) at 5.10µg/ml and *T. cordifolia* showed antiplasmodial activity at 36.85µg/ml. *C. bonducella* extract is found to be more efficacious when compared to *T. cordifolia* and interestingly showed greater potency against the CQ-resistant K1 strain than against the CQ-sensitive 3D7 strain.

Further the plants are non-toxic shows promising extracts ranged as evidenced by their good CC50 values (Table 1).

Table 1: *In vitro* antiplasmodial activity of two plant extracts

Plant/standard drug	IC_{50} against *Plasmodium falciparum* 3D7 chloroquine sensitive strain	IC_{50} against *Plasmodium falciparum* K1 chloroquine resistant strain	CC_{50} against VERO cell line
C. bonducella extract	27.45 µg/ml	5.10µg/ml	>500 µg/ml
T. cordifolia extract	19.65 µg/ml	36.85µg/ml	>500 µg/ml
Chloroquine	8.2nM	880nM	-

6. Discussion

North-Eastern part of India is very rich in biodiversity and there are numerous plants found naturally growing in wild across the region as

medicinal properties of these plants have been evaluated for various ailments in the past and there are many which has contributed a lot in the development of drugs from the natural products. As malarial parasites still lack the effective ideal treatment and search for newer antimalarial is still going on, the proposed objective wishes to explore the flora of Assam and in this context two plants have been selected on the basis of their medicinal properties documented in the indigenous system. An evidence-based approach regarding the efficacy and effectiveness of antimalarial plants that are used traditionally by people of Assam has not been scientifically studied so far, therefore keeping that in mind, this study was undertaken to evaluate the perpetuated efficacy of the plants, so as to validate its use in the traditional medicine system of these people.

The yield of particular plants varies from plant to plant in different geographical region as such there is a merit to explore the plants in North Eastern part of India. The yield of extraction is also affected by the polarity of solvents used, pH, temperature, extraction time and composition of the sample, etc. Extraction efficiency of plants depends on chemical nature of phytoconstituents, the extraction method used, the solvent used, as well as the sample particle size present [7].

In earlier studies the plant extracts have been studied against different diseases [8, 9] and also as antimalarial [10, 11]. The qualitative phytochemical study on the leaf extract of *C. bonducella* and *T. cordifolia* revealed the presence of several major chemical constituents, but tannins, saponins and steroids were present in abundance in both the plant. It is likely that the potent antimalarial activity of methanol extracts may be due to the presence of either of these bioactive constituents. It is apparent from literature that these plant secondary metabolites have also been used as a primary source of treatment of malaria for many centuries and they also exhibit significant antiplasmodial activities [12].

In this study, *C. bonducella* showed better efficacy for chloroquine sensitive and resistant parasite *Pf* 3D7 and K1 respectively. Crude extract of *C. bonducella* showed promising activity against *Pf* K1 (IC50 Pf3D7 & K1: 27.45 µg/ml & 5.10µg/ml respectively) while *T. cordifolia* showed moderate antiplasmodial activity (IC50 Pf3D7 & K1: 19.65 & 36.85µg/ml respectively). Simonsen *et al*., (2001) reported that the stem ethanol extract of *T. cordifolia* is poorly antiplasmodial (IC50 Pf3D7: 62 µg/mL). In the present study it was found that methanolic leaf extract of *Tinospora cordifolia* is moderately antiplasmodial against 3D7 strain of *Pf. C. bonducella* extract is found to be more efficacious when compared to *T. cordifolia*.

Our findings are not in the line with the earlier works that have been reported in any *in vitro* system though some of them have studied in *in vivo* system [13]. Further investigation in *in vivo* system is warranted to authenticate its efficacy in broader aspects.

7. Conclusion

From the study, it may be concluded that *C. bonducella* and *T. cordifolia* leaf extract possesses a significant *in vitro* antiplasmodial effects on both chloroquine sensitive and resistant strain of *Pf*. However, to draw a complete picture of the plant's potential efficacy the *in vivo* study has to be carried out further. Nevertheless, these findings lend support to the traditional use of these plants as antimalarial with a hope that refining of plant extract and preparation in a holistic manner may contribute to the development of a suitable herbal medicine with a low risk of resistance and toxicity.

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References

1. WHO. World malaria report, 2018. Geneva: World Health Organization.
2. https://nvbdcp.gov.in/WriteReadData/l892s/5232542721532941542.pdf, 2016 Operational Manual for Malaria Elimination in India
3. Dua VK, Dev V, Phookan S, Gupta NC, Sharma VP, Subbarao SK. Multi-drug Resistant Plasmodium Falciparum Malaria in Assam, India: Timing of Recurrence and Anti-Malarial Drug Concentrations in Whole Blood. American Journal of Tropical Medicine and Hygiene. 2003; 69:555-7.
4. Shah NK, Dhillon GPS, Dash AP, Arora U, Meshnick SR, Valecha N. Antimalarial drug resistance of Plasmodium falciparum in India: changes over time and space. Lancet Infectious Diseases. 2011; 11:57-64.
5. Newman DJ, Cragg GM. Natural products as sources of new drug over the 30 years from 1981 to 2010. Journal of Natural Products. 2016; 75:311-315.
6. Saxena S, Pant N, Jain DC, Bhakuni RS. Antimalarial agents from plant sources. Current Science. 2003; 85:9.
7. Stalikas CD. Extraction, separation, and detection methods for phenolic acids and flavonoids. Journal of Separation Science. 2007; 30:3268-3295.

8. Gogoi S, Yadav AK. *In vitro* and *in vivo* anthelmintic effects of *Caesalpinia bonducella* (L.) Roxb. leaf extract on *Hymenolepis diminuta* (Cestoda) and *Syphacia obvelata* (Nematoda). Journal of Intercultural Ethnopharmacology. 2017; 5:427-433.

9. Billah MM, Islam R, Khatun H *et al*. Antibacterial, antidiarrhoeal and cytotoxic activities of methanol extract and its fractions of *Caesalpinia bonducella* (L.) Roxb leaves. BMC Complementary and Alternative Medicine. 2013; 13:101.

10. Nondo RS, Erasto P, Moshi MJ, Zacharia A, Masimba PJ, Kidukuli AW. *In vivo* antimalarial activity of extracts of Tanzanian medicinal plants used for the treatment of malaria. Journal of Advanced Pharmaceutical Technology and Research. 2016; 7:59-63.

11. Simonsen HT, Nordskjold JB, Smitt UW, Nyman U, Palpu P, Joshi P *et al*. *In vitro* screening of Indian medicinal plants for antiplasmodial activity. Journal of Ethnopharmacology. 2001; 74:195-204.

12. Pan WH, Xu XY, Shi N, Tsang SW, Zhang HJ. Antimalarial Activity of Plant Metabolites. International Journal of Molecular Sciences. 2018; 19:13-82.

13. Kaushik NK, Bagavan A, Rahuman AA, Mohanakrishnan D, Kamaraj C, Elango G *et al*. Antiplasmodial potential of selected medicinal plants from eastern Ghats of South India. Experimental Parasitology. 2013; 134:26-32.