universität wien

DISSERTATION

Titel der Dissertation

Prevalence and phylogeny of *Plasmodium ovale* and *P. malariae* in Bangladesh.

Subtitle:
A novel PCR technique in comparison with standard PCR methods for the prevalence screening of *Plasmodium* sp. in Bangladesh, with a main focus on the distribution and phylogeny of *P. ovale*, *P. malariae*, and *P. knowlesi*, as well as the extended short term culture of *P. vivax* under field conditions.

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1 Declarations

“I declare in lieu of an oath that I have written this doctoral thesis myself and that I have not used any sources or resources other than stated for its preparation. I further declare that I have clearly indicated all direct and indirect quotations. This doctoral thesis has not been submitted elsewhere for examination purposes.”

2 Table of Contents

3 Abbrevations

ACT	Artemisinin-based combination therapy
AD	Anno Domini
ARDS	Acute respiratory distress syndrome
BC	Before Christ
bp	base pair
CDC	Centers for Disease Control and Prevention
CHTs	Chittagong Hill Tracts
cox1	cytochrome c oxidase 1
cox3	cytochrome c oxidase 3
Csp	circumsporozoite protein
Cytb	cytochrome b
DNA	Deoxyribonucleic acid
e.g.	exempli gratia
ELISA	enzyme-linked immunosorbant assay
HRP2	histidin rich protein 2
ICDDR,B	International Centre for Diarrhoeal Disease Research, Bangladesh
kb	kilobase
LSU rRNA	large subunit ribosomal RNA
MARIB	Malaria Research Initiative Bandarban
MSP-1	Merozoite surface protein-1
NGO	Non-governmental organization
PCR	polymerase chain reaction
pLDH	parasite lactate dehydrogenase

PoRBP2	Plasmodium ovale reticulocyte binding protein 2
PoTRA	Plasmodium ovale tryptophan rich antigen gene
RDT	rapid diagnostic test
RPMI	Roswell Park Memorial Institute Medium
SSU rRNA	small subunit ribosomal RNA
UNDP	United Nations Development Programme
WHO	World Health Organization

4 Introduction

4.1 Malaria

Malaria is a mosquito-borne disease caused by protozoan parasites of the genus *Plasmodium*. More than 3.2 billion people worldwide live in risk of infections with malaria parasites (Fig. 1). 1.2 billion thereof live in areas with high malaria risk (WHO 2010c). It is estimated that the number of cases of malaria rose from 233 million in 2000 to 244 million in 2005 but decreased to 225 million in 2009. The number of deaths due to malaria is estimated to have

decreased from 985 000 in 2000 to 781 000 in 2009. The majority of those malaria cases occurred in Africa (> 13.2 million), followed by South-East Asia (> 2.7 million), Eastern Mediterranean regions (> 1 million), the Americas (561,000) and the Western Pacific region (247,669) (WHO 2010c).

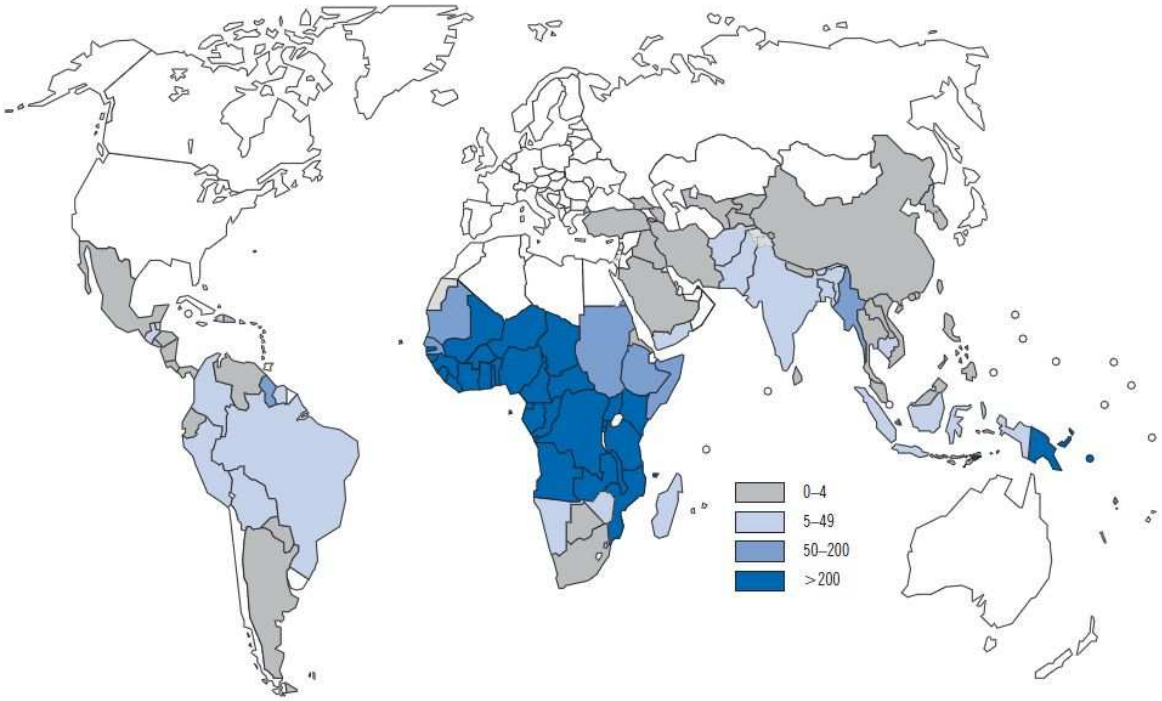

Fig. 1: Estimated malaria incidences per 1,000 persons (Source: WHO 2008).

Traditionally four malaria parasites are recognized in human, but in the last years molecular epidemiological and phylogenetic studies added another two to a total of six species. The systematic status of those parasites is given below according to Adl et al. (2005).

Domain: Eukaryota
Kingdom: Chromalveolata ADL et al., 2005
Superphylum: Alveolata CAVALIER-SMITH, 1991
Phylum: Apicomplexa LEVINE, 1980 emend. ADL et al. 2005
Class: Aconoidasida MEHLHORN, PETERS and HABERKORN, 1980
Order: Haemospororida DANILEWSKY, 1885
Genus: Plasmodium MARCHIAFAVA AND CELLI, 1885
Species:
Plasmodium falciparum
Plasmodium vivax
Plasmodium malariae
Plasmodium ovale curtisi
Plasmodium ovale wallikeri
Plasmodium knowlesi

4.2 History of malaria

The genus *Plasmodium* is a product of thousands of years of evolution, which still is proceeding. Species of this genus are known in almost all strictly terrestrial vertebrates possessing more or less strict host specificity (Coatney et al. 1971).
Diseases with malaria-like symptoms were first described by ancient Chinese and Egyptians (hieroglyphs), but it is not definitely clear if those were caused by malaria parasites (Warrel and Giles 2002). In ancient Greece (about 400 BC) Hippocrates was the first malarologist, describing various clinical symptoms like fevers (Coatney et al. 1971). About 30 AD Aulus Cornelius Celsus, a Roman encyclopedist, described two different kinds of tertian fever in “De Medicina”. Galen of Pergamon documented (probably malaria related) jaundice and seasonal fever in the summer.
In the 17th century a bitter extract of the bark of the chinchona tree, used by the indigenous Peruvian population against malaria-like fevers, was introduced to Europe by the Jesuits.

Chinchona bark was first described in Heyden's "Discours et Aris sur les fluis de ventre" in Antwerp in 1643 (Coatney et al. 1971). In 1775 Torti documented that cinchona bark is not effective against all kind of fever diseases. Giovanni Maria Lancisi described several malaria-like epidemics in Italy and found black pigments in human spleens and brains. Furthermore, he came up with the connection between insects and malaria. The importance of malaria and its therapy increased during the years of colonialization.

In 1831 Boyle introduced the swamp theory and named the disease mal'aria (bad air) and paludiena. Malaria was also called ague or marsh fever because of its emergence in areas close to marshland or swamps (Reiter 2004). In 1848 Meckel described the occurring of black pigments in protoplasmic masses but it took until 1879 when Afanasiev mentioned that these bodies are causing diseases.

In 1880 Charles Louis Alphonse Laveran was the first scientist who described *P. falciparum* gametocytes in malaria patients, but most scientists did not pay attention because of the belief that malaria is caused by bacteria (Warrel and Gilles 2002). In 1897 Mac Callum observed fertilization in bird malaria and later in *P. falciparum* at the John's Hopkins University in Baltimore (Coatney et al. 1971). Ronald Ross documented in 1896 the exflagelation of *P. falciparum* in the stomach of *Anopheles* mosquitoes, and later described the sporogonic cycle of a bird malaria parasite *P. relictum*. Further on, Theobald Smith mentioned mosquitoes as vectors of malaria (Russell 1955). Amico Bignami (1898) infected volunteers with *P. falciparum*, and in 1899 Grassi, Bignami and Bastianelli were able to complete the cycle of *P. falciparum*, and Bastianelli and Bignami those of *P. vivax*. Those results were summarized by Grassi in 1900.

The genus *Plasmodium* was first mentioned by Marchiafava and Celli in 1885. However, the systematics in those times were chaotic, and so several names were used for this genus: Haemamoeba, Oscillaria, Laverania and Haemomonas (Coatney et al. 1971). *P. malariae* was named by Charles Louis Alphonse Laveran in 1881. Grassi and Feletti (1890) described *P. vivax* and named this parasite *Haemamoeba vivax*. *Plasmodium falciparum* was named *Haematozoon falciparum* by Welch in 1897.

At the beginning of the 20th century 3 *Plasmodium* parasites were recognized to cause malaria in human. First vector control trials were started in Havana/Cuba by Gorgas in 1900 and continued at the building of the Panama Canal in 1907, which enabled the completion of this channel. Between the 1930's and 1940's the first insecticides were introduced. In these decades primate malaria studies started, and Shortt discovered pre-erythrocytic stages of *Plasmodium* parasites in 1948. Furthermore, several scientists discovered numerous

malaria parasites in monkeys and the first evidence of the zoonotic impact of simian malaria parasites emerged (Coatney et al. 1971).

It took until 1976 when Willam Trager and James Jensen established the cultivation of *P. falciparum* (Trager and Jensen 1976). Modifications of this technique are still in use and are essential for the *in vitro* diagnosis of drug resistances and sensitivity assays in *P. falciparum* and other malaria parasites.

4.3 Life cycle of *Plasmodium* sp.

4.3.1 Asexual life cycle

With the mosquito drawing a blood meal spindle shaped sporozoites are introduced into the body within the saliva. Some sporozoites are destroyed by the host's macrophages; others enter the lymphatic system, and still others the blood vessels of its intermediate host (Vaughan et al. 2008; Yamauchi et al. 2007). Within 70 min the sporozoites reach the liver via the blood stream and invade hepatocytes (Cerami et al. 1992). At the preerythrocytic schizogony in the liver each sporozoite develops into a trophozoite and multiplies to exo-erytrhrocyctic schizonts (Fig. 2). These schizonts grow to a size of 40-60 µM after 5-7 days with up to 50,000 daughter cells. The outer surface is increasing. There the nuclei attach to a portion of plasma and detach as merozoite from the schizont (Cerami et al. 1992; Warrel and Giles 2002; Döngens 1988). Most *Plasmodium* species have the tendency to repeat the liver schizogeny - with the exception of *P. falciparum* (blood schizogony).

The prepatence period ends with the start of the erythrocytic schizogony which needs 24-72 hours. Haemozoin results at the degradation of haemoglobin and forms the malaria pigment (Dorn et al. 1955). The merozoites invade erythrocytes of special stages. Each *Plasmodium* species has its own preferences: e.g. *P. malariae* prefers older erythrocytes, whereas *P. vivax* invades retikulocytes. Within the red blood cells merozoites develop into ring forms (young trophozoites), trophozoites, pre-schizonts and schizonts. Each mature schizont inhabits 8-24 merozoites which are released when the erythrocyte ruptures. At most *Plasmodium* species the erythrocytic schizogony is synchronized and all infected red blood cells rupture within a time frame of 1-3 hours. Fever attacks are caused due to the host's immune response to this synchronized system (Warrel and Giles 2002). The merozoites invade new erythrocytes and the erythrocytic cycle restarts. To slip through re-

sponses of the immune system this synchronization system is needed, and the invasion of new erythrocytes needs 20 seconds only (Döngens 1988). Merozoites are not able to invade liver cells and are thus only able to restart the erythrocytic schizogony. A host can inhibit up to 10^{13} parasites (Greenwood et al. 2008).

After a species-specific time frame (e.g. 1-3 weeks at *P. falciparum*) certain parasites start differentiation at blood schizogony and develop into extra-cellular, non-pathogenic gametocytes. *P. vivax* develops gametocytes soon after the release of the liver schizogony, whereas *P. falciparum* has its gametocyte peak about one week after the asexual erythrocytic peak (Pukrittayakamee et al. 2008, Miller et al. 2002). Gametocytes can be found in the peripheral blood from weeks up to months. The proportion between the female macrogametocytes and the male microgametocytes is 4:1.

4.3.2 Sexual reproduction (Sporogony)

At blood meal the definitive host, the *Anopheles* mosquito, intakes macro- and microgametocytes. They find their way to the gut of the mosquito host where sporogony is initiated. Mikrogametocytes exflagellate to haploid male gametes and fuse with female gametes into diploid zygotes. Within 18 hours they develop into ookinets with a size of 18x3 µM (Döngens 1998). Until this development stage sporogony can also occur in inappropriate hosts like species of the genus *Culex*.

The ookinet changes its shape to a round form in cells of the midgut. Parasites grow to oocysts within 10 days. Up to 1,000 active sporozoites are released at the rupture of the oocyst (Marsh and Makani 2004). Via the hemolymph they reach the salivary glands of the mosquito, invade, and reach a new intermediate host with the saliva at the next blood meal of the insect. Sporozoites can survive up to 50 days in the salivary glands. Mosquitoes often harbour a huge load of sporozoites and can so infect several intermediate hosts (Döngens 1988). Sporogony is correlated with the outside temperature and differs between different *Plasmodium* species. At temperatures below 20°C there is no sporogony at *P. falciparum* (Döngens 1988). However, mosquitoes benefit from infections with *Plasmodium* and vice versa. Infected insects were observed to have a higher survival rate and an increased rate of blood feeding (Barillas-Mury and Kumar 2005; Ferguson and Read 2004).

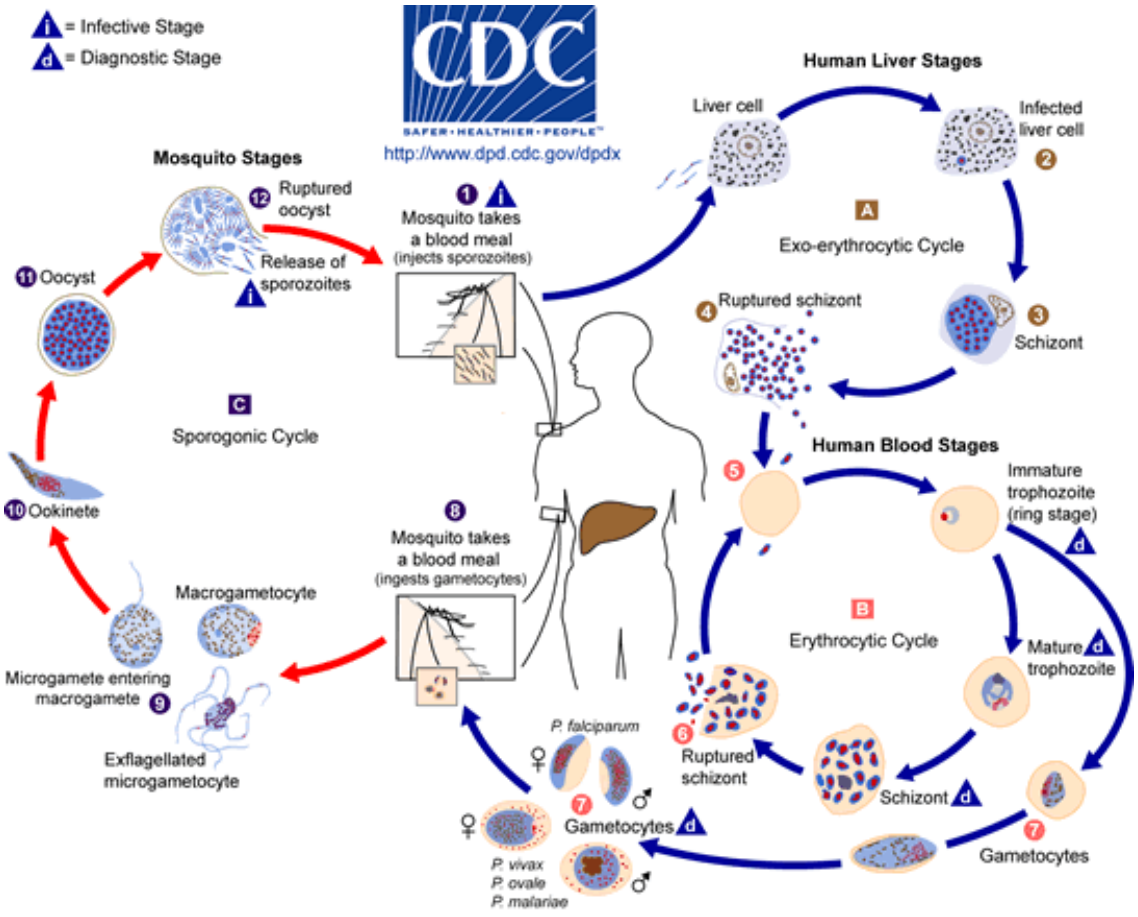

Fig. 2: Life cycle of *Plasmodium* sp. (Source: http://www.cdc.gov/malaria/about/biology/).

4.4 *Plasmodium falciparum* (Welch, 1897)

P. falciparum is known as the causative of Malaria tropica, which is the most dangerous form of human malaria with a high rate of complications and mortality. More than 90% of all malaria infections worldwide were caused by this pathogen in 2006 (WHO, 2008). *P. falciparum* is distributed in tropical and sub-tropical regions worldwide (Fig. 3) (Coatney 1971). Especially in sub-Saharan Africa *P. falciparum* is the predominant malaria-causing species, if compared to other parts of the world. However, *P. falciparum* was used to be common in the South-Eastern USA, the Mediterranean region and the Balkans, but was eliminated with eradication programs, advances in health systems and better economic conditions (Coatney 1971, Wernsdorfer 2002). Its distribution is dependent of temperature and height. *P. falciparum* was found in Kenya, Bolivia and Tajikistan at 2,600 m (Polumordvinov 1945, Hackett 1945, Garnham 1948).

P. falciparum seems to be the evolutionary youngest species causing malaria in human because it causes a high rate of mortality and damage in its intermediate host, the human being (Coatney 1971).

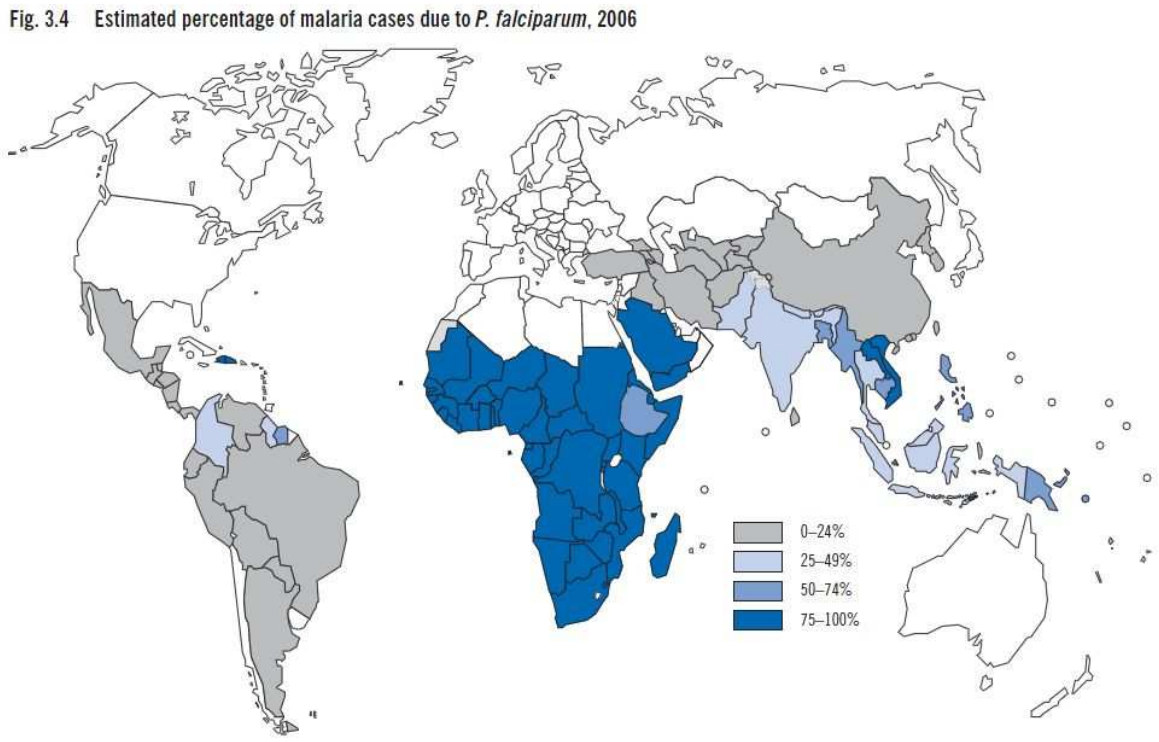

Fig. 3: Estimated percentage of infections with *P. falciparum* in 2006 (Source: WHO, 2008).

4.4.1 Morphological features

The asexual cycle of *P. falciparum* takes 48 h at an imperfect synchronized erythrocytic schizogony (Döngens 1988). This parasite is common for multiple invasions of the erythrocytes and high parasite densities (Shute 1956). In comparison to other human malaria parasites its young ring forms are smaller and infected host cells keep normal in size (Fig. 4). Shortly after the hair-like ring forms Maurer's dots are formed which are not really, but better visible after staining with special techniques (Coatney 1971). Trophozoites are rarely found at microscopic analysis, because of their tendency to stick on capillaries of inner organs or the brain. Mature schizonts contain 8 to more than 20 merozoites (usually 16) which are seldom seen in the peripheral blood (CDC). Adult gametocytes are sickle, banana-like or crescent shaped and appear about 10 days after the asexual forms in the peripheral blood. Macrogametocytes are longer than microgametocytes, with intensive blue colour and compact chromatin. Microgametocytes are sausage-shaped, reddish in colour and the chromatin is diffuse.

The extraerythrocytic schizogony in the liver generates a single generation only. In comparison to other human malaria species mature schizonts are large in size and contain a

larger number of small merozoites (Shortt et al. 1951). The extraerythrocytic schizogony is rapid and takes 6 days only.

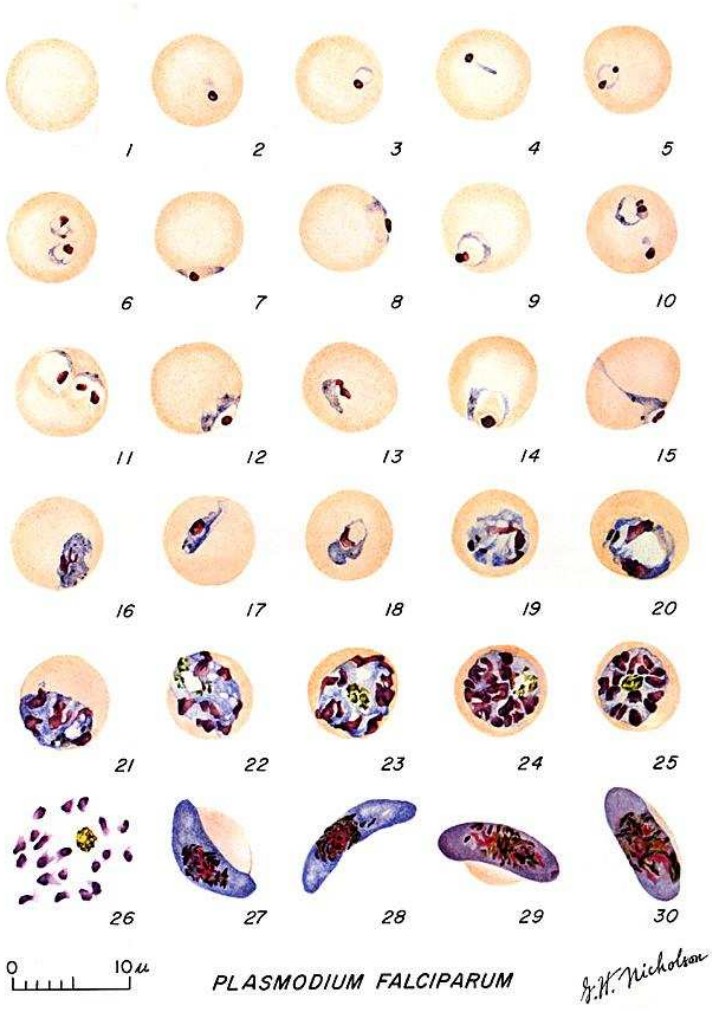

Fig. 4: Erythrocytic schizogony of *P. falciparum* (Source: Coatney 1971).

4.4.2 Pathogenesis

Only a very short overview is given on the pathogenesis of *P. falciparum*. After an incubation period of 7-12 days initial symptoms appear. In general initial and uncomplicated malaria symptoms are unspecific and multifaceted, like fever, headache, muscle ache, runny nose, fatigue, vomiting, nausea, chills, anaemia and splenomegaly. Fever caused by falciparum malaria is more often of the tertian than the quotidian type (Coatney 1971). Most cases in highly risky malaria endemic countries are uncomplicated, based on the patient's semi-immunity status. In high-endemic areas severe malaria is primarily found in children and pregnant woman. However, if patients never had malaria before (e.g. in a low-endemic malaria area) infections can cause severe malaria in adults and children. According to the

WHO, severe malaria is present if one or more of the symptoms listed in Tab.1 and Tab.2 are present. The most life-threatening complications in severe malaria are cerebral malaria, severe anaemia and respiratory distress.

Tab. 1: Defining criteria for severe malaria (WHO 1990)

Defining Criteria	**Finding**
Cerebral malaria (unrousable coma)	Unrousable coma not attributable to any other cause in a patient with falciparum malaria. Coma should persist at least 30 minutes after a generalized convulsion to make the distinction from transient post-ictal coma.
Severe normocytic anemia	Normocytic anemia with hematocrit < 15% or hemoglobin < 5 g/dL in the presence of parasitemia > 10,000 parasites per µL.
Renal failure	Urine output < 400 mL in 24 hours in adults, or 12 mL per kg in children, failing to improve after rehydration, and with serum creatinine > 265 µmol/L (3 mg/dL)
Pulmonary edema, ARDS	Clinical criterion
Hypoglycemia	Glucose < 2.2 mmol/L (< 40 mg/dL)
Circulatory collapse, shock	Hypotension (systolic blood pressure < 50 mm Hg in children 1-5 years old; < 70 mm Hg in adults)
Spontaneous bleeding, DIC	Spontaneous bleeding from gums, nose, GI tract or other sites, with laboratory evidence of DIC
Repeated generalized seizures	More than 2 observed seizures
Acidemia or acidosis	Arterial pH < 7.25, plasma bicarbonate < 15 mmol/L
Malarial haemoglobinuria	Macroscopic haemoglobinuria

Tab. 2: Additional criteria for severe malaria (based on WHO 2000)

Additional Criteria	**Finding**
Impaired consciousness but rousable	Impaired consciousness less marked than unrousable coma, can localize a painful stimulus
Prostration and extreme weakness	Patient unable to sit or walk, with no other obvious neurological explanation
Hyperparasitemia	High parasite densities are associated with increased risk of severe disease but is affected by the immune status (more than 5% parasitemia in non-immune patients)
Jaundice	Total bilirubin > 50 µmol/L (> 3 mg/dL)
Hyperpyrexia	Prolonged temperature > 40 °C
Post-mortem evidence of severe malaria	Neuropathologic evidence of venules and capillaries packed with erythrocytes containing malarial parasites

4.5 *Plasmodium vivax* (Grassi and Feletti, 1890)

P. vivax is distributed in subtropical and tropical regions worldwide and the dominant human malaria species in temperate climates (Fig. 5). Approximately 130-145 million vivax malaria infections are estimated per year with 2.6 billion people living in risk regions (Baird 2007). *P. vivax* is discussed to be the most prevalent malaria parasite worldwide because of its high population density in Asia and its dominance in Middle and South America, where up to 70% of all malaria cases are caused by this parasite (Coatney 1971; Price et al. 2007; CDCb). Brumpt (1949) described the “benign tertian mystery” in West Africa because of the absence of vivax malaria. This is caused by the lack of the Duffy blood group antigen (Fy9) in 90% of the population in West-, Central-, and East Africa (Rosenberg 2007). *P. vivax* is dependent on this antigen to penetrate the erythrocyte. Like at *P. falciparum* this parasite’s distribution is limited by temperature because of its need of at least 15°C for sporogony. However, until the 20th century *P. vivax* was endemic in Middle Europe (e.g. Austria and Germany) (Wernsdorfer 2002; Köhler and Köhler 2008).

P. vivax is very closely related to *P. simium*, a simian malaria parasite in New World monkeys in Brazil. Molecular based phylogenetic analysis revealed that both are almost identical (Cornejo and Escalante 2007). Furthermore, *P. vivax* is closely related to *P. cynomolgi*, *P. simiovale* and even *P. knowlesi* and it is estimated that the origins of *P. vivax* were in Old World Monkeys (*Macaca*) before its switch to the human host (Cornejo and Escalante 2007).

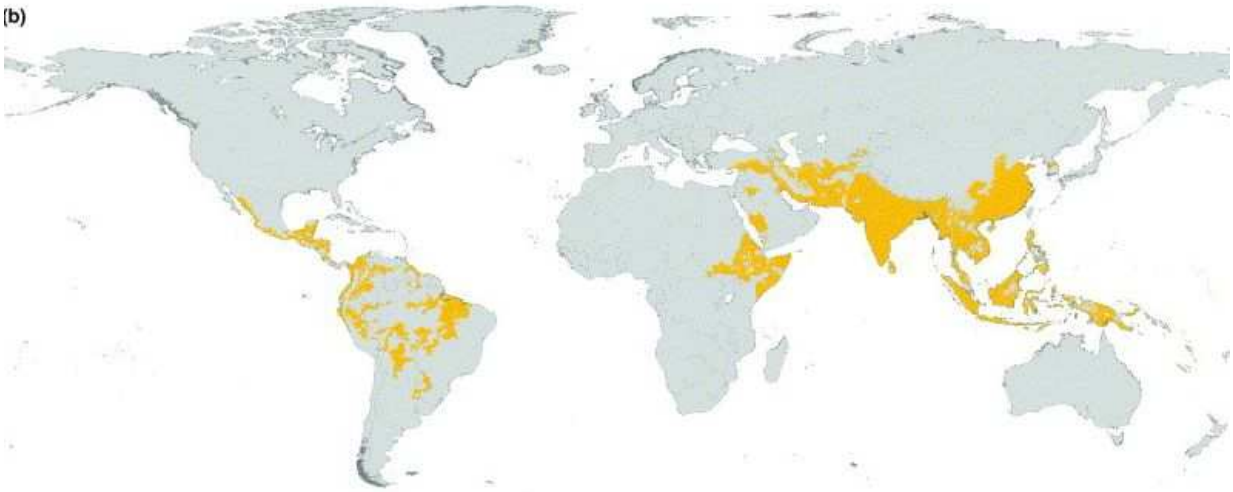

Fig. 5: Global distribution of *P. vivax* (Source: Guerra et al. 2007).

4.5.1 Morphological Features

The erythrocytic schizogony takes 48 h. Merozoites have a preference to invade reticulocytes. Young ring forms present a deep red nucleus of 1/3 in diameter of the erythrocyte (Fig. 6). Multiple invasions in one red blood cell are common (Coatney 1971). In mature trophozoites the cytoplasma is amoeboid in shape, Schueffner's dots and the invaded erythrocyte possess twice the size of a normal red blood cell. Schizonts are large in size and contain 16 (12-24) merozoites (Coatney 1971, Döngens 1988). Soon after the start of blood schizogony gametocytes can be found in the host's peripheral blood. Gametocytes are round and compact. Microgametocytes fill the whole erythrocyte, the nuclei are peripheral, and the plasma is diffuse. Macrogametocytes are bigger than a normal erythrocyte, and the cytoplasma is compact and eccentric (CDC) in shape.

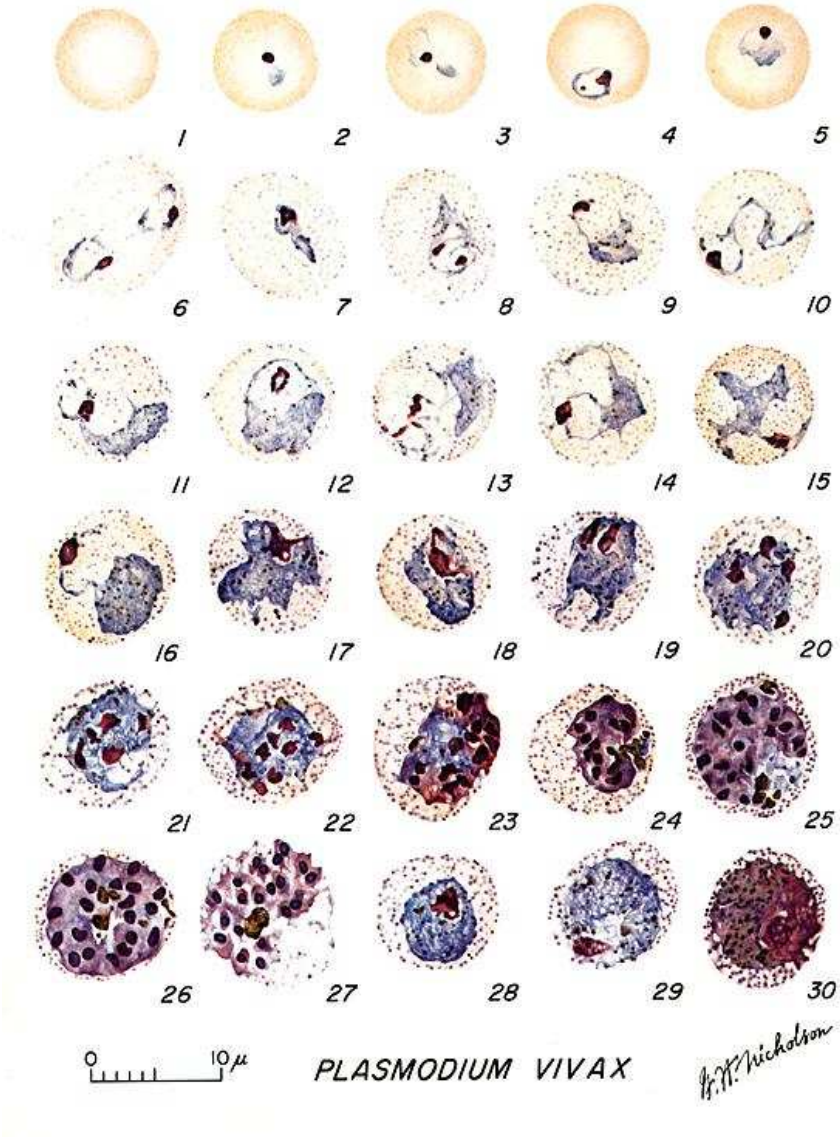

Fig. 6: Asexual, erythrocytic life cycle of *P. vivax* (Source: Coatney 1971).

4.5.2 Pathogenesis

The time of liver schizogony until the start of the blood schizogony (= prepatent time) takes 8-27 days and is dependent on the vivax variation type (Coatney 1971). *P. vivax* is known as a causative of tertian malaria with upcoming fever intervals every 48 hours, but initial fever can occur unspecific (Döngens 1988). Each fever peak emerges at the end of a synchronized blood schizogony round. At the rupture of the red blood cells not only merozoites but also toxic antigens are released (e.g. lectinase is released and inhibits the cellular respiration within the mitochondria). Fever peaks may emerge every 24 hours (Malaria duplicata, Malaria quotidiana), if two parasite populations in different schizogony intervals are present (Döngens 1988).

Symptoms of vivax malaria are general weakness, headache, body pain, chills and fever up to 41°C. Fever occurs in malaria tertiana typically every 48 hours. Fever periods take 6-12 hours and fever attacks recurr 12-15x (Coatney et al. 1971). Attacks of sweating are common when the fever decreases. Typically vivax malaria patients have no fever and feel well the day after a fever peak. Hepatosplenomegaly, anemia and bilirubinemia may be present. Not all sporozoites divide at the liver schizogony. Some do not divide or grow when entering the hepatocytes. The so called hypnozoites are dormant within the liver cell and cause relapses. Early relapses may occur within the first two months after a weathered vivax malaria, whereas at late relapses may emerge up to 3 years after the primary infection.

Normally the prognosis at vivax malaria is good. Complicated or severe vivax malaria cases are reported rarely and may cause retinal haemorrhage, ARDS, cerebral anaemia, renal failure and jaundice (Choi et al. 2004; Kocher et al. 2005).

4.6 *Plasmodium ovale* (Stephens, 1922)

4.6.1 History

Because of its morphological similarity to the more prevalent malaria parasite *P. vivax* it is very difficult to date the first documentation of *P. ovale*. The first suspected case was reported by Craig in 1900 in American soldiers who were based on the Philippines. They presented tertian fever and the parasites differed from *P. vivax* at microscopical analysis. In 1914 Emin reported a variant form of *P. vivax* in pilgrims in Camaran at the Red Sea. He named this variant form *P. vivax minuta*, which was renamed as *P. camaranese* by Ziemann in 1915. Macfie and Ingram (1917) observed pronounced Schüffner's stripplling in parasites resembling quartan *P. malariae* infections in a child at the Gold Coast in Africa. In 1918 Stephens, who worked at the Liverpool School of Tropical Medicine, found malaria parasites with fimbriated edges and an oval shape. In 1922 he named those parasites *P. ovale* but this discovery was not accepted by scientists for years (reviewed by Coatney 1971; Collins and Jeffery 2005).

The ancestral home of *P. ovale* is suggested to be tropical Africa. In 1949 Brumpt reviewed *P. ovale* cases documented by then and recognized that out of 105 cases only 14 were diagnosed outside of Africa. Lysenko and Benjaev (1966) analyzed the distribution of

P. ovale and reported that outside Africa *P. ovale* can only be found on the Philippines and New Guinea and based the limited distribution on host-vector relationships, climate, vegetation, host susceptibility and the relation to simian malaria. The highest prevalence of *P. ovale* was examined in equatorial forests and savannahs in Africa. Similar vegetation and climate conditions can be found in the Western Pacific region (Coatney 1971).

Several discussions came up if *P. ovale* is a zoonotic disease because of its rare occurrence in human (Coatney 1971). Since then tools for phylogenetic analyses changed from morphological features to molecular based techniques (see Chapter xx), but the discussions of the zoonotic potential of *P. ovale* are still going on. Duval (2009) analyzed several conserved gene regions of malaria parasites in chimpanzees (*Pan troglodytes troglodytes*) and was able to confirm the presence of *P. ovale* in those animal hosts.

Different strains and variations of *P. ovale* have been known for decades, and *P. ovale* parasites were found in several countries in South-East Asia. Based on molecular tools two genetically distinct variations of *P. ovale* parasites were reported by several authors (detailed in PCR chapter). Because of those dimorphic characters *P. ovale* was split in two species by the group around Sutherland in 2010, and named *P. ovale curtisi* (to honour Christopher F. Curtis) and *P. ovale wallikeri* (to honour David Walliker). The differentiation into two species was based on the perfect segregation of five gene loci, the evidence of sympatric distribution in Africa and Myanmar, no recombination or mating of the two species and differences in the *cyt b* gene (highly conserved region). Furthermore only minor differences in *dhfr-ts* sequences can be observed in *P. falciparum*, *P. vivax* and *P. malariae*, but in the case of *P. ovale wallikeri* and *P. ovale curtisi* those sequences are significantly different. Cross mating experiments, which normally are essential for the determination of a new species, are almost impossible to perform in malaria parasites (Sutherland et al. 2010). The sympatric distribution of *P. ovale wallikeri* and *P. ovale curtisi* in Africa was further confirmed (Oguike et al. 2011). However, there is lack of knowledge about the sympatric distribution of those parasites in Asia.

4.6.2 Distribution and Epidemiology

P. ovale has been known for decades to have its main distribution area and to be relatively common in West African countries (like Nigeria, Ghana, Sierra Leone, Liberia and Gambia). Its distribution spreads through Central Africa (where prevalences are less) to the East

African coast (e.g. Mozambique) (Coatney 1971). In Africa most cases were documented in children below the age of 8 years from savannah areas and less in forested regions (Lagan 1962; Mueller 2007). Microscopy based studies revealed a prevalence of up to 10% in West Africa (Garnham 1966). In Uganda a prevalence of 2.6% was reported in children between 1-4 years, compared to 0.7% in adults (> 20 years) (Onori 1967). However, it has to be mentioned that the focus of most studies was on children and the true prevalence may vary (Coatney 1971).

Outside Africa the prevalence of *P. ovale* is normally low (3-5%). Before molecular diagnostic tools were introduced for the detection of malaria parasites, *P. ovale* was reported rarely on the Philippines, Papua New Guinea, Indonesian Islands (e.g. Flores) and some South-East Asian countries (Mueller et al. 2007). Until now this malaria causative has never been reported from South- or Central America.
Infections with *P. ovale* are often asymptomatic and parasite densities are frequently very low. Mixed infections with other *Plasmodium* species can be observed frequently. Therefore more sensitive techniques than microscopy are needed for epidemiological studies (Mueller et al. 2007). PCR-based epidemiological studies made it possible that *P. ovale* was reported in almost all South-East Asian countries, India and Yemen (e.g. Mueller et al. 2007). In 1998 Zhou et al. observed a prevalence of 3.8% at the Thai-Myanmar border. In Myanmar *P. ovale* was observed several times with prevalence of up to 6.1% in Tanintharyi Division (Kawamoto et al. 1999; Win et al. 2002). A low prevalence (< 4%) was reported from Cambodia, Thailand, Vietnam and Malaysia (Incardona et al. 2005; Putaporntip et al. 2009; Gleason et al. 1970; Kawamoto et al. 1996; Singh et al. 2004; Lim et al. 2010). Single cases were further observed in India and Sri Lanka (Wickremasinghe et al. 2008). Further reports of *P. ovale* were documented from China, the Philippines, Indonesia, Thailand, Vietnam, Laos and Myanmar (reviewed in Win et al. 2002).

Originally *P. ovale* was known as a human parasite only. In experimental studies *P. ovale curtisi* was successfully cultivated in intact and splenectomised chimpanzees (reviewed in Coatney 1971, Sutherland et al. 2010). By now chimpanzees were also reported as natural hosts of *P. ovale* (Duval et al. 2009).
P. ovale has been reported to be transmitted by the following vectors: *Anopheles albimanus, A. atroparvus, A. freeborni, A. maculates, A. quadrimaculatus, A. superpictus, A. stephensi, A. gambiae, A. funestus* and *A. dirus* (Coatney 1971; Collins and Jeffrey 2005).

4.6.3 Life Cycle

The asexual erythrocytic life cycle of *P. ovale* is of tertian kind and takes 48 hours. Merozoites have a preference to invade reticulocytes (Collins and Jeffrey 2005). Multiple infections in one erythrocyte are common. Young ring forms have an eccentric, prominent, circular nucleus and sturdy cytoplasm surrounding a vacuole (Fig. 7). The parasitized erythrocyte is enlarged. Early in blood schizogony Schüffner's dots emerge which become more prominent in parasite growth. Overall Schüffnerization is more prominent as in *P. vivax* (Coatney 1971). During growth the amount of cytoplasm increases and the vacuole disappears. Older trophozoites fill almost half of the host cell of oval shape and are fimbriated. Sometimes band-like forms can be observed. Pigmentation increases within schizogony. First dust-like grains are build, later compact beads and thereafter yellowish brown-stained patches. The cytoplasm is transparent and pale with masses of stippling. The nuclei are enlarged and 2-4 times of the size of those in a young ring. Schizonts possess 8 (6-14) merozoites. At relapses and at experimental infections in chimpanzees 12-16 merozoites can be observed in a heavily enlarged host cell (Coatney 1971, Collins and Jeffrey 2005).
The sexual cycle of *P. ovale* takes around 49 hours. The cytoplasm of macrogametocytes stains blue, whereas the eccentric nuclei stain red. They fill the host erythrocyte completely. Prominent beads of pigments are present and Schüffner's strippling is ring-shaped around the parasite. The cytoplasm of microgametocytes stains lighter blue and possesses pigment beads. The nucleus is very large and fills 50% of the parasite. The parasite is surrounded with stippling.

Liver schizogony takes about nine days, but some parasites stay dormant as hypnozoites in the liver and a second generation can occur 19 days after. According to Coatney (1971) it can be divided into three stages.

1) The nuclear multiplication progresses rapidly with no evident merozoite formation
2) Merozoites appear at the edges of the parasitized host cell and nucleus division takes place elsewhere in the parasite
3) Schizonts mature completely and rupture

Furthermore, liver schizogony differs from those of *P. falciparum* and *P. vivax* by a definite limiting periplast, tangential adjusted peripheral nuclear bars, and a minor hypertrophy of the host cell's nucleus (Coatney 1971).

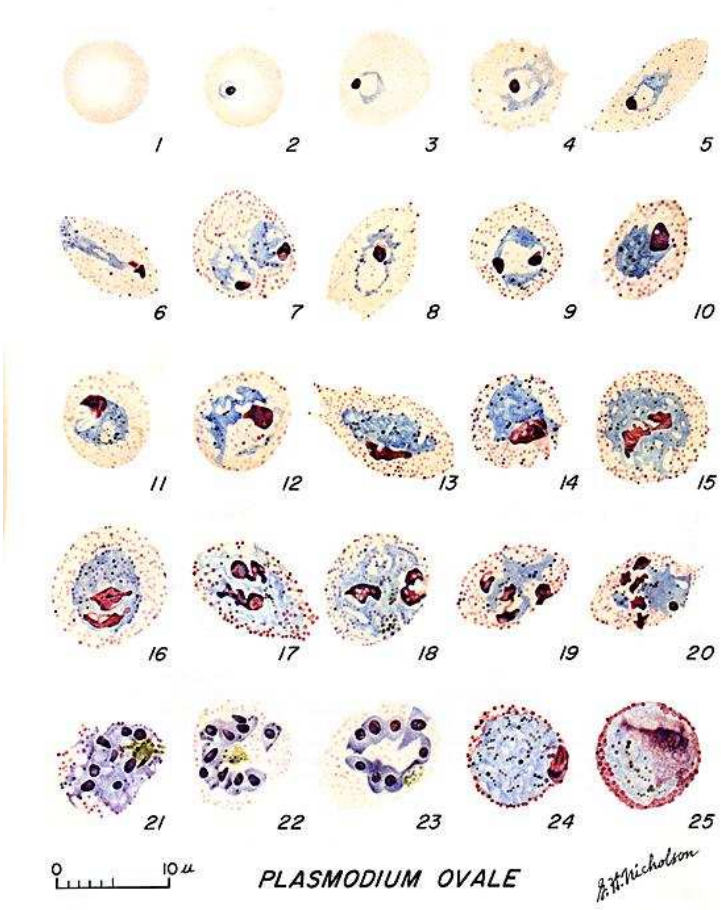

Fig. 7: Asexual, erythrocytic life cycle of *P. ovale* (Source: Coatney 1971).

4.6.4 Pathogenesis

In general infections with *P. ovale* are less severe than those with other human malaria parasites and cause mild clinical courses. Incubation period normally takes 11-18 days and fever may arise 17-18 days post infection (Coatney 1971). Nausea, resistant headache, chills and splenomegaly are common symptoms. Rigors are rarely observed. As at vivax malaria fever with temperatures up to 40.6 °C emerge every 34-61 hours (median = 49 hours) for 6-10 hours. Peroxysms emerge 1-22 times at experimental studies with the "Donaldson" strain (mean = 10x). The fever decreases at the peaks of peroxysms (James et al. 1949). The maximal parasite density is lower than in infections with *P. vivax* and *P. falciparum* with 380-27,600 parasites/µl (reviewed in Collins and Jeffrey 2005). Parasites can be observed in the blood for 29-91 days, although clinical symptoms decrease after ~17 days.

Relapses are common but asymptomatic in most cases. Around 17-255 days after the end of the first attack relapses can occur. Some authors reported delayed entries of first attacks at latent infections but it is suggested that those are relapses occurring 1.3-4 years post infection (reviewed in Coatney 1971; Collins and Jeffrey 2005).

Immunological studies revealed that semi-immunity to fresh infections with *P. ovale* can be observed in patients who had ovale malaria before. Semi-immunity was even reported between different strains like the Western Pacific "Donaldson"-strain and the West African "Liberian"-strain (Coatney 1971). However, no semi-immunity is present at fresh infections with other human *Plasmodium* species like *P. falciparum, P. vivax* and *P. malariae*. Garnham (1966) mentioned that the structure of antigens in *P. ovale* seems to be homogenous.
In West-Africa *P. vivax* is not present in Duffy-blood-group negative populations. However, *P. ovale* possess its highest prevalence worldwide in those areas and it is estimated that there is a negative interaction with *P. vivax* (Mueller et al. 2007).

4.7 *Plasmodium malariae* (Grassi and Feletti 1890)

The causative of malariae malaria, *P. malariae*, was first discovered in the blood of an Algerian patient by Laveran in 1880. Laveran described 8 merozoites, a rosette formation, and a central body of pigments, which are typical morphological features of *P. malariae* (Coatney 1971). This cosmopolitan malaria parasite is distributed in sub-Saharan Africa, South-East Asia, western Pacific islands and in Central- and South America (Collins 2007). *P. malariae* was formerly present in parts of Europe and the southern parts of the USA. The distribution of this pathogen is variable and spotty, and limited to a minimal temperature of at least 15°C, which is needed for sporogony within the mosquito host. Furthermore *P. malariae* coincides with *P. falciparum*, and co-infections of both are very common (Collins 2007).
Epidemiological studies documented high prevalence (15-30%) of *P. malariae* in Africa, Papua New Guinea and the Western Pacific, and infrequent observations (1-2%) in Asia, the Middle East, Central- and South America (Mueller et al. 2007).

P. malariae is assumed to be the oldest *Plasmodium* parasite in human, because of its high degree of adaption to its host. This parasite survives and stays longer in the blood than other malaria species and causes little discomfort. Opposing seasonal fluctuation between *P. malariae* and *P. falciparum* is known and Coatney (1971) described the situation of *P. malariae* as: “learned to wait in the wings”.

For decades it is assumed that *P. malariae* possesses animal reservoirs in tropical regions. In Africa chimpanzees are parasitized by *P. rhodhainii*, which is morphologically very similar to *P. malariae* (Coatney 1971). In Asia no simian hosts of *P. malariae* are known. *P. inui* is a quartan simian parasite of Old World Monkeys in India and Malaysia, but is morphologically different from *P. malariae*. In South America a morphologically similar simian parasite of New World monkeys, *P. brasilianum*, is known. This parasite was not distinguishable from *P. malariae* at molecular phylogenetic analyses (Tazi et al. 2011, Escalante et al. 1995). It is estimated that *P. brasilianum* came from man to the monkeys, as vice versa (Coatney 1971).

However, genetic and symptomatic differences within various strains of *P. malariae* are known. In China and at the Thai/Myanmar border phylogenetic analysis of *P. malariae* revealed differences within SSU rRNA sequences in variant forms of this parasite (Liu et al. 1998).

4.7.1 Morphological features

P. malariae sporozoites invade the liver fast within one hour post infectionem (Collins 2007). Liver schizogony takes 15 days. Enlargement of host cells and its nucleus, peripheral and internal vacuoles, absence of cytomeres, large clefts, and plaques in mature liver schizonts are typical for *P. malariae* liver schizogony (Coatney 1971).

P. malariae merozoites prefer to invade older red blood cells. Blood schizogony takes 72 hours, and parasites grow more slowly in comparison to other human malaria parasites. Young ring forms of this parasite build no true ring (Fig. 8). Ring stages fill 1/4 - 1/3 of the erythrocyte. Sometimes a second chromatin is build. Mature trophozoites are sometimes (typical for *P. malariae*) ribbon-shaped – the so called band-forms. Pigments in 30-50 granules are of dark-brown colour. The nuclei of schizonts build a rosette in the periphery of the cell. Mature schizonts are pigmented prominently and inhabit 8 (6-14) merozoites. After several weeks of blood infection gametocytes emerge. Those fill the non-enlarged erythrocyte, are round or oval in shape, and their pigment granula are scattered. Young gametocytes are morphologically very similar to asexual trophozoites. Mature macrogame-

tocytes of *P. malariae* fill the red blood cell completely, have compact cytoplasma and a small, eccentric nucleus (Coatey 1971). Adult microgametocytes possess a light-bluish cytoplasma and a diffuse nucleus. Normally more microgametocytes than macrogametocytes are present.

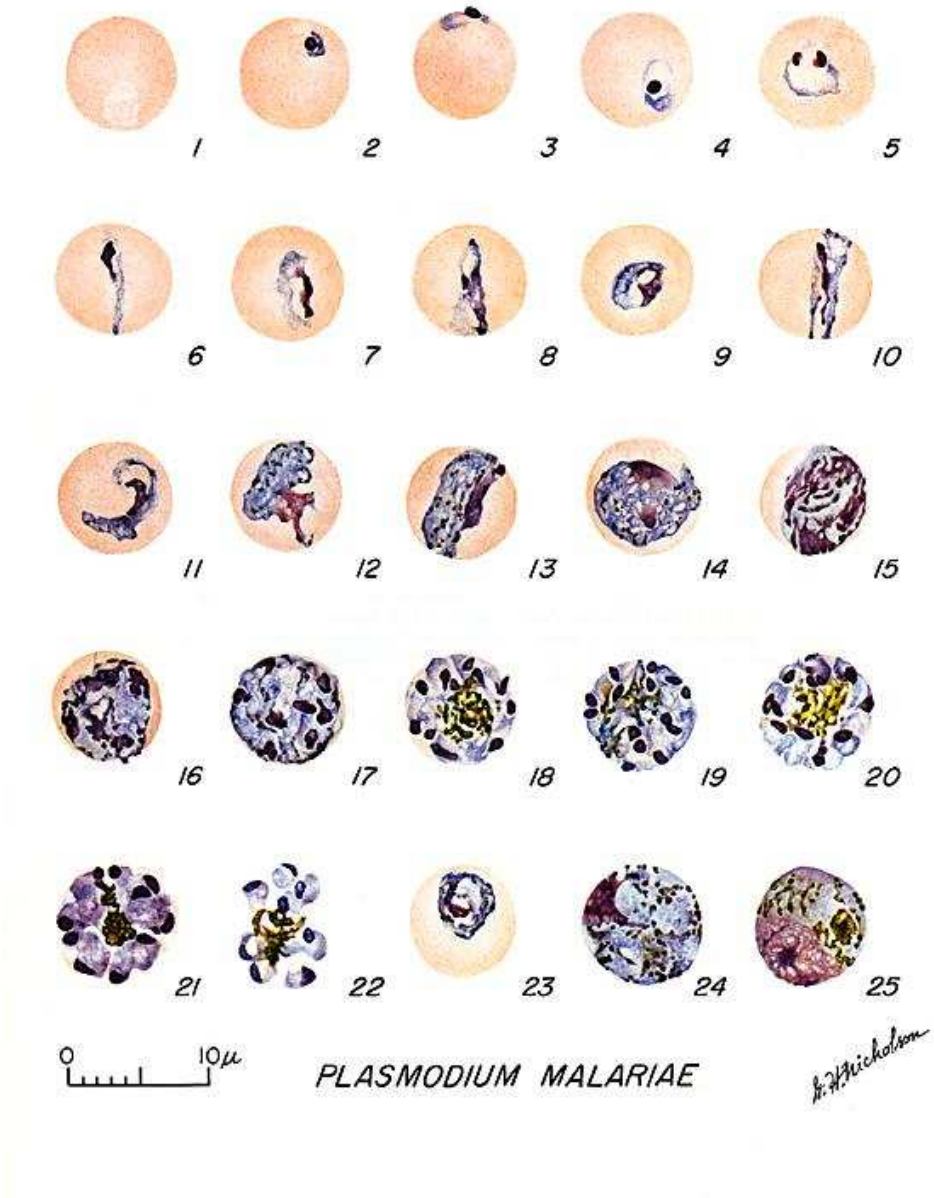

Fig. 8: Morphological features of *P. malariae* (Source: Coatney 1971).

4.7.2 Pathogenesis

Depending on the *P. malariae* strain prepatent periods take 20-35 days (Döngens 1988). *P. malariae* is the causative of malariae malaria, which is relatively benign with a synchronous 72 hour interval. Quartana duplicata occurs if 2 populations of schizonts are coexisting, and present an interval of 2 days fever and 1 day free of fever (Döngens 1988).

Shortly after the segmentation of the schizonts fever starts and reaches its peak (up to 40°C) at the rupture of the erythrocytes. Chills are typical within the increase of fever and take 13-195 minutes (mean = 53 min) (Coatney 1971). At *P. malariae* infections the parasitemia is lower (in comparison to *P. falciparum* or *P. vivax*) because of several factors like less merozoites in blood schizogony and longer erythrocytic schizogony cycle times. Parasitemias were reported to be between 1,648/µl and 49,680/µl (mean = 8,875/µl), and normally less than 0.25% of the erythrocytes are infected. The duration of an infection can be extremely long. Renal diseases are not uncommon and may end fatal (Collins 2007, Eiam-Ong et al. 2003).

Recrudescence is a well known phenomenon of *P. malariae*. This parasite does not relapse from persistent liver hypnozoites (Collins 2007). The origins of recrudescences are blood parasites in very low density (premunity). Recrudescences are discussed to be induced by stress. Latent chronic infections were reported with duration of more than 10 years. Because of those circumstances in some countries former quartana malaria patients are not allowed to donate blood (Döngens 1988).

4.8 *Plasmodium knowlesi* Sinton and Mulligan, 1932

Until very recently *P. knowlesi* was known as a simian parasite only. The distribution of this pathogen is strictly limited to South-East Asia because of those of both its intermediate and final hosts (Fig. 9). Until 1971 *P. knowlesi* was known to be endemic from peninsular Malaysia to the Philippines, and northwards up to Taiwan (Coatney 1971; Lambrecht et al. 1961; Yokogawa et al. 1941). Meanwhile it was documented in several other South-East Asian countries (e.g. Myanmar, Thailand).

The intermediate hosts of *P. knowlesi* are long tailed macaques (*M. fascicularis*), pig tailed macaques (*M. nemestrina*), and mitred leaf monkeys (*Presbytis melalophos*). In these natural hosts *P. knowlesi* cause mild infections with low parasite density (Cox-Singh and Singh 2008a). This parasite can be experimentally inoculated into several other primates (e.g. rhesus monkeys). In rhesus monkeys infections often cause deadly full blown malaria.

The vectors of *P. knowlesi* are mosquitoes of the *Anopheles leucosphyrus* group (e.g. *A. latens*, *A. cracens*) (Cox-Singh and Singh 2008a; Vythilingam et al. 2008).

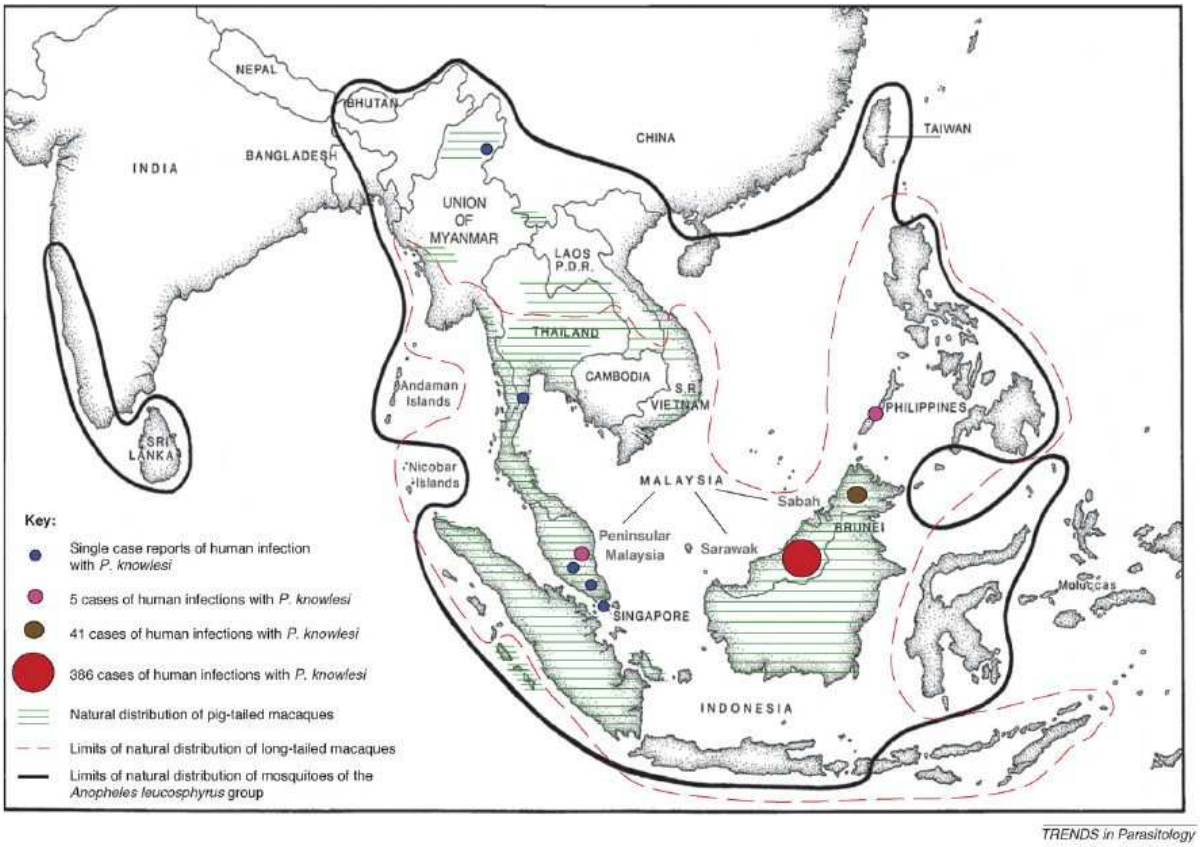

Fig. 9: Distribution of *P. knowlesi* (Source: Cox-Singh and Singh 2008a)

It might be that Franchini was the first one who described *P. knowlesi* in the blood of its simian host *Silenus cynomolgus* (*Macaca fascicularis*) in 1927. In 1931 Campbell and Napier found this parasite in *M. fascicularis* and were able to inoculate the pathogen to other macaques, of which one was a rhesus monkey developing fulminate malaria (Coatney 1971). In 1932 Knowles and Das Gupta described the blood forms of *P. knowlesi* and inoculated it into human volunteers. *P. knowlesi* was named to honour Dr. R. Knowles. In the upcoming years discussions came up about the loss of virulence in continued man-to-man transfer, and that previous infection with *P. vivax* lead to partial resistance against *P. knowlesi* in man (reviewed in Coatney 1971). *P. knowlesi* was further used therapeutically in human at general paresis and neurosyphilis. After first successes research was discontinued because of the death of some participants (Kantele et al. 2011). Several variations of *P. knowlesi* are known, and some of those might be less pathogenic in rhesus monkeys.

4.8.1 Morphology

The quotidian asexual cycle of *P. knowlesi* needs 24 hours only. In man young ring forms reach large numbers in the circulation blood. The nucleus is spherical and prominent (Fig.

10). One or more accessory chromatins are common. Overall young ring forms resemble those of *P. falciparum* (Lee et al. 2009). Late trophozoits possess non-amoeboid shape and fill ½ or more of the red blood cell. Band forms may appear. From then on *P. knowlesi* resembles *P. malariae* (Lee et al. 2009). Pigmented dark grains are formed and the parasite becomes more compact. The nucleus increases in size and "Sinton and Mulligon's stippling" (stippling in erythrocyte) can be observed. Schizonts contain 10 (up to 16) merozoites. Mature schizonts fill the parasitized red blood cell and pigment is visible as yellowish-black masses, which sometimes form a single mass. Gametocytes grow more slowly than asexual forms and can be differentiated by colour (Coatney 1971). At macrogametocytes the erythrocyte is enlarged, the cytoplasm is of blue and the eccentric nucleus of deep pink color. Scattered black pigment granules can be seen. In comparison microgamteocytes possess a pink-stained cytoplasm and a big, darker pink nucleus. Exoerythrocytic schizogony takes place in parenchymal cells of the liver and takes less than 120 hours (Coatney 1971).

4.8.2 Pathogenesis

After an incubation period of 9-12 days daily non-specific fever and chills emerge. Symptoms look like those of closely related *P. vivax*, but are more severe. Initial fever of 39°C is common, and fever peaks may reach up to 40-41°C appearing daily for about 10 days. Parasite densities are rarely over 100 parasites/10,000 RBCs, but in severe cases hyperparasitemia is common (Coatney et al. 1971). Because of the circumstances that the parasite invades all stages of red blood cells and an asynchronous blood, schizogony parasitemias can be extremely high with up to 10-15% of infected erythrocytes in fatal cases. Furthermore pyrexia, tachypnea, tachycardia, myalgia, rigors, malaise, abdominal pain, breathlessness, and cough were reported (Daneshvar et al. 2009). Thrombocytopenia and mild hepatic dysfunction are common. In adults anaemia is rarely observed. However, in children very low haemoglobin levels and anaemia were observed in 69% of all knowlesi patients (Barber et al. 2011).

Relapses (or recrudescences) can occur. Low levels of gametocytes can be found in human blood (Lee et al. 2009).

In 7% of human infections the parasite causes severe knowlesi malaria with a mortality rate of 1.8% (reviewed in Kantele et al. 2011). Respiratory distress is correlated with parasite density. Sequestrations of infected erythrocytes were observed in capillaries of heart, kidneys and brains (Cox-Singh et al. 2010).

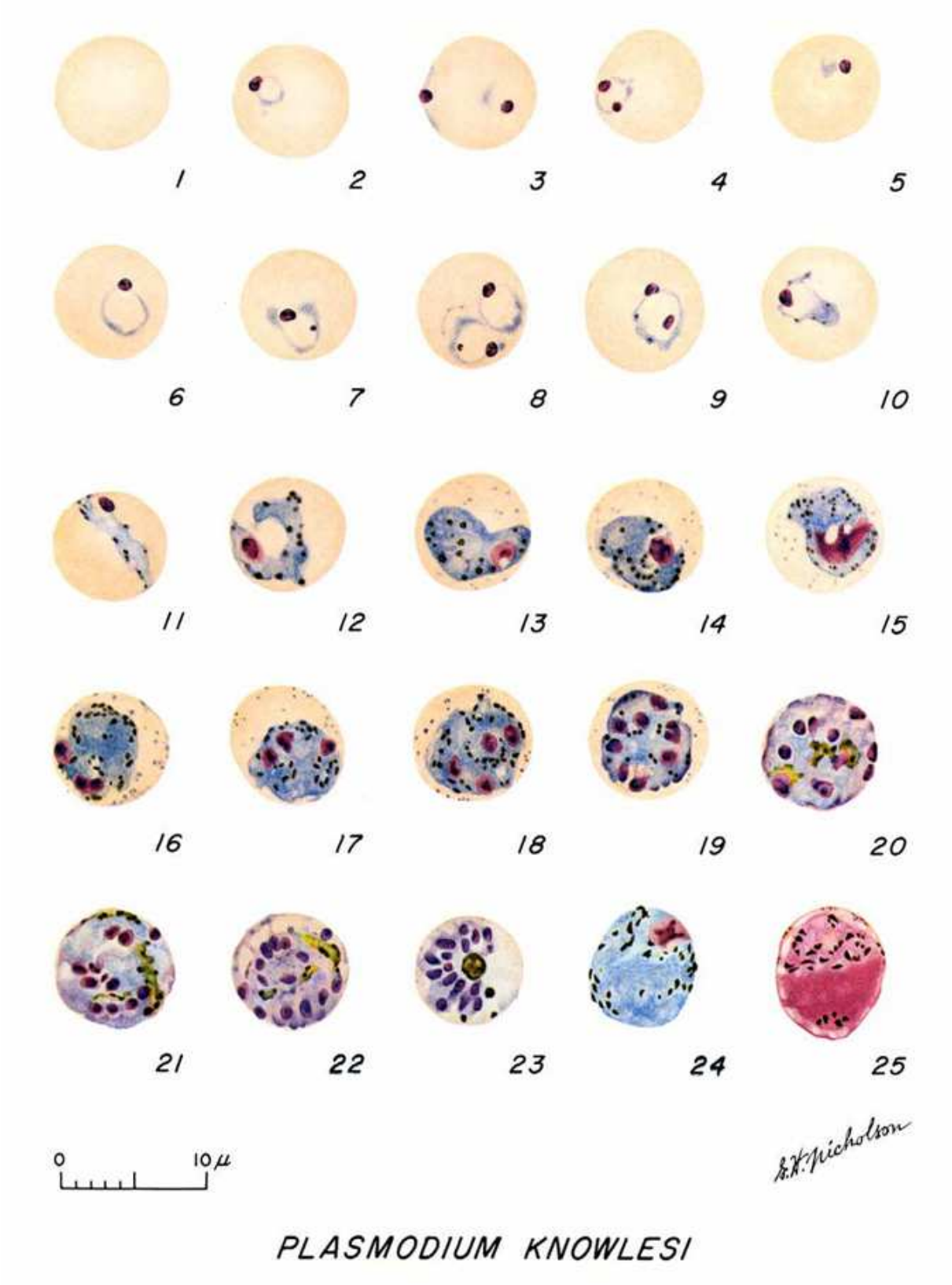

Fig. 10: Blood cycle of *P. knowlesi* (Source: Coatney 1971)

4.8.3 Epidemiology

In 1965 the first human case of a *P. knowlesi* was reported in a male American soldier working in the rainforests of Peninsular Malaysia (Chin et al. 1965). The patient documented anorexia, fatigue, nausea, chills, fever and sweating as initial symptoms. The patient returned to the USA. At the first microscopic blood analysis rings only were observed, whereas at the second analysis band forms were observed and misdiagnosed as *P. malariae*. However, blood was referred to the CDC and was there diagnosed as *P. knowlesi* accidentally. In 1971 a second suspected case was reported from Peninsular Malaysia (reviewed in Kantele et al. 2011).

However, it took until 2004 when 120 human *P. knowlesi* cases were diagnosed with molecular based tools by the Kuching group around Balbir Singh and Janet Cox-Singh. Since those reports many other cases were reported at epidemiological studies in South-East Asia, and in tourists (Tab. 3).

Tab. 3: Reported *P. knowlesi* cases in human (revised after Kantele et al. 2011)

Country	Local	Tourists
Malaysia/Borneo	707 [a]	4 [b]
Brunei/Borneo		1
Indonesia/Borneo	1	1
Malaysia/Peninsular	89	1
Thailand	11	1 [c]
Myanmar	33	
Vietnam	5	
Philippines	5	1
unknown		1
Overall	857	9

[a] including 137 cases reported by Barber et al. (2011).

[b] including 1 case documented by Hoosen and Show (2011).

[c] case reported by Berry et al. (2011)

4.9 Diagnosis of malaria

4.9.1 Microscopy

Clinical diagnosis of malaria is the least expensive form, but it is inaccurate and often leads to misdiagnosis due to confusion with other tropical diseases (Wongsrichanalai et al. 2007). Microscopy still remains the gold standard in malaria diagnosis (WHO 2009b). Malaria diagnosis by microscopy is based on detecting and identifying malaria parasites to species level in blood films or thick smears. For thin smears a blood drop of the finger prick is spread on a grease-free microscopic slide, air dried and fixed with methanol before staining with Giemsa. For thick films a capillary blood drop is spread and air dried. Afterwards the red blood cells are lysed with water to remove the haemoglobin, and stained with Giemsa. Thick films are more sensitive with a sensitivity of 4-20 parasites/µl in highly expertise laboratories, and 50-100 parasites/µl under field conditions (Payne 1988; Dowling and Shute 1966; WHO 1988; Milne et al. 1994). Regarding the specificity thin films (diagnosis to species level) are more accurate compared to thick films.

Malaria microscopy is cost-effective and provides results within a short time period. Furthermore, it allows an accurate diagnosis up to species level, gives information about the parasite density, and the result has a direct impact on the patient's treatment (WHO 2005b). Microscopes are available in most villages and small laboratories (WHO 2010b). However, certain problems are well known; microscopists need accurate training to be able to accurately diagnose malaria to species level – even at low parasitemias; unfortunately, the training is often insufficient due to limited resources.

4.9.2 Rapid Diagnostic Tests (RDTs)

Within the last decades several antigen detection techniques for the diagnosis of malaria were employed. RDTs in form of card tests, cassettes or dipsticks are simple to use, easily transportable, relatively cheap, rapid, specific and more or less sensitive (White 2003). Furthermore, RDTs can be stored at high temperatures, which are common in malaria endemic countries. More than 25 million RDTs were used for malaria diagnosis in 2005 (WHO 2006). RDTs are based on the detection of malaria specific antigens in the blood, namely histidin rich protein 2 (HRP2), pLDH (parasite lactate dehydrogenase) and aldolase.

HRP2 based RDTs are specific for *P. falciparum* and the most common used RDTs. Its sensitivity was described to be similar to microscopy (White and Silamut 1989; Tjitra et al. 2001). However, HRP2 clears very slowly and RDTs remain positive for up to one month after successful falciparum malaria treatment (Mayxay et al. 2001). Furthermore, this test remains negative in patients with rare antigen variations of HRP2 (Baker et al. 2005).

pLDH-RDTs are antigen-capture tests based on monoclonal antibodies to pLDH, and are used for the diagnosis of *P. falciparum* (*Pf*LDH) or as pan-malaria test (WHO 2009). pLDH rapidly clears from blood, and the immunochromatographic tests reveal negative results within days after successful treatment. Aldolase based RDTs are used for pan-malaria diagnosis, but are less sensitive than other RDTs (Cho et al. 2011).

In conclusion, RDTs can not be considered as gold standard in malaria diagnosis, but are an essential diagnostic tool in the field outside of health facilities, where no microscope is available.

4.9.3 Molecular tools for the diagnosis of *Plasmodium* sp.

With the development of the polymerase chain reaction (PCR) technique a new era in the diagnosis of *Plasmodium* sp. was initiated. This method was developed by Kary Mullis in 1983 and is a scientific technique which allows the amplification of a single or few copies of DNA to millions of copies of a partial DNA sequence (Bartlett and Sterling, 2003). A PCR consists of cycles of repeated heating and cooling steps (Denaturation, Annealing and Extension) to melt DNA and replicate DNA with the use of enzymes (= polymerase). Therefore short DNA-fragments (oligonucleotids) - the so called primers - are needed to serve as starting points for DNA synthesis (Fig 11).

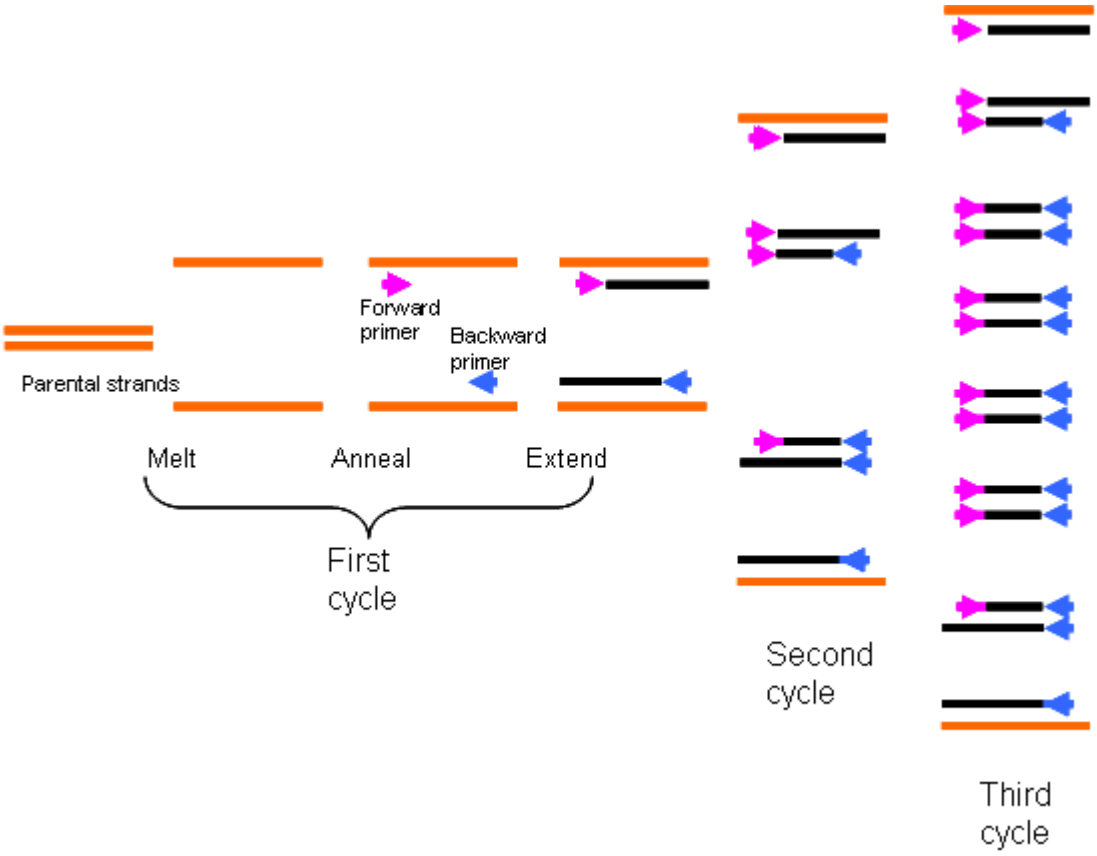

Fig 11: Principles of a PCR reaction (Source: http://www.obgynacademy.com/basicsciences/fetology/genetics/)

4.9.3.1 Diagnosis of *Plasmodium* sp. by nested PCR of the SSU rRNA gene

4.9.3.1.1 NP-1993

Genes of the small subunit ribosomal RNA (SSU rRNA) are highly conserved regions, which allow not only a discrimination of different *Plasmodium* species, but can also be used for phylogenetic characterization of a wide range of different *Plasmodium* (Snounou and Singh 2002). For the diagnosis of human malaria parasites in 1993 the group around Snounou developed a nested PCR technique (NP-1993 protocol) binding in the SSU rRNA gene (Snounou et al. 1993). Soon this technique became one of the most used and standardized PCR techniques for the detection and determination of different human malaria parasites. The lower limits of detection were reported to be between a single parasite in 10 µl blood (0.000002% parasitemia) and 6 parasites/µl (Snounou and Singh 2002, Singh et al. 1999). The NP-1993 protocol seemed to be perfectly suitable for epidemiological studies. In the NP-1993 species-specific rPLU5 and rPLU6 were used for the Nest 1 PCR reaction amplifying a product of about 1,200 bp in size (Snounou 1993). For species determi-

nation the primers rFAL1 and rFAL2 for *P. falciparum* (206 bp), rVIV1 and rVIV2 for *P. vivax* (121 bp), rMAL1 and rMAL2 for *P. malariae* (145 bp) and rOVA1 and rOVA2 for *P. ovale* (787 bp) were used.

Several modifications of the NP-1993 protocol followed (Singh et al. 1996, Cox-Singh et al. 1997). In 1999 the group around Balbir Singh updated the NP-1993 protocol (Singh et al. 1999). Instead of the genus-specific Nest 1 primer rPLU6 they used the primer rPLU1. The Nest 1 product resulting at the usage of the primers rPLU1 and rPLU5 has a size of 1,670 bp, and allowed the introduction of a genus-specific Nest 2 PCR with the primers rPLU3 and rPLU4 (240 bp). Instead of five PCRs only two PCRs were needed to detect if a blood sample is positive for any species of *Plasmodium* or not.

4.9.3.1.2 NP-2002 – The problem in the diagnosis of *P. ovale* becomes obvious

It became obvious that the NP-1993 had some limitations in the diagnosis of *P. ovale*. Some patient samples which were positive for *P. ovale* in microscopy gave negative results within the nested PCR and so the protocol was updated in 2002 (Snounou and Singh 2002). The primers for the Nest2 species analysis of *P. ovale* were changed to a genus-specific primer rPLU2 combined with rOVA1. Furthermore, the NP-2002 involved the genus-specific Nest2 primers rPLU2 and rPLU3 (Fig. 12).

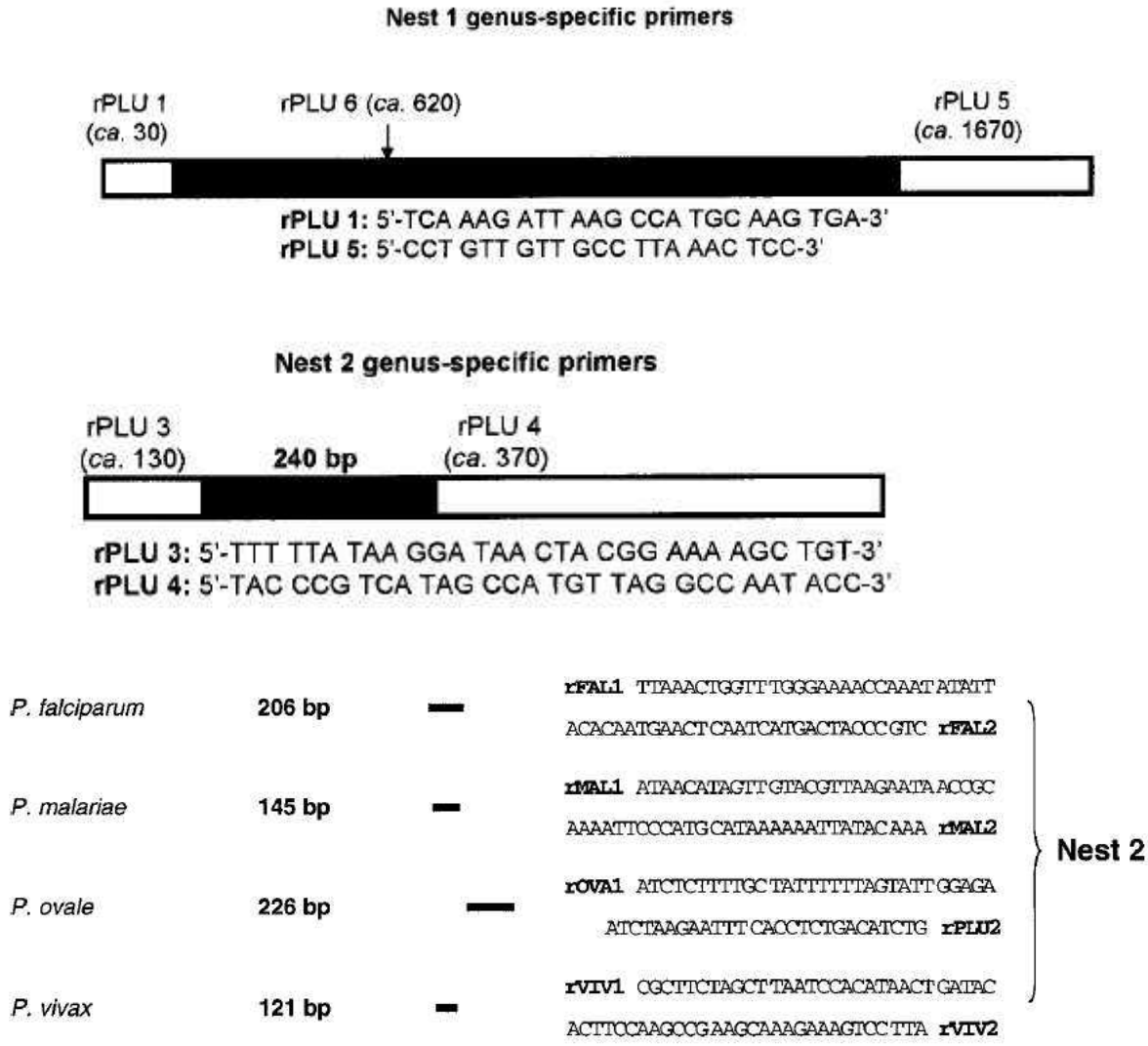

Fig. 12: Schematic representation of the primers used in the nested PCR of the SSU rRNA gene according to the NP-2002 protocol (Snounou and Singh, 2002; Singh et al. 1999)

4.9.3.1.3 NP-2005 – The problem with *P. ovale* proceeds

Until 2005 more than 14 different protocols were published for the diagnosis of *P. ovale* and it became obvious that *P. ovale* has to be divided into the classic and the variant type. In 2005 the group around Calderaro introduced the NP-2005 protocol. Instead of the use of the rPLU2 and rOVA1 primers they recommended the use of two primer pairs: rOVA1v and rOVA2v for the diagnosis of the variant type of *P. ovale* and rOVA1 and rOVA2 (NP-1993 primers) for the determination of the classic form of *P. ovale*. A comparison of the ovale-specific primers NP-1993, NP-2002 and NP-2005 is given in Fig. 13.

Age (yr)	Country of origin	Country visited	Latency[a] (mo)	Parasitemia (%)	Microscopy result[b]	PCR result				
						Genus-specific PCR (NP-2002)	*P. ovale*, NP-1993[c]	*P. ovale*, NP-2002	*P. ovale*, NP-2005	Other species, NP-2002[b]
40	Italy	Senegal	5	–	–	+	+	+	+	–
4	Ghana	Ghana	NA[e]	<0.01	Pf	+	+	+	+	–
NA	Italy	Africa[d]	24	0.064	Pv	+	+	+	+	–
24	Mozambique	Mozambique	NA	0.2	Pv	+	+	+	+	–
NA	Italy	NA	NA	0.1	Pv	+	+	+	+	–
27	NA	NA	NA	<0.1	Pv	+	+	+	+	–
22	Italy	Ghana	NA	0.14	Pv	+	+	+	+	–
26	Cameroon	Cameroon	5	<0.01	Po	+	+	+	+	–
30	Cameroon	Cameroon	3	0.12	Po	+	+	+	+	–
35	Nigeria	Nigeria	0.1	0.24	Po	+	+	+	+	–
25	Burkina Faso	Burkina Faso	0.25	<0.01	*Plasmodium* spp.	+	+	+	+	Pf + Pm
10	Ivory Coast	Ivory Coast	2	0.5	Pf	+	+	+	+	Pf
26	Ivory Coast	Ivory Coast	NA	<0.1	Pv	+	+	+	+	–
29	NA	N/A	NA	<0.004	Pv or Po	+	+	+	+	–
19	Burkina Faso	Burkina Faso	NA	<0.001	Pf	+	+	+	+	Pf + Pm
42	Italy	Tanzania	NA	<0.1	Po	+	–	+	+	–
51	Ghana	Ghana	NA	0.3	Pv	+	–	+	+	–
63	Italy	Unknown	NA	0.2	Po	+	–	+	+	–
24	Cameroon	Cameroon	6	0.07	Po	+	–	+	+	–
23	Cameroon	Cameroon	NA	0.2	Po	+	–	+	+	–
30	Ivory Coast	Ivory Coast	NA	0.037	Po	+	–	–	+	–
29	Cameroon	Cameroon	5	–	–	+	–	–	+	–
3	Burkina Faso	Burkina Faso	NA	–	–	+	–	–	+	–

Fig. 13: Comparison of different NP-protocols for the diagnosis of *P. ovale* (Source: Calderaro et al. 2005).

4.9.3.1.4 *P. knowlesi* – Problems in the diagnosis of this newly recognized malaria parasite in human

Within the last years *P. knowlesi*, an originally classified simian parasite in South-East Asia, was recognized as the fifth human malaria parasite (White 2008). Especially the Kuching group around Balbir Singh and Janet Cox-Singh worked with this parasite and its molecular detection. For the diagnosis of *P. knowlesi* primers Pmk8 and Pmk9r were developed which bind within the region of the NP-2002 Nest 1 product (Singh 2004). However, this primer pair was found to cross-react with *P. vivax* genomic DNA within the ssrRNA-S gene (Fig. 14). A new set of primers, namely PkF1140 and PkR1550 was designed which can be used for secondary PCR reactions after primary amplification with rPLU1 and rPLU5 (Fig. 15) (Imwong et al. 2010).

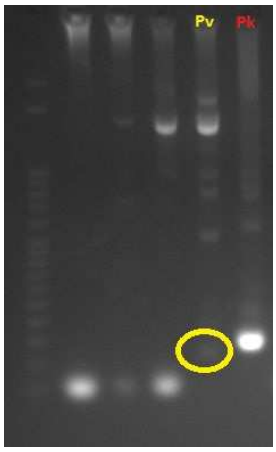

Fig. 14: Visualized amplicons of the primers Pmk8 and Pmk9r, not only binding *P. knowlesi* (Pk) but also *P. vivax* (Pv)

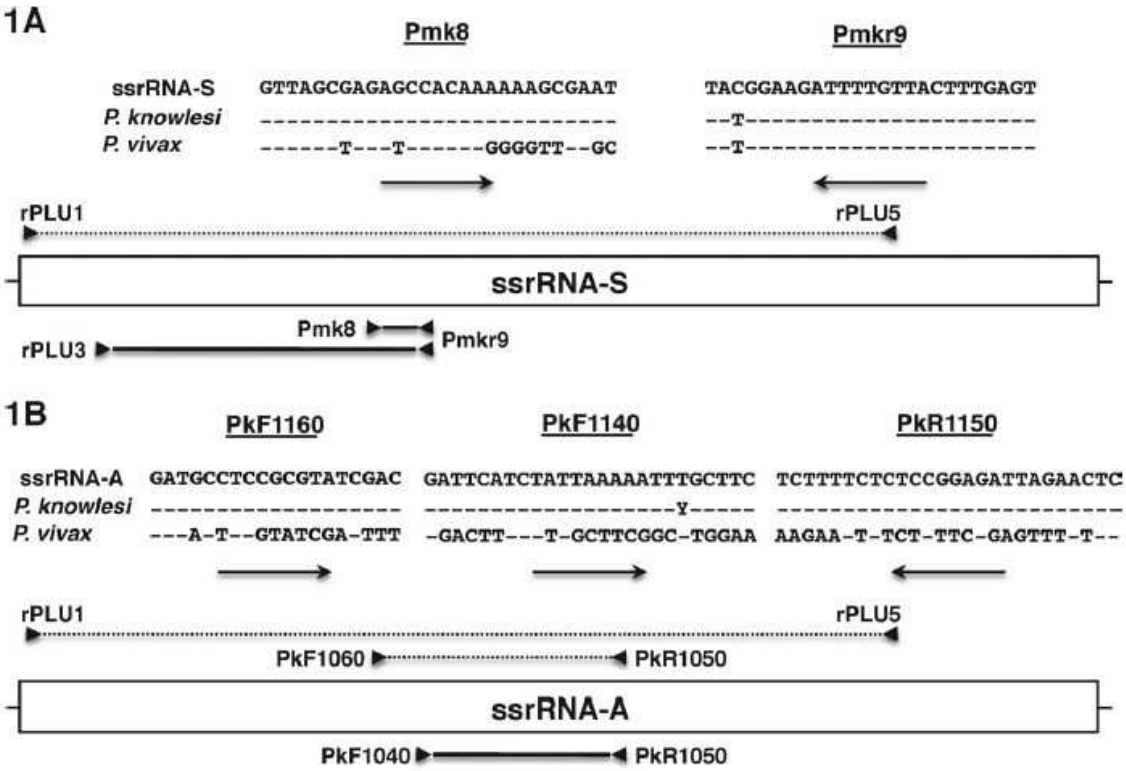

Fig. 15: Primers for diagnosis of *P. knowlesi* within the ssrRNA genes (Source: Imwong et al. 2010).

4.10 Molecular phylogeny of *Plasmodium* sp. using the mitochondrial genome

Phylogenetic studies of the genus *Plasmodium* are often based on genes of the mitochondrial DNA. Mitochondrial genomes of different systematic groups vary in structure, size and organization. Those of plants are known to be very large (up to 2,400 kb), whereas the mitochondrial genome of *Plasmodium falciparum* is the smallest one with only 6 kb (Knoop and Müller 2009). The mitochondrial genome of malaria parasites is repeated tandemly and linear (Wilson and Williamson 1997). It encodes the small- and large subunit rRNA (SSU rRNA and LSU rRNA) genes and only 3 protein-coding genes: cytochrome c oxidase 1 (cox1) and 3 (cox3), and cytochrome b (cyt b) (reviewed in Hikosaka et al. 2011).

Many studies have examined the phylogeny of *Plasmodium* in the last years, and revealed new conclusions when compared to the morphologically based systematic before the introduction of molecular techniques. Only a short overview of the newest and most important approaches in phylogeny of *Plasmodium* based on mitochondrial genes is mentioned below.

Recent studies revealed that most simian parasites (e.g. *P. knowlesi*) are closely related to *P. vivax*, although their morphology differs (Fig.18; LeClerk 2004). For example, *P. simiovale* looks very similar to *P. ovale* and was named after their morphological compatibility but genetic analysis revealed that *P. simiovale* is closely related to *P. vivax* and not *P. ovale* (Fig. 16 and Fig. 19). Furthermore the zoonotic potential of human malaria parasites was made visible by way of molecular analysis. Duval et al. (2010) confirmed the presence of *P. falciparum*, *P. ovale* and *P. malariae* in African Great Apes (Fig. 16). *P. brasilianum*, a simian parasite of New World monkeys, was found to be genetically identical to *P. malariae* (Fig. 17; Tazi et al. 2011, Escalante et al. 1995). It was shown that several malaria species can switch hosts, and this had an impact on the relatively quick evolutional diversification of malaria lineages (Hayakawa et al. 2008). Molecular techniques also allowed the introduction of the 5th and "6th" human malaria parasite. In South-East Asia *P. knowlesi* was diagnosed in humans with molecular tools, and based on its molecular dimorphisms *P. ovale* was divided into two new species, namely *P. ovale wallikeri* and *P. ovale curtisi* (Fig 19; Sutherland et al. 2010).

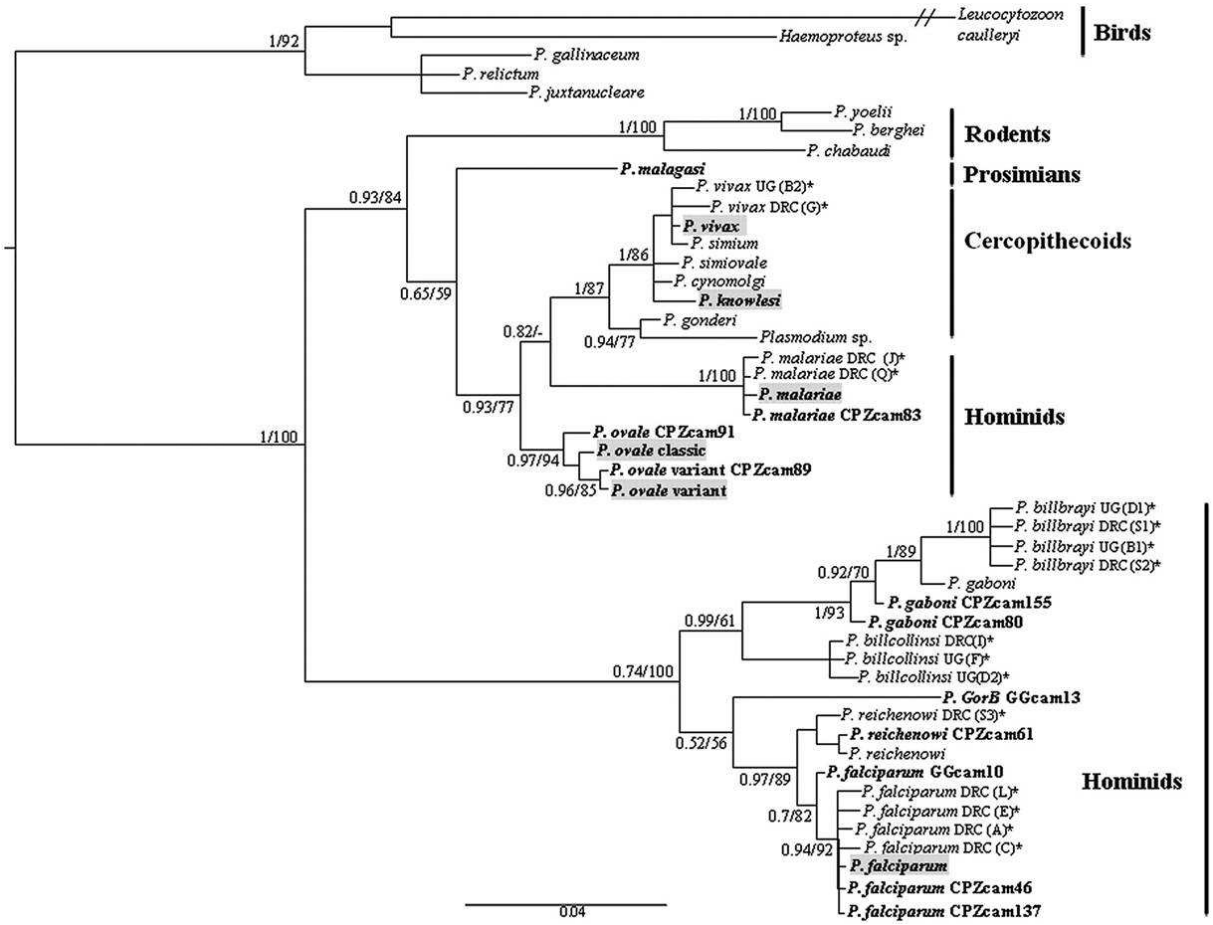

Fig 16: Phylogeny of malaria parasites based on cyt b and cox1 sequences (Bayesian Values/maximum likelihood) (Duval et al. 2010).

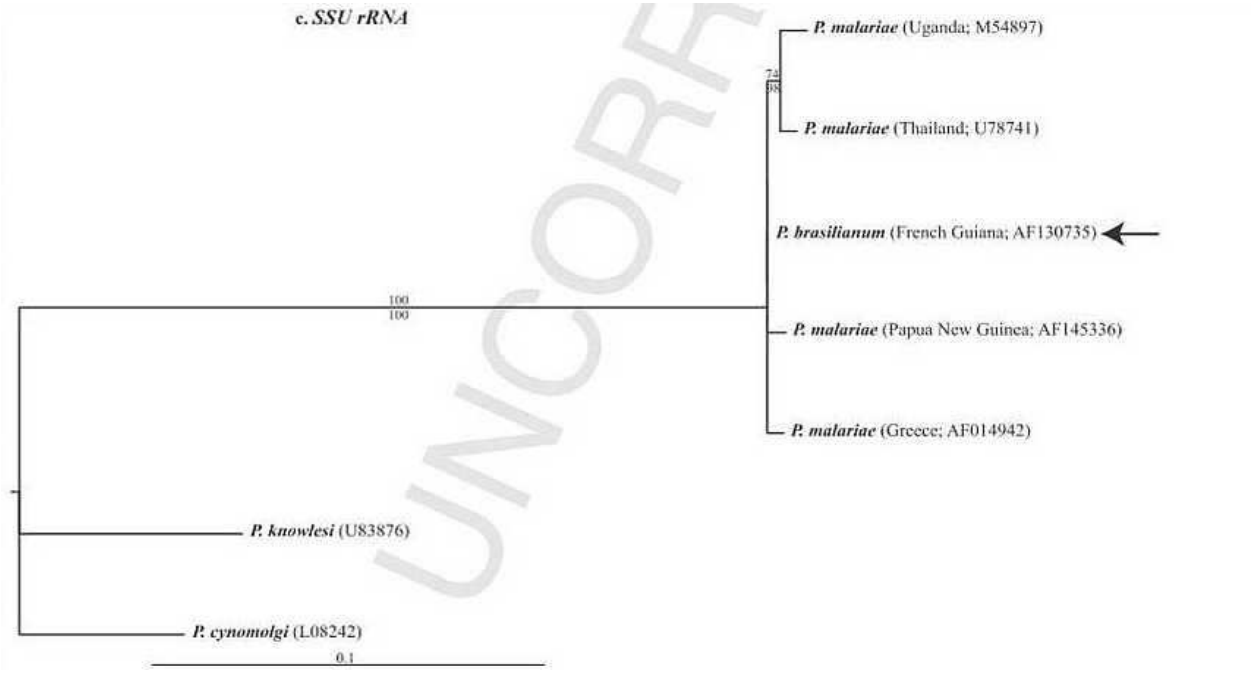

Fig 17: Phylogenetic relationship among *P. malariae* and *P. brasilianum* based on the SSU rRNA gene (Tazi et al. 2011).

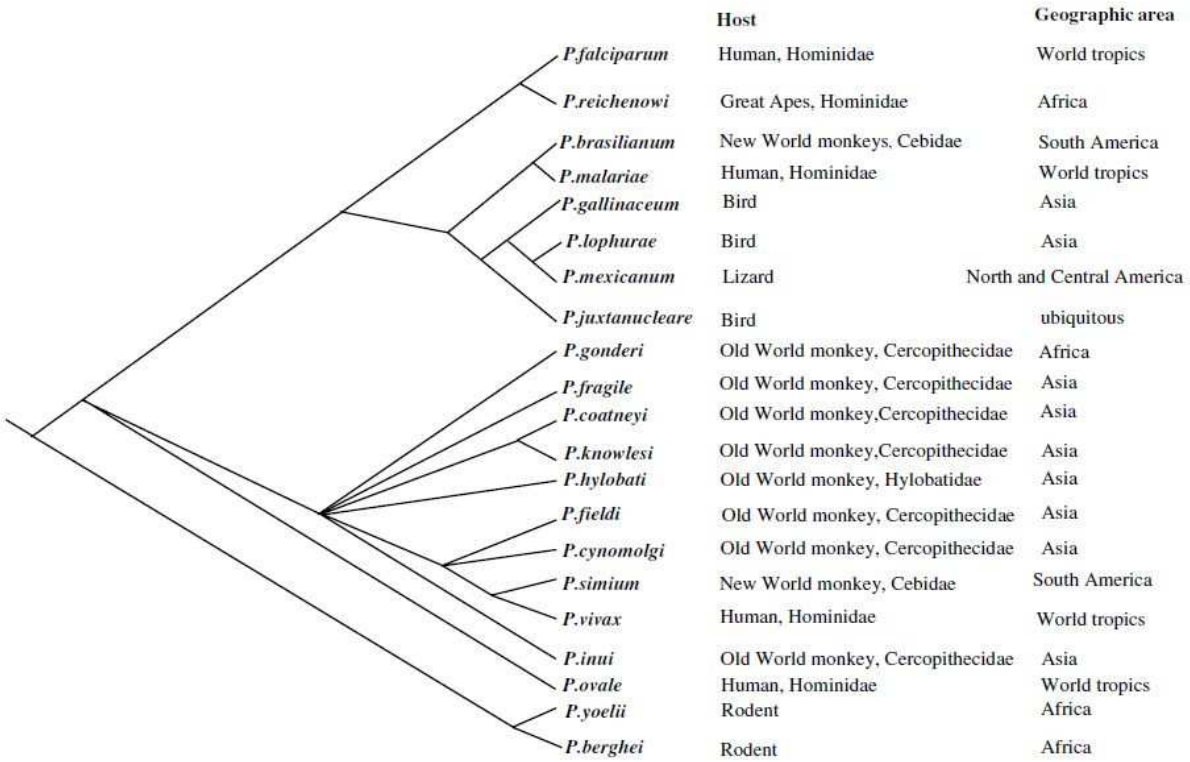

Fig. 18: SSU rRNA based phylogeny of the genus plasmodium with a cladistic consensus tree (Leclerk et al. 2004).

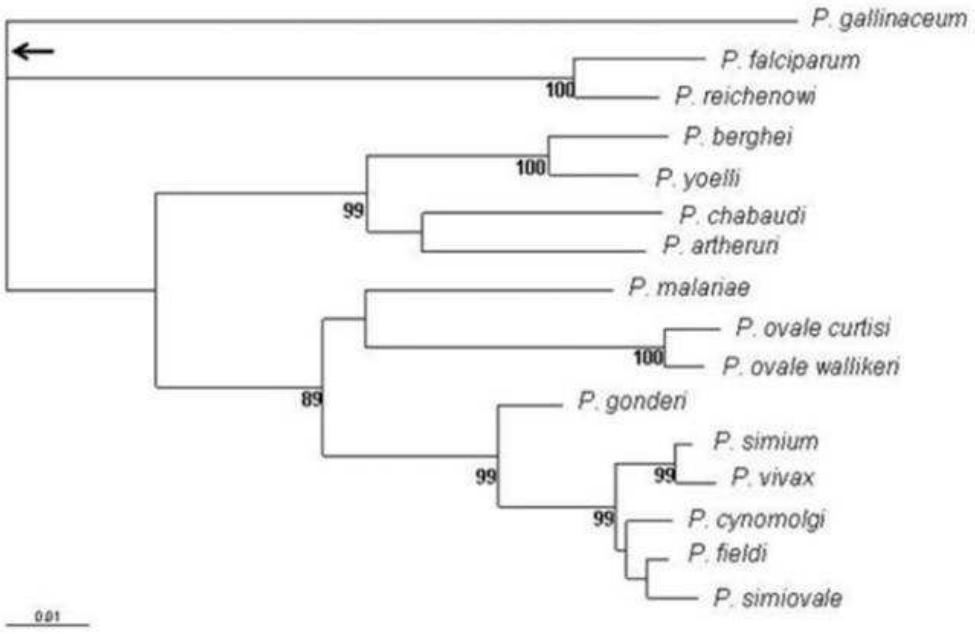

Fig. 19: Unrooted phylogenetic tree based on the mitochondrial cyt b gene (single optimum bifurcating tree) (Sutherland et al. 2010).

4.11 Malaria treatment, drug resistances and drug susceptibility

Table 4 gives an overview on the drugs used for the treatment of malaria parasites.

Tab. 4: Drugs recommended for malaria treatment (Source: Kasper et al. 2005)

Drug	Uncomplicated Malaria (Oral)	Severe Malaria[a] (Parenteral)
Chloroquine[b]	10 mg of base/kg followed by 10 mg/kg at 24 h and 5 mg/kg at 48 h *or* by 5 mg/kg at 12, 24, and 36 h (total dose, 25 mg/kg); for *P. vivax* or *P. ovale*, primaquine (0.25 mg of base/kg per day for 14 days[d]) added for radical cure	10 mg of base/kg by constant-rate infusion over 8 h followed by 15 mg/kg over 24 h *or* by 3.5 mg of base/kg by IM or SC injection every 6 h (total dose, 25 mg/kg)[f]
Amodiaquine[b]	15 mg of base/kg followed by 10 mg/kg per day at 24 and 48 h (total dose, 35 mg/kg)	—
Sulfadoxine/ pyrimethamine[b]	25/1.25 mg/kg, single oral dose (3 tablets for adults)	—
Mefloquine[b]	15 mg/kg followed 8–12 h later by second dose of 10 mg/kg	—
Quinine	10 mg of salt/kg q8h for 7 days combined with tetracycline[e] (4 mg/kg qid) or doxycycline (3 mg/kg once daily) or clindamycin (10 mg/kg bid) for 7 days	20 mg of salt/kg by IV infusion over 4 h[f] followed by 10 mg/kg infused over 2–8 h every 8 h
Quinidine gluconate	—	10 mg of base/kg by constant-rate infusion over 1–2 h followed by 0.02 mg/kg per min, with ECG monitoring[g]
Artesunate	In combination with 25 mg of mefloquine/kg, 12 mg/kg given in divided doses over 3–5 days (e.g., 4 mg/kg for 3 days or 4 mg/kg followed by 2 mg/kg per day for 4 days); if used alone or in combination with clindamycin or doxycycline, give for 7 days (usually 4 mg/kg initially followed by 2 mg/kg daily)	2.4 mg/kg IV or IM stat followed by 1.2 mg/kg at 12 and 24 h and then daily (or 2.4 mg/kg once daily)
Artemether	Same regimen as for artesunate	3.2 mg/kg IM stat followed by 1.6 mg/kg per day
Atovaquone-proguanil (Malarone)	For adults >40 kg, each dose comprises 4 tablets (each tablet containing atovaquone 250 mg and proguanil 100 mg) taken once daily for 3 days with food	—
Artemether-lumefantrine	For adults ≥35 kg, each dose comprises 4 tablets (each tablet containing artemether 20 mg and lumefantrine 120 mg) at 0, 8, 24, 36, 48, and 60 h, taken after food	—

Multidrug resistances in *Plasmodium falciparum* compromise malaria treatment and control. By now resistances to chloroquine are widespread around the tropics. Resistances to mefloquine and sulfadoxine/pyrimethamin are observed frequently (Astelbauer and

Walochnik 2011, Marma et al. 2010). Recently first reports of resistances to artemisinine and artesunate-mefloquine were documented (Noedl et al. 2008; Noedl et al. 2009; Noedl et al. 2010; Dondorp et al. 2009; Wongsrichanlai and Meshnick 2008).

Chloroquine can be used for the treatment of blood forms of *P. vivax* and *P. ovale*. In areas where chloroquine-resistances are known in *P. vivax* ACTs should be used. Amodiaquine and mefloquine are also effective against *P. vivax* blood forms. For the treatment of hypnozoites of *P. vivax* and *P. ovale* primaquine should be given for 14 days to patients without G6PD-deficiency only. Tavenoquine also possesses activity against hypnozoites (reviewed in Astelbauer and Walochnik 2011).

To cure malaria caused by *P. malariae* chloroquine can be used, although care should be taken. First reports of resistances in *P. malariae* to chloroquine were examined in Indonesia (Maguire et al. 2002).

Until now human *P. knowlesi* malaria cases were treated with chloroquine, quinine + doxycycline, mefloquine, and atvaquone + proguanil (Kantele et al. 2011, Kantele et al. 2008). A recent study documented full activity of chloroquine against *P. knowlesi* (Daneshvar et al. 2010).

To test the efficacy of drugs *in vitro* cultivation of *Plasmodium* sp. is an essential tool (WHO 2007). *P. falciparum* is easy to cultivate – even under field site conditions. Trager and Jensen established the cultivation of this pathogen in 1976 and modified protocols of this technique are still in use.

However, until now there are no *in vitro* cultivation techniques for *P. vivax* allowing exponential parasite growth for at least 72 hours, which would be necessary to test the efficacy of slow acting drugs. Various parasite species possess different preferences to its host cells – e.g. *P. vivax* and *P. ovale* prefer the invasion of reticulocytes, whereas *P. malariae* shows a tendency to invade older erythrocytes. The problems regarding the cultivation of *P. vivax* are discussed in Chapter 8.5. Several authors reported limited success in the cultivation of *P. ovale* and short time cultivation of *P. malariae* (Ringwald et al. 1997; Tanariya and Pasuralertsakal 1994). Recent studies used modified schizont maturation assays for the cultivation of *P. malariae* and *P. ovale* (Siswantoro et al. 2011).

4.12 Bangladesh

The people's republic of Bangladesh is a South Asian country (20° and 27°N and longitudes 88° and 93°E) with an area of 147,569 km^2. It is almost entirely surrounded by India (West Bengal, Assam, Meghalaya, Tripura and Mizoram), shares a 193 km border with Myanmar in the Southeast and the Bay of Bengal (Indian Ocean) in the South. Bangladesh has a population of 164.4 million people of whom 60% are below the age of 25 years. The population density is 1,100 people/km^2 (8^{th} densest in the world). 98% of the population are Bengali with Bangla as mother tongue and 90% of the population are Muslims. The total fertility rate is high at 2.5 children per woman. Two thirds of the population are farmers and 75% of the Bangladeshi population live outside larger cities. The literacy rate is increasing, but is still low at 53.5% in 2007 (UNDP 2009). For both male and female life expectancy is 63 years (WHO 2005).

Bangladesh is a Northern Tropic country with seasonal climate: warm and humid monsoon from June to October, mild winter from October to March, and hot, humid summers from March to June.

4.12.1 The Chittagong Hill Tracts and the District of Bandarban

Van Schendel et al. (2001) described the area as following: "South Eastern Bangladesh consists of two different distinct geographical parts. On the one hand there is a long narrow plain along the Bay of Bengal, on the other hand there is a mountainous region with hills up to 1,200 m – the so called Chittagong Hill Tracts (CHTs)". The CHTs have an area of 13,180 km^2 and can be divided into the districts Bandarban, Rangamati and Khagrachhari.

The boundary between the plain area in the West and the hilly region in the East is not only a geographic one. There is also a cultural division of the South Asian and the South East Asian cultural rims. Most of the plain areas in Bangladesh are inhabited by Bengalis, whereas the CHTs are home to different non-Bengali ethnical minorities like Marma, Chakma, Tripura and Mru (Tab. 5) (van Schendel et al. 2001). There is also a cultural differentiation within the various ethnical groups (e.g. food, religion).

Tab. 5: Population of the Chittagong Hill Tracts (Source: van Schendel et al. 2001)

Group	Main religion	Population (1981)
Chakma	Buddhism	230,000
Taungchengya	Buddhism	20,000
Marma	Buddhism	120,000
Sak	Buddhism	1,500
Khyeng	Community religion	1,500
Tripura	Hinduism	40,000
Riang/Brong	Hinduism	10,000
Mru	Community religion	20,000
Khumi	Community religion	1,000
Bawn	Christianity	8,000
Pangkhua	Christianity	2,000
Lushai/Mizo	Christianity	1,000
Bengali	Islam	290,000
Total		**745,000**

The district of Bandarban itself is located in the Southeast of the Chittagong Hill Tracts (Chittagong Division). Bandarban District comprises an area of 4,479 km^2 and is bounded by Arakan (Myanmar) and the river Naf to the south, Arakan and Rangamati district to the east, Rangamati District to the north, and Chittagong and Cox's Bazar district to the west. Administratively it is divided into 7 upazilas (sub-districts). Those 7 subdistricts are Bandarban, Alikadom, Naikhongchari, Ruma, Thanchi, Rowangchari and Lama. The area of Bandarban capitol is inhabited by approximately 32,000 people and is 51.80 km^2 in size. Bandarban is not only the remotest district of the country, but also is the least populated one and its natural environment harbours a high variety of plants and animals in its rainforests.

The Malaria Research Initiative Bandarban (MARIB) - a field site of the Medical University of Vienna (in collaboration with different other organisations e.g. WHO,

ICDDR,B) - is based at the Bandarban Sadar Hospital in Bandarban (Fig. 20). The Bandarban Sadar Hospital is a district hospital with outpatient and inpatient facilities. There are 100 beds in inpatient departments and more than 10 physicians work at the hospital. The majority of severe malaria patients within the region are referred to this hospital, and about 4,000 falciparum malaria cases are treated there every year.

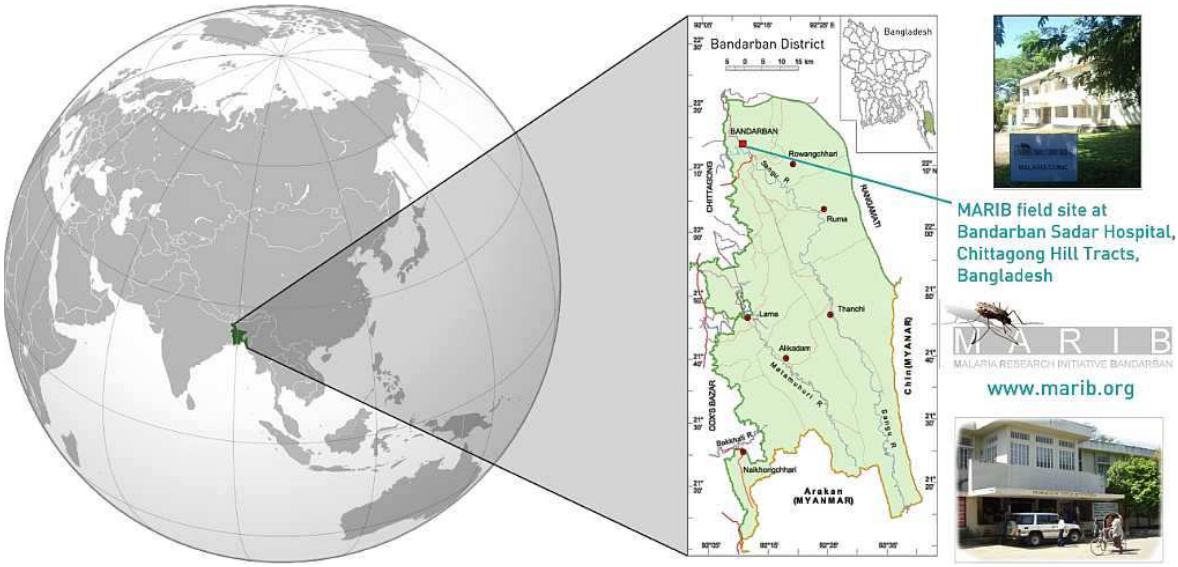

Fig. 20: Location of the MARIB field site in Bandarban District/Bangladesh (map by Markus Fally©)

4.13 Malaria Situation in Bangladesh

In Bangladesh 55 million people bear low or high risk of being infected with malaria parasites, and 11.4 million thereof reside in high risk areas. In 2009 more than 569,000 suspected malaria cases were documented, of which 79,853 were confirmed. Blood of over 553,000 patients was analyzed by either microscopy or RDT. Of those 63,873 were diagnosed positive for malaria by RDTs (38,670) and microscopy (25,203). Only two species were officially reported from Bangladesh in 2009, namely *P. falciparum* (18,242 confirmed cases) and *P. vivax* (6,853 confirmed cases). In comparison to the years before, mortality decreased and caused 47 deaths in 2009 (WHO 2010c).

Eradication programs in the 1970s eliminated malaria in 51 out of 63 districts in Bangladesh. In the 1990s malaria re-emerged and became one of the major health problems in rural Bangladesh. Malaria is concentrated in 13 hilly and forested districts along the borders to Myanmar and India, where 98% of all fatal cases had their origin. In the late 1990s approximately 900,000 clinical malaria cases were documented, of which only 70,000 were confirmed by microscopy (WHO 1999). Because of a lack of surveillance and information regarding this disease, the malaria situation in Bangladesh was underestimated (Bangali et al. 2000; Faiz et al. 2002). In 2005 over 240,000 clinical malaria cases were documented; of those 48,121 were clinically confirmed and 501 were of fatal outcome. Because of different types of occupation (e.g. hunters, wood cutters) more men than women (ratio 54.9% to 45.1%) were affected. *P. falciparum* is the dominant *Plasmodium* species in Bangladesh causing about 70% of all malaria cases (WHO 2005). In 2005 WHO concludes that the malaria situation in Bangladesh and especially in the Chittagong Hill Tracts is getting worse. Tab. 6 gives an overview on the malaria situation in the District of Bandarban. Resistances to chloroquine and sulfadoxine/pyrimethamine are common in those malaria endemic areas, and the Ministry of Public Health introduced an artemisinin-based combination therapy as first line treatment of uncomplicated falciparum malaria in Bangladesh (WHO 2005; Attlmayr et al. 2006; Thriemer et al. 2006). A recent surveillance study based on the diagnosis of malaria with RDTs examined a countrywide prevalence of *P. falciparum* of 3.58%, and a prevalence of 0.21% was observed of *P. vivax* (Haque et al., 2009).

Until the start of this thesis project – which was the first PCR corrected cross-sectional malaria surveillance in Bangladesh – virtually all malaria cases were diagnosed as *P. falci-*

parum or *P. vivax* (5-8). *P. malariae* was first documented in Bangladesh in 2004 with a prevalence of 1% in the Chittagong Hill Tracts (van der Broek et al. 2004). In 2010 one severe *P. malariae* case was reported from Chittagong (Rahman et al. 2010). However, before the start of our studies, neither *P. ovale* nor *P. knowlesi* were discussed or documented to be endemic in Bangladesh.

Table 6: Officially reported malaria cases of microscopically confirmed malaria in Bandarban District from 2003-2008 (does not include malaria diagnosed by NGOs, private laboratories or self-treatment).

Year	**Malaria slides examined**	**Microscopically confirmed malaria**	***Plasmodium vivax***	***Plasmodium falciparum***	**Severe Malaria**	**Deaths**
2003	39,075	17,357	2,001 (11,53%)	15,356 (88,47%)	8,451	108
2004	37,742	14,460	1,735 (12,00%)	12,725 (88,00%)	7,424	81
2005	35,505	12,921	1,318 (10,20%)	11,603 (89,80%)	7,291	88
2006	29,943	9,483	1,131 (11,93%)	8,352 (88,07%)	8,620	71
2007	30,859	8,507	1,149 (13,5%)	7,338 (86,26%) + Mixed infections 20 (0,24%)	8,616	53
2008	47,216	9,333	1,387 (14,9%)	7,691 (82,4%) Mixed infections 255 (2,7%)	2,883	11

5 Aims

The primary aims of this study were to determine the distribution, prevalence and phylogeny of the less common human malaria parasites *P. ovale*, *P. malariae* and *P. knowlesi* in the Chittagong Hill Tracts in Bangladesh, and to establish new techniques for malaria diagnosis and *P. vivax* cultivation.

The first aim was to examine the distribution of *P. ovale*, *P. malariae* and *P. knowlesi* in fever patients in Bangladesh. For this purpose a standardized nested PCR binding in conserved regions of the mitochondrial SSU rRNA genes was employed to classify positive malaria samples collected on filter paper to species level.

The second aim was to establish a novel PCR technique using blood spots on filter paper which not only allows a faster implementation, but also results in a higher sensitivity compared to standard PCR tools. For this purpose the value of Direct PCR was examined and compared to a standard PCR technology.

The third and most important aim was to examine the distribution of a newly discovered pathogen in Bangladesh (in 2010 the formerly known species *P. ovale* was divided by the group around Sutherland et al. into *P. ovale curtisi* -classic form- and *P. ovale wallikeri* -variant form) and to analyze the phylogeny of these parasites for the first time in South Asia. To determine the presence of these pathogens, more than 2,000 blood samples of asymptomatic participants and febrile patients were analyzed with different PCR techniques (different protocols at the SSU rRNA gene, PoTRA gene) and compared to each other. *P. ovale* positive monoinfections were further examined by analysis of partial sequences of the SSU rRNA, Cox1 and Porbp2 genes, compared to each other and to previously published sequences. Furthermore, a closer look at clinical syndromes of infections with *P. ovale* was taken.

The fourth aim of this thesis was to analyse the distribution and phylogeny of *P. malariae* and *P. knowlesi* in the district of Bandarban by way of PCR and sequence analysis in more than 2,000 blood samples collected on filter papers.

The fifth goal of this study was to establish a permanent cultivation technique of *P. vivax* under field conditions. More than 90 different cultivation techniques were employed and compared to each other.

6 Studies

6.1 Status of Peer Reviewed Publications

FUEHRER HP, STARZENGRUBER P, SWOBODA P, KHAN WA, MATT J, LEY B, THRIEMER K, HAQUE R, YUNUS EB, HOSSAIN SM, WALOCHNIK J, NOEDL H. **Indigenous *Plasmodium ovale* malaria in Bangladesh.** Am J Trop Med Hyg. 2010 Jul;83(1):75-8.
Impact Factor (2010): 2.446

FUEHRER HP, FALLY MA, HABLER VE, STARZENGRUBER P, SWOBODA P, NOEDL H. **A novel nested Direct PCR technique for malaria diagnosis from filter paper samples.** J Clin Microbiol. 2011 Apr;49(4):1628-30.
Impact Factor (2010): 4.22

FUEHRER HP, HABLER VE, FALLY MA, STARZENGRUBER P, SWOBODA P, KHAN WA, BLÖSCHL I, NOEDL H. **Variations of *Plasmodium ovale* in Bangladesh: First evidence of the sympatric distribution of *P. ovale curtisi* and *P. ovale wallikeri* in South Asia.** J Infect Dis. – submitted – Dec. 21st 2011 (JID-S-11-02921).
Impact Factor (2010): 6.228

Studies not submitted by now:

Molecular epidemiology of *Plasmodium malariae* and *P. knowlesi* in Bangladesh. (Chapter 6.5).

A highly sensitive in vitro-assay for the detection and quantification of *Plasmodium vivax* parasite biomass after the usage of different cultivation methods (Chapter 6.6).

Further congress presentations and posters are listed at the end of according chapters and within the CV.

6.2 Indigenous *Plasmodium ovale* malaria in Bangladesh

Hans-Peter Fuehrer, Peter Starzengruber, Paul Swoboda, Wasif Ali Khan, Julia Matt, Benedikt Ley, Kamala Thriemer, Rashidul Haque, Emran Bin Yunus, Shah Monir Hossain, Julia Walochnik, and Harald Noedl*

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6.2.1 Abstract

In spite of the high prevalence of malaria in Southeastern Bangladesh there remains a significant shortage of information regarding the presence of three out of five human malaria parasites: *Plasmodium ovale, P. malariae,* and *P. knowlesi*. The presence of *P. ovale* and *P. knowlesi* has never previously been reported from Bangladesh. We used a genus- and species-specific nested PCR, targeting highly conserved regions of the small subunit ribosomal RNA (SSU rRNA) gene to investigate the presence of malaria parasites in a total number of 379 patient samples in a survey of febrile illnesses in the Chittagong Hill Tracts in Southeastern Bangladesh. We identified the first cases of *P. ovale* in Bangladesh. They were confirmed by sequence analysis. 189 out of 379 samples (49.9%; 95%CI: 44.9-54.9%) were positive for *Plasmodium* sp. by PCR. *P. falciparum* monoinfections accounted for 68.3% (61.3-74.5%), followed by *P. vivax* (15.3%; 10.9-21.2%), *P. malariae* (1.6%; 0.5-4.6%), *P. ovale* (1.6%; 0.5-4.6%), and mixed infections (13.2%; 9.1-18.8%). We found no evidence of *P. knowlesi* in this region.

6.2.2 Introduction

Almost one million people die of malaria every year and recent reports of emerging artemisinin resistance in Southeast Asia will add another challenge to malaria control efforts.[1,2,3] High standards in the diagnosis of the malaria-causing *Plasmodium* species are essential to control and adequately treat malaria. In spite of its known limitations microscopy remains the gold standard of malaria diagnosis in the field and frequently not even microscopy is available in resource-limited environments. This may lead to a significant underestimation of the true malaria burden, especially of less prevalent and less documented species such as *P. ovale*, *P. malariae,* and *P. knowlesi* in Asia.

The Chittagong Hill Tracts in Southeastern Bangladesh are known to be highly endemic for *P. falciparum* with reported resistance to chloroquine and sulfadoxine/pyrimethamine.[4] Virtually all malaria infections were previously attributed either to *P. falciparum* or *P. vivax*.[5-8] Recent surveillance studies based on the diagnosis with rapid diagnostic tests (RDTs) indicate *P. falciparum* as the dominant species in malaria-endemic districts of Bangladesh with a country-wide prevalence of 3.58% (as compared to only 0.21% for *P. vivax*).[9] The existence of infections with *P. malariae* with a prevalence of 1% in Bangladesh was first reported from a study conducted in the Chittagong Hill Tracts.[11] Both *P. ovale* and *P. malariae* are typically found at very low prevalences in Southeast Asia.[12] *P. ovale* is known to be endemic in sub-Saharan Africa, the Middle East, Irian Jaya and Papua New Guinea, but following the availability of PCR-based techniques for the diagnosis of malaria this parasite has recently also been reported from a number of countries in South and Southeast Asia.[13-17] So far infections with *P. ovale* had not been reported from Bangladesh.

In recent years, human cases of *P. knowlesi* infections have been reported from Southeast Asia, especially by the Kuching group in Malaysia and although originally classified as a simian malaria parasite *P. knowlesi* is now generally recognized as the fifth human malaria species.[18] PCR-confirmed cases have been documented in Malaysia, Thailand, Myanmar, Singapore, and the Philippines.[19-25] *P. knowlesi* has a daily (quotidian) asexual cycle potentially resulting in life-threatening hyperparasitemia and hepatorenal dysfunction. A number of fatal cases have been reported from Malaysia.[20] Identification of *P. knowlesi* solely based on microscopy remains difficult.[19,26] Several Macaque species, including the traditional hosts of *P. knowlesi*, have their habitats in Bangladesh, and populations of critically endangered *Macaca fascicularis* are known to be endemic in the very southeastern parts of the country.[27] The limited distribution of mosquitoes of the *Anopheles leucosphyrus* group,

the only known vector of *P. knowlesi*, restrict the current distribution of *P. knowlesi* to a limited area in Southeast and parts of South Asia, including the Chittagong Hill Tracts in Bangladesh.[29,30]

The primary aim of this study was to establish the prevalence of all 5 malaria species among febrile patients in the Chittagong Hill Tracts in Bangladesh with special emphasis on the 3 rare malaria species.

6.2.3 Materials and Methods

Study population. Diagnostic samples were collected from febrile patients in the course of field surveys in Bandarban District in the Chittagong Hill Tracts in 2007/2008 and a hospital-based survey at the MARIB (Malaria Research Initiative Bandarban) field site in 2008/2009. Male and female volunteers of any age with acute fever or a history of fever within the past 72 hours were included in this study. Venous blood was only drawn from patients 8 years or older. Written informed consent was obtained from all study participants or their legal representatives before blood samples were collected. The study protocol was reviewed and approved by the Ethical Review Committee of the International Centre for Diarrhoeal Disease Research, Bangladesh.

Malaria diagnosis. Thick and thin blood smears were prepared and examined in duplicate by two expert microscopists blinded to each other's results after staining with Giemsa (Merck KGaA®, Darmstadt, Germany). In thick films 200 oil-immersion fields were screened before declaring a slide negative. If the parasite count on the thick film was too numerous to count, the number of parasites per 2000 red blood cells was counted on the thin film**.** RDTs (FalciVax®, Zephyr Biomedicals, India) based on the detection of *P. falciparum*-specific histidine-rich protein 2 (HRP2) and *P. vivax*-specific lactate dehydrogenase (pLDH) were used in 357 patients.

Blood sample collection for PCR. From all patients over the age of 8 years 100 µL of venous whole blood and from children below 8 years 2 drops of finger prick blood were transferred onto filter paper (903™ Schleicher & Schuell BioScience GmbH, Dassel, Germany). A total of 379 filter papers were prepared in duplicate, air dried at room temperature, and stored in airtight containers at 4°C until further processing by PCR.

DNA Isolation and Purification. A modified chelex-based DNA extraction method using the InstaGene™ Whole Blood Kit (Bio-Rad Laboratories, Hercules, CA) was employed for the extraction and purification of *Plasmodium* DNA from the blood spots on filter paper.

To ensure comparable quantities of blood, samples of exactly 4 mm in diameter were punched out of the blood spots. Blood spots were soaked overnight in 100 µL phosphate-buffered saline (PBS) at 4°C, and the DNA extraction was performed on the following day as previously described.[32,33] The resulting supernatant was purified with InstaGene Matrix twice.

Parasite detection by nested PCR. Nested PCR assays were performed as described previously.[34,35] The specific primers bind in highly conserved regions of the small subunit ribosomal RNA (SSU rRNA) gene which was used for the detection of genus and/or species specific *Plasmodium* DNA.

In the nested PCR we used the genus-specific primers rPLU1 and rPLU5 for the first and rPLU3 and rPLU4 for the second PCR. Whenever this genus-specific nested PCR gave positive results species-specific "Nest 2" PCRs were performed for species determination using the following internal primer pairs: rFAL1 and rFAL2 for *P. falciparum*, rVIV1 and rVIV2 for *P. vivax*, rMAL1 and rMAL2 for *P. malariae*, rOVA1 and rPLU2 for *P. ovale* (classical type) and Pmk8 and Pmkr9 for *P. knowlesi*.[19,34,35] The oligonucleotides were obtained from Microsynth (Microsynth AG, Balgach, Switzerland).

We used a template of 5 µL DNA in a 50 µL reaction (GoTaq PCR Core System, Promega, Madison, USA) for the first amplification and 5 µL Nest 1 product in 50 µL reactions for the amplification in Nest 2 (GoTaq PCR Core System). In all experiments a negative control of water and known positive controls were run with the samples. The PCR products of Nest 2 amplifications were analyzed by gel electrophoresis with 2% agarose and ethidium bromide staining. The individual interpreting the PCR results was blinded to the results of microscopy and RDT.

Sequence analysis. The amplicons of the *P. ovale*-positive samples from the nested PCR where further analyzed by sequencing for species confirmation. PCR products were purified using the Xact DNA Gel extraction Kit (GenXpress, Wiener Neudorf, Austria) and sequenced using the BigDye® sequencing kit and an automatic 310 ABI PRISM sequencer (PE Applied Biosystems, Germany).

6.2.4 Results

A total number of 189 out of 379 (49.9%; 95% confidence interval: 44.9-54.9%) filter papers from patients with febrile illnesses gave positive results for *Plasmodium* spp. with genus-specific primers by nested PCR. All 159 samples classified as positive by microscopic examination were confirmed as being positive. In addition, 30 samples which were

diagnosed as negative by microscopy, were positive in the genus-specific nested PCR. Species-specific PCR showed that out of 189 *Plasmodium* spp. positive samples, 154 (81.5%; 95%CI: 75.3-86.4%) were positive for *P. falciparum*, 50 (26.5%; 95%CI: 20.7-33.2%) for *P. vivax*, 7 (3.7%; 95%CI: 1.8-7.4%) for *P. malariae*, and 3 for *P. ovale* (1.6%; 95%CI: 0.5-4.6%). All samples tested negative for *P. knowlesi*.

We found 164 (86.8%; 95%CI: 81.2-91.1%) monoinfections and 25 (13.2%; 95%CI: 9.1-18.8%) mixed infections. 129 (68.3%; 95%CI: 61.3-74.5%) patients presented with *P. falciparum*, 29 (15.3%; 95%CI: 10.9-21.2%) with *P. vivax*, 3 (1.6%; 95%CI: 0.5-4.6%) with *P. malariae*, and 3 (1.6%; 95%CI: 0.5-4.6%) with *P. ovale* monoinfections. In addition, 21 (11.1%; 95%CI: 7.4-16.4%) patient samples contained DNA of both *P. falciparum* and *P. vivax*, 2 (1.1%; 95%CI: 0.3-3.8%) of *P. falciparum* and *P. malariae* and 2 (1.1%; 95%CI: 0.3-3.8%) triple infections with *P. falciparum*, *P. vivax*, and *P. malariae* (Table 1). One patient presented with *P. ovale* twice in the course of this study. Based on the PCR results 30 (7.9%; 95%CI: 5.5-10.8%) microscopy slides were classified as false negative and none as false positive. In comparison to the 25 PCR-confirmed samples with mixed malaria infections only 9 (36%) were read as positive for mixed infections in microscopy and only 6 (24%) were diagnosed as mixed infections by RDT.

All three samples positive for *P. ovale* in PCR were proven by DNA sequencing and after unblinding were also found positive for *P. ovale* on microscopical re-examination.

6.2.5 Discussion

For 2006, the World Health Organization estimated almost 3 million malaria cases with 15,000 deaths in Bangladesh in mostly unconfirmed cases. Among microscopy-confirmed malaria infections, *P. falciparum* was the dominant species causing more than 70% of all malaria cases.[1] A recent report based on microscopic diagnosis indicated, that 70.3% of all malaria cases in the Chittagong Hill Tracts were caused by *P. falciparum*, 29.6% by *P. vivax* and only 0.01% were read as mixed infections.[7] A study using microscopic and molecular methods for diagnosis conducted between 2000 and 2002 revealed that of those slides considered positive for *Plasmodium* spp., 84% were *P. falciparum* monoinfections or mixed infections, 15% *P. vivax* and 1% *P. malariae*.[11] Our study shows comparable results for *P. falciparum* with 81.5% (of which 68.3% were monoinfections) and *P. vivax* with 26.5% (of which 15.3% were monoinfections). However, none of the earlier studies ever reported any cases of *P. ovale*.

We report the first three cases of *P. ovale* in Bangladesh. All of them were monoinfections and originated from the Chittagong Hill Tracts. Two of the positive samples found in this survey were seen in the same patient, who originally tested negative in the RDT but was diagnosed with *P. malariae* based on microscopy (assuming that *P. ovale* was not an option as it had never been seen in Bangladesh before). Two months after being treated for his suspected *P. malariae* infection the patient returned with signs and symptoms consistent with malaria. This sample was confirmed to be *P. ovale* by molecular techniques, suggesting a relapse with *P. ovale*.

The nested PCR assay employed in this study is highly sensitive with a documented limit of detection of 6 parasites/µL.[34] Using the primer pair rOVA1/rPLU2, we selectively screened for the so called classic type of *P. ovale*.[36] *P. ovale* was found in 1.6% of the *Plasmodium*-positive samples. *P. ovale* had previously been reported from nearby Rakhine State in Myanmar. It has also been reported from other parts of Myanmar and a surprisingly high prevalence of up to 6.1% in Tanintharyi Division in 1996.[12,37] In the same study *P. malariae* was reported from 15,2% of all cases. Other PCR-based studies in Southeast Asia report up to 4% of *P. ovale* malaria in Northeastern Cambodia and 1.03% in Thailand.[17,23,36]

The impact of newly emerging pathogens in public health is increasing. Although our data do not confirm the presence of *P. knowlesi* in Bangladesh, our knowledge of the reservoir, vectors, and ecology of this malaria parasite indicate that Southeastern Bangladesh may be a an environment in which *P. knowlesi* is likely to be found. Human infections are easily mistaken for *P. falciparum* infections when the parasites are in the stage of young trophozoites.[29] In studies based on microscopic diagnosis it may also easily be mistaken for the morphologically very similar parasite *P. malariae*.[26] This parasite should therefore always be considered whenever patients are from or report a travel history to remote areas of Southeast Asia and are diagnosed with *P. malariae* malaria based on microscopic examination.[28,31]

Relative to microscopy, molecular methods not only tend to improve the sensitivity and specificity of diagnostic studies, they also result in higher estimates of malaria prevalence, particularly for the rare species *P. malariae* and *P. ovale*, and in higher rates of mixed infections.[10] Studies conducted in Lao PDR (23.1%), and Northwestern Thailand (23-24%) in which molecular techniques were used all suggested the presence of high numbers of mixed infections.[23,36] In our survey, we found 25 cases (13.2% of all positive samples) of mixed infections, of which the combination of *P. falciparum* and *P. vivax* was most fre-

quently seen. Triple infections with *P. falciparum*, *P. vivax*, and *P. malariae* are rare and were found in only two patients coming from the same small village (Nathogri, Rowanchari Subdistrict).

Accurate diagnosis of *Plasmodium* spp. is essential for optimizing malaria treatment guidelines. Further studies assessing the prevalence of the rare species *P. ovale*, *P. malariae*, and *P. knowlesi* in South Asia are therefore urgently needed to better understand the species distribution and to allow for adapting treatment strategies.

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Table 1: Comparison of malaria diagnosis by nested PCR, Microscopy and FalciVax®-RDT

	Neg	Pf	Pv	Pm	Po	Pk	Pf+Pv	Pf+Pm	Pf+Pv+Pm
PCR[a]	190	129	29	3	3	0	21	2	2
Microscopy[a]	220	122	29	3	0	0	6	0	0
RDT[b]	228	108	15	-	-	-	6	-	-

* neg = negative; Pf = *Plasmodium falciparum*; Pv = *P. vivax*; Pm = *P. malariae*; Po = *P. ovale*; Pk = *P. knowlesi*; Pf+Pv = *P. falciparum* + *P. vivax*; Pf +Pm = *P. falciparum* + *P. malariae*; Pf+Pv+Pm = *P. falciparum* + *P. vivax* + *P. malariae*

[a] $n_{PCR} = n_{microscopy} = 379$

[b] $n_{RDT} = 357$; Diagnosis of Pm, Po, Pk are not possible with FalciVax®-RDTs

6.3 A novel nested Direct PCR technique for malaria diagnosis from filter paper samples

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Running title: Detection of *Plasmodium* spp. with Direct nested PCR

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6.3.1 Abstract

The use of Direct nested PCR enables the detection of *Plasmodium* spp. from blood samples collected on filter papers without requiring the time-consuming procedures associated with DNA extraction. Direct PCR provides a rapid, highly sensitive, and cost effective alternative to diagnosing malaria on filter paper samples by standard nested PCR.

6.3.2 Introduction

Malaria remains a major global health burden with an estimated death toll of almost 900,000 every year.[11] Recent reports of newly emerging artemisinin resistance and the emergence of endemic populations of a number of "new", potentially human pathongenic *Plasmodium* species such as *P. knowlesi* as well as a variety of *P. ovale* parasites in Asia mean that there is an urgent need for new techniques to provide rapid and highly accurate diagnosis to adequately treat and control malaria.[3, 5, 9]

The use of Direct PCR allows for PCR amplifications without any prior DNA extraction and purification steps. The Phusion® blood DNA polymerase used in the assay is reported to lead to a 25-fold lower error rate in comparison with common *Thermus aquaticus* polymerase.[2]

The aim of this study was to adapt this novel technique for use in the rapid lab-based diagnosis of *Plasmodium* spp. and validate the sensitivity in comparison to conventional nested PCR and microscopy.[6,8]

6.3.3 Methods

Patient samples were collected between 2007 and 2009 at the MARIB (Malaria Research Initiative Bandarban) center in Bandarban, Chittagong Hill Tracts, Bangladesh, as part of a hospital and field-based fever survey. Written informed consent was obtained from all study participants or their legal representatives and the study protocol was approved by the respective ethical review committee.

From all participating patients aged 8 years and above, 100 µl venous blood was drawn. From patients below this age, 2 drops of finger-prick blood were collected and transferred onto filter paper (903™ Schleicher & Schuell BioScience GmBH, Dassel, Germany) in duplicate. Filter papers were air dried at room temperature and stored airtight at 4°C until further processing. A total number of 140 filter paper samples was included in the evaluation.

Direct nested PCR. A blood spot 2 mm in diameter was punched out of each filter paper sample and washed with 30 µl double distilled water at 50°C for 3 minutes. The water was removed and the PCR mix (Phusion® Blood Direct PCR Kit, Finnzymes OY, Espo, Finnland) was added directly to the sample. A modified standard nested PCR protocol was used for the evaluation of genus- and species-specific *Plasmodium* DNA within the highly

conserved regions of the small subunit ribosomal RNA (SSU rRNA) gene.[6, 7, 8] Following primers were used: rPLU1/rPLU5 for the Nest 1 reactions and rPLU3/rPLU4 for the genus-specific Nest 2 amplifications. Whenever the genus-specific Nest 2 PCR revealed positive results, species-specific Nest 2 primers were used to determine the species: rFAL1/rFAL2 (*P. falciparum*), rVIV1/rVIV2 (*P. vivax*), rMAL1/rMAL2 (*P. malariae*), rOVA1/rPLU2 (*P. ovale*) and Pmk8/Pmkr9 (*P. knowlesi*). All oligonucleotide primers were obtained from Microsynth (Microsynth AG, Balgach, Switzerland).

A 50 µl Nest 1 reaction was set including 25 µl 2x Phusion® Blood PCR Buffer (which included 200 µM dNTPs and 3mM $MgCl_2$), 1 µl (2 U) Phusion® Blood DNA Polymerase, and 5 µl of each primer (rPLU1 and rPLU5 – 10 µM) according to the manufacturer's manual[2]. The DNA was denatured at 98°C for 4 min, followed by 25 cycles of amplification (annealing: 65°C for 2 min, extension: 72°C for 2 min, denaturation: 94°C for 1 min). After 25 cycles the final extension was set at 72°C for 4 min using an Eppendorf Mastercycler Personal (Eppendorf AG, Hamburg, Germany). The annealing temperature was determined using the Tm calculator on the manufacturer's website (https://www.finnzymes.fi/tm_determination.html).[2] The resulting Nest 1 PCR product was centrifuged at 1,000 x g for 3 minutes. 2.5 µl Nest 1 products (same in standard nested PCR and direct nested PCR) were used in 25 µl Nest 2 amplifications (GoTaq PCR Core System, Promega, Madison, USA).

Known positive control samples and nuclease free water as negative control were run with each PCR amplification. Nest 2 PCR products were analyzed by gel-electrophoresis with 2% agarose and ethidium bromide staining.

Standard nested PCR-technique. A modified chelex-based method using an InstaGene™ Whole Blood Kit (Bio-Rad Laboratories, Hercules, CA) was used to extract DNA from blood spots on filter paper. A blood spot of 4 mm in diameter was punched out and soaked overnight in 100 µl phosphate-buffered saline (PBS) at 4°C. DNA extraction was performed on the following day as described previously.[1] All samples were purified twice with the InstaGene matrix and stored at -20°C until further processing.

A template of 5 µl was used in a 50 µl Nest 1 reaction (GoTaq PCR Core System, Promega, Madison, USA) under the following conditions: 5 µl of each primer (10 µM), 125 µM of each dNTP, 2 mM of $MgCl_2$ and 1 U of GoTaq® DNA-polymerase.

Nest 2 reactions and further procedures (with the exception of the centrifugation step of the Direct PCR Nest 1 product) were identical to the standard - and Direct nested PCR techniques discussed above.

Microscopy. Thick and thin smears were prepared in duplicate from each patient's blood and stained with Giemsa (Merck KGaA®, Darmstadt, Germany). Each slide was examined by two expert microscopists blinded to each other's results. In thick films, 200 oil-immersion fields were evaluated before a sample was declared negative and to rule out mixed infections. On thin films the parasite count was established per 2000 red blood cells.

6.3.4 Results and Discussion.

The level of detection was determined in double-blinded fashion (each step blinded to the results of each other: microscopy, DNA extraction, PCRs, and gel electrophoresis) using filter papers with 100 µl blood spots with known parasitemia obtained from the K1 (1 parasite/µl – 250,000 parasites/µl) and 3D7 *Plasmodium falciparum* strains (1 parasite/µl – 290,000 parasites/µl), as well as a *Plasmodium vivax* isolate (1 parasite/µl – 30,000 parasites/µl),. The lowest parasitemia reliably resulting in positive results was 3 parasites/µl for the *Plasmodium vivax* isolate and the K1 strain isolate, and 5 parasites/µl for the 3D7 laboratory strain.

Using Direct nested PCR 95 of 140 field isolates gave positive results with genus-specific primers as compared to 92 of 140 using standard nested PCR and 89 of 140 using microscopic determination (Table 2). Based on a total of 640 Nest 2 PCRs (genus and species) a sensitivity of 99.8%, a specificity of 96%, a positive predictive value (PPV) of 90.9% and a negative predictive value (NPV) of 99.7% in comparison to standard nested PCR were calculated (Table 1). All field isolates giving positive results for malaria parasites in microscopy remained positive in Direct nested PCR. The limitations in terms of specificity of the primers in the detection of *P. ovale* and *P. knowlesi* has previously been discussed.[4, 9]

Although microscopy remains the gold standard for malaria diagnosis the limit of detection may significantly differ between microscopists and has previously been estimated at a parasitemia of 50-100 parasites/µl under field conditions.[10] Despite their known limitations, microscopy and/or Rapid Diagnostic Tests (RDTs) remain the primary techniques of malaria diagnosis. However, in the past decades the improvement of molecular diagnostic tools (e.g. PCR, real-time PCR) has resulted in the availability of far more sensitive tools.

With only 3 parasites/µl the novel assay is likely to be slightly more sensitive than standard nested PCR with its limit of detection of 6 parasites/µl.[6] The calculated value for the specificity (96%) and the PPV (90.9%) relative to standard PCR (PCR corrected microscopy) may possibly under-/overestimate the true specificity as the higher proportion of positive samples found by Direct PCR could possibly also be a result of the higher sensitivity of the new assay.

Certainly the biggest advantage of Direct PCR is the fact that the extraction and purification of DNA from filter paper can be omitted, resulting in an overall saving in time of approximately 2 hours, plus the overnight DNA extraction step, which in our eyes justifies the slightly higher price of each single direct Nest 1 PCR reaction (~ 2.1 US$) in comparison to the standard Nest 1 PCR (1.7 US$) for the DNA extraction and Nest 1 reaction of one sample. At the same time the collection of filter papers is a practical way of sampling, storing, and transporting diagnostic blood samples. This technique is not limited to screening for malaria parasite species, it might equally be employed for genotyping, drug resistance research, as well as for the diagnosis of other blood pathogens. We therefore conclude that Direct PCR in combination with the collection of blood samples on filter paper provides a rapid, highly sensitive, and cost effective alternative for malaria diagnosis.

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Table 1: Comparison of *Plasmodium* sp. diagnosis by standard nested PCR and by Direct nested PCR.

	Standard nested PCR									Total
Direct PCR	Neg	Pf	Pv	Pm	Po	Pk	Pf+Pv	Pf+Pm	Pf+Pv+Pm	
Neg	45[a]									45
Pf		59					1			60
Pv	1		6							7
Pm	1			2						3
Po	1				4					5
Pk						0				0
Pf+Pv		4					7			11
Pf+Pm		1		2				2		5
Pv+Pm			2							2
Pf+Pv+Pm									1	1
Pf+Pv+Pm+Po									1	1
Total	48	64	8	4	4	0	8	2	2	140

* neg = negative; Pf = *Plasmodium falciparum*; Pv = *P. vivax*; Pm = *P. malariae*; Po = *P. ovale*; Pk = *P. knowlesi*; Pf+Pv = *P. falciparum* + *P. vivax*; Pf +Pm = *P. falciparum* + *P. malariae*; Pf+Pv+Pm = *P. falciparum* + *P. vivax* + *P. malariae;* Pf+Pv+Pm+Po = *P. falciparum* + *P. vivax* + *P. malariae* + *P. ovale.*

[a] including 2 samples negative in standard nested PCR, which gave positive results in genus-Direct nested PCR but remained negative in the species direct nested PCRs and after repeating the genus-Direct nested PCRs.

Table 2: Comparison of malaria diagnosis by Direct nested PCR, nested PCR and Microscopy with the inclusion of all samples (n = 140) and only those negative in microscopy or with a parasitemia of 200/µl or below (n = 61).

	Overall			**Parasitemia ≤ 200/µl or not detected with microscopy**		
	Direct nested PCR	**nested PCR**	**Microscopy**	**Direct nested PCR**	**nested PCR**	**Microscopy**
neg	45	48	51	45	48	51
Pf	60	65	72	5	4	9
Pv	7	8	9	1	1	1
Pm	3	3	2	1	1	0
Po	5	4	3	2	1	0
Pf + Pv	11	8	3	4	4	0
Pf + Pm	5	2	0	2	1	0
Pf + Pv + Pm	1	2	0	0	1	0
Pf + Pv + Pm + Po	1	0	0	1	0	0
Pv + Pm	2	0	0	0	0	0
Pk	0	0	0	0	0	0

* neg = negative; Pf = *Plasmodium falciparum*; Pv = *P. vivax*; Pm = *P. malariae*; Po = *P. ovale*; Pk = *P. knowlesi*

6.4 *Plasmodium ovale* in Bangladesh: genetic diversity and the first evidence of the sympatric distribution of *P. ovale curtisi* and *P. ovale wallikeri* in South Asia

Running head: *Plasmodium ovale* in Bangladesh

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6.4.1 Abstract

Background. In spite of the high prevalence of malaria in Bangladesh and other South Asian countries, there remains a substantial shortage of knowledge about the less common human malaria parasites. Recent studies indicate that *P. ovale* itself, is made up of two species, namely *Plasmodium ovale wallikeri* and *Plasmodium ovale curtisi*.

Methods. Genus- and species-specific nested PCR analysis of the small subunit ribosomal RNA (*SSU rRNA*) gene was used to detect *Plasmodium ovale* infections in 2,246 diagnostic samples. *Plasmodium ovale* infections were further differentiated by nested PCR of the *potra* gene and multilocus analysis of the *cox1*, *porbp2* and the *SSU rRNA* genes.

Results. Both *P. ovale curtisi* and *P. ovale wallikeri* occur sympatrically in the Chittagong Hill Tracts and are associated with a mild or asymptomatic symptom complex. The pathogens can be differentiated by nested PCRs targeting the *SSU rRNA* and *potra* genes, and display dimorphism in multilocus analysis.

Conclusions. We report the first evidence of sympatric *P. ovale curtisi* and *P. ovale wallikeri* in South Asia and within a relatively confined study area of less than 5,000 km^2. High rates of mixed infections, the emergence of "new" human malaria species and the evidence of zoonotic capability call for optimized diagnostic strategies for a new eradication era.

Keywords

Plasmodium ovale curtisi, *Plasmodium ovale wallikeri*, Bangladesh, *cox1*, *porbp2*, *SSU rRNA*, *potra*

6.4.2 Background

Since the introduction of molecular tools the number of parasite species known to cause human malaria increased from formerly 4 to 6 species within just a few years; namely: *Plasmodium falciparum, P. vivax, P. malariae, P. knowlesi, P. ovale curtisi* (classic type) and *P. ovale wallikeri* (variant type) [1, 2]. Because most scientific efforts are focused on *P. falciparum* and *P. vivax* only, there is a lack of knowledge regarding the less common *Plasmodium* species, including the recently divided species *P. ovale* [1].

Because of their tertian periodicity and their morphological resemblance *P. ovale* and *P. vivax* are frequently confused [3]. Until fairly recently the distribution pattern of *P. ovale* was considered to be limited to tropical regions in sub-Saharan Africa, Papua New Guinea, parts of Indonesia (e.g. Timor, Flores, West-Papua) and the Philippines [4].With the introduction of polymerase chain reaction (PCR)-based techniques, which allow a faster and more accurate differentiation of *Plasmodium* species than microscopy, *P. ovale* was found to have a far wider distribution than previously anticipated. In the meantime *P. ovale* has been documented in nearly all countries of South and South-East Asia, including the South Asian countries Bangladesh, India and Sri Lanka [5, 6, 7]. However, information about this parasite from Asia remains limited [4].

Difficulties in the diagnosis of *P. ovale* are not limited to microscopy. Even molecular analysis has previously resulted in inconsistent results. In 1993 Snounou et al. introduced a species-specific nested PCR technique targeting conserved regions of the small subunit ribosomal RNA (*SSU rRNA*) gene which quickly emerged as a standard methodology in the molecular identification of *Plasmodium* species [8]. “Species”-specific primers rOVA1 and rOVA2 (NP 1993) were used for the diagnosis of ovale malaria. However, selected samples positive for *P. ovale* by microscopy consistently gave negative results by PCR. Because of known sequence variations the set of primers was adapted in 2002 (NP2002) [9]. In spite of the use of a genus-specific primer, namely rPLU2, in combination with rOVA1 a number of microscopically confirmed samples remained negative prompting the differentiation of the classic form of *P. ovale* (binding at the NP1993 primers) and the variant type. In 2005 specific primers rOVA1v and rOVA2v were designed to diagnose variant forms of *P. ovale* [10]. Several studies suggested that the genetic polymorphisms are not limited to the *SSU rRNA*. Documented differences between the classic and variant types of *P. ovale* include the *cytb* gene (encoding the cytochrome b protein), genes encoding lactate dehydrogenase (*LDH*) and ookinete surface antigens, *Cox1* (cytochrome c oxi-

dase 1 gene), *Porbp2* (Plasmodium ovale reticulocyte binding protein 2 gene), *PoTRA* (Plasmodium ovale tryptophan rich antigen gene), *pog3p* (encoding the glyceraldehyde-3-phosphatase) and *podhfr-ts* (encoding the dihydrofolate reductase-thymidylate synthase) [1, 11, 12, 13, 14].

Thus genetic polymorphisms of *P. ovale* led to the introduction and segregation of *P. ovale* into two species, namely *P. ovale curtisi* (former classic type) and *P. ovale wallikeri* (former variant type) in 2010 [1].

After the first description of *P. ovale* in Bangladesh and the first report of a relapse caused by this pathogen in the Chittagong Hill Tracts we hypothesized that *P. ovale curtisi* and *P. ovale wallikeri* may both be endemic and occur sympatrically in the Chittagong Hill Tracts in Bangladesh [5, 15]. To address this hypothesis over 2,200 blood samples from Bangladesh were analyzed using a variety of PCR techniques (e.g. *SSU rRNA* gene) for the differentiation of *P. ovale curtisi* and *P. ovale wallikeri*. Multilocus genetic analyses were performed to explore genetic variations of *P. ovale* and correlated with clinical, parasite and demographic data to provide evidence of both species in South Asia and their sympatric distribution within a confined area.

6.4.3 Methods

Study area and population. The district of Bandarban comprises an area of of 4,479 km^2 and is located in the very southeast of Bangladesh bordering Myanmar. Bandarban is part of the Chittagong Hill Tracts which are known to be endemic for malaria with the highest malaria prevalence in Bangladesh. The district itself is divided in 7 subdistricts, namely Bandarban, Alikadom, Naikhongchari, Ruma, Thanchi, Rowangchari and Lama (Fig. 1). In the course of field surveys and a hospital-based fever survey at the MARIB (Malaria Research Initiative Bandarban) field site blood samples from 1,867 asymptomatic and 379 febrile patients were collected in the Bandarban District in 2007 and 2008. Patients presenting with fever or reporting fever within the last 72 hours were included in the fever survey, whereas asymptomatic participants took part in the asymptomatic prevalence study. Written informed consent was obtained from each participant or their legal representatives before blood collection. The study protocol was reviewed and approved by the respective ethical review committee.

Malaria diagnosis by microscopy and sample collection. For malaria diagnosis thin and thick blood films were prepared and stained with Giemsa (Merck KGaA, Darmstadt, Ger-

many). The slides were examined in duplicate by two expert microscopists blinded to each other's result. At least 200 oil immersion fields were screened in thick smears before defining a slide negative. From each participant 8 years or older 100 µl venous blood were collected, whereas two drops of finger prick blood were taken from children < 8 years, and transferred on filter paper (903; Schleicher&Schuell, BioScience GmbH, Dassel, Germany). Filter paper samples were air dried at room temperature, sealed airtight and stored until further processing.

DNA-isolation and preliminary PCR analysis. DNA was extracted from blood spots on filter paper using a modified chelex-based technique [5, 16, 17]. To obtain a subset of *P. ovale* positive DNA samples a total of 2,246 DNA samples were examined for *Plasmodium* infections using nested PCR assays of the small subunit ribosomal RNA (*SSU rRNA*) gene as described previously [8, 9, 18]. *Plasmodium* genus-specific primers rPLU1 and rPLU5 were used for the Nest 1 PCR amplifications and rPLU3 and rPLU4 for Nest 2 PCRs under conditions described previously [5]. If genus-specific Nest 2 reactions revealed positive results following species-specific primers were used for Nest 2 PCR amplifications: rFAL1 + rFAL2 (*P. falciparum*), rVIV1 + rVIV2 (*P. vivax*), rMAL1 + rMAL2 (*P. malariae*), rOVA1 and rOVA2 (*P. ovale* – NP1993 primers), rOVA1 + rPLU2 (*P. ovale* – NP2002 primers), rOVA1var + rOVA2var (*P. ovale* variation primers) and Pmk8 + Pmkr9 (*P. knowlesi*) [8, 9, 10, 19]. All conventional PCR amplifications were performed using an Eppendorf Mastercycler Personal (Eppendorf AG, Hamburg, Germany) and analyzed by gel electrophoresis.

PoTRA-PCRs (Plasmodium ovale tryptophan rich antigen gene). Samples testing positive for *P. ovale* within the preliminary PCR amplifications were analyzed with *PoTRA*-Nested PCRs for the discrimination and confirmation of *P. ovale wallikeri* (PoW) and *P. ovale curtisi* (PoC) using the primers PoTRA fwd3 + PoTRA rev3 and PoTRA fwd5 + PoTRA rev5 as described previously [1, 20].

Genetic diversity of *P. ovale*. Confirmed *P. ovale* monoinfections were also characterized using following genes: *Cox1* (cytochrome c oxidase 1 gene), *Porbp2* (Plasmodium ovale reticulocyte binding protein 2 gene), and *SSU rRNA* gene.

***Cox1*.** Haemasporidia-specific Nest 1 primers cox1a + cox1b and Nest 2 primers cox1c + cox1d were used to amplify a 964-bp fragment with conditions described previously [14]. Cox1c + cox1d were used for sequencing.

***PoRBP2*.** PoRBP2fwd + PoRBP2rev were used to obtain Nest 1 amplifications within the *Plasmodium ovale* reticulocyte binding protein 2 gene. For sequencing the internal primers PoRBP2TMfwd and PoRBP2TMrev were used [20].

***SSU rRNA*.** Two sets of primers, rPLU1 + rPLU5, were used for the Nest 1 reaction as in the standard nested PCR described above. Because of the limited amount of DNA resulting from the Nest 1 reaction, we chose the internal genus-specific primers rPLU2 + rPLU3 which were also used for sequencing.

Sequence analysis. All amplicons of the *P. ovale*-positive PCR products from monoinfections were purified with ExoSap-IT® (GE Healthcare, Buckinghamshire, UK) to remove incorporated nucleotides and primers, and sequenced using the BigDye Sequencing Kit and an automatic 310 ABI PRISM sequencer (PE Applied Biosystems, Weiterstadt, Germany). From each patient sample and gene locus three amplicons were sequenced with forward and reverse primers discussed above. Sequences were aligned using ClustalX v. 2.0.12 software [21]. The alignment had 737 positions of which 18 were excluded from the analyses as they showed variations in the sequence length. Calculations of models for sequence evolution were performed with jModeltest v. 0.1.1. [22] resulting in the selection of the model HKY [31] and a gamma distribution, allowing rate variation between sites. Bayesian analyses were performed using MrBayes v. 3.1.2 [23]. The runs were started with random trees and performed for 5 million generations each with four Markov chains and a sampling frequency of one of hundred per generation. Data sampled from the generations preceding the stationary of the Markov chain (25%) were discarded before calculating the consensus tree. A Neighbor-joining [32] dendrogram was calculated with ClustalX v. 2.0.12 using p-distances (1000 bootstrap replicates) [21]. Tree analyses was performed with the *cox1* sequences of the newly retrieved samples and *P. gonderi* as an outgroup.

6.4.4 Results

P. ovale in Bangladesh

Out of 2,246 diagnostic samples 740 tested positive for *Plasmodium* spp. by PCR-corrected microscopy. A total number of 23 out of 2,246 (1.02%; 95% confidence interval (CI) = 0.7-1.5%) filter papers gave positive results for *P. ovale* within the preliminary PCR examination of the *SSU rRNA* gene (Tab. 1). Among these 10 (0.45%; CI = 0.3-0.8) were classified as *P. ovale curtisi*, 12 (0.53%; CI = 0.3-0.9%) as *P. ovale wallikeri* and one patient presented both *P. ovale curtisi* and *P. ovale wallikeri* (0.04%; CI = 0-0.2%). Monoinfections with *P. ovale curtisi* were diagnosed in 4 (36.4%) blood samples, whereas mixed

infections were present in 7 (63.6%) (Tab. 2). *P. ovale wallikeri* mono-infections were diagnosed in 6 (46.1%) participants, and mixed infections were documented in 7 (53.9%). These results confirm the sympatric distribution of *P. ovale curtisi* and *P. ovale wallikeri* in a single district with both *P. ovale* species being documented in 4 of 7 subdistricts (Fig. 1).

The gender ratio between female and male ovale malaria-infected persons was 11 (47.8%) to 12 (52.2%). Twenty two of 23 (95.7%) participants reported to have been infected with *Plasmodium* spp. in the past.

***P. ovale* in symptomatic patients**

Four of 379 (1.05%; CI = 0.4-2.7%) blood samples collected from symptomatic patients presenting with febrile illnesses (fever within the last 72 hours) were positive for *P. ovale*, out of which 1 (0.26%; CI = 0.1-1.5%) was determined to be *P. ovale curtisi* and 3 (0.79%; CI = 0.3-2.3%) gave positive results for *P. ovale wallikeri*.

The 4 symptomatic ovale malaria patients had parasite densities between 280 and 6,680 parasites/µl and tested positive for malaria by microscopy. However, none were diagnosed as *P. ovale*, as during enrolment *P. ovale* was not known to be endemic in Bangladesh (Tab. 1).

***P. ovale* in asymptomatic participants**

Among asymptomatic participants 19 of 1,867 (1.02%, CI = 0.7-1.6%) filter papers tested positive for *P. ovale*, out of which 9 (0.48%; CI = 0.3-0.9%) each were classified as *P. ovale curtisi* and *P. ovale wallikeri*, respectively, and one patient (0.05%, CI = 0-0.5%) presenting with both. Overall 551 participants tested positive for malaria by PCR-corrected microscopy with *P. ovale* contributing 19 (3.44%; CI = 2.2-5.3%) cases.

Comparison of diagnostic methods

Within the *SSU rRNA* gene three standardized pairs of primers were tested. The classic NP-1993 primers rOVA1-rOVA2 resulted in positive results for 11 specimens (Tab. 2) and were found to be specific for *P. ovale curtisi*. rOVA1-rOVA2 gave positive results in 18 isolates. The variant primers rOVA1v-rOVA2v gave positive results in 13 specimens of *P. ovale wallikeri*. The *PoTRA* PCR was established after the differentiation of the two *P. ovale* species amplified fragments with sizes of 317 bp for *P. ovale curtisi* and 245 bp for *P. ovale wallikeri*, and allowed for a discrimination of the two species.

Genetic diversity of *P. ovale*

To confirm the presence and genetic diversity of *P. ovale* in Bangladesh we investigated two mitochondrial genes (*cox1* and *SSU rRNA*) and one nuclear gene (*Porbp2*) in blood samples with monoinfections (Tab. 3). Sequence analysis of these genes allowed for a reliable discrimination of *P. ovale curtisi* and *P. ovale wallikeri*.

Partial *Cox1* gene sequences of 10 isolates confirmed the PCR results within the *SSU rRNA* gene. Six samples of *P. ovale wallikeri* were identical to human and chimpanzee variant parasite strains and 4 *P. ovale curtisi* were identical to those of classic *P. ovale* isolates (Tab. 3). Dimorphism between the two species was found in 12 loci, but no variability of the sequences within each *P. ovale* species was observed.

On the basis of partial sequences of the *ssrRNA* gene the classic or variant status of 8 isolates was determined. Six *P. ovale wallikeri* and 3 *P. ovale curtisi* possessed dimorphic characters (Tab. 4). All 3 *P. ovale curtisi* samples were identical to an isolate from Guinea-Bissau (GQ183068). Furthermore *P. ovale wallikeri* isolates showed intraspecific variability and were comparable to isolates from Thailand, Sao Tome and Cameroon (Fig. 2).

Eight samples (5 *P. ovale wallikeri* and 3 *P. ovale curtisi*) were compared based on partial sequences of the *Porbp2* gene and dimorphism was observed in 21 loci (Tab. 4). Two *P. ovale wallikeri* samples Po2 and Po3 (JF894423 and JF894424), which were identical in the partial sequences of the *Cox1* and *SSU rRNA* genes, varied with one substitution from those of other *P. ovale wallikeri* sequences.

6.4.5 Discussion

Although for almost a century *P. ovale* has essentially been known as the African malaria parasite, more recently Asian prevalence rates similar to those in Africa seem to suggest a much more global distribution.

The Chittagong Hill Tracts in Bangladesh are known to be highly endemic for *P. falciparum* with known resistance to sulfadoxine/pyrimethamine and chloroquine [24]. Until very recently the diagnosis of malaria in this rural area was limited to *P. falciparum* and *P. vivax*, largely because based on microscopic and rapid diagnostic tests neither *P. ovale* nor *P. malariae* were known to be endemic in this region [5, 25]. With 81.5% (of which 68.3% were monoinfections) a recent molecular study implicated *P. falciparum* as the dominant species in the Chittagong Hill Tracts, followed by *P. vivax* with 26.5% (of which 15.3% were monoinfections), *P. malariae* with 3.7% and *P. ovale* (variant type) with 1.6% [5]. Using the primer pairs selected for the differentiation of the variant and classic *P. ovale*

types we found *P. ovale* in 3.44% of all malaria-positive asymptomatic participants (*P. ovale wallikeri* and *P. ovale curtisi:* 1.63% each, and one patient infected with both pathogens) and in 2.12% of all symptomatic malaria-positive patients (1.59% *P. ovale wallikeri* and 0.53% *P. ovale curtisi*) [10]. Previous studies conducted in Rhakine State in Myanmar described *P. ovale* prevalence rates of up to 6.1% [26, 27].
For Southeast Asia prevalences of 1.03% in Thailand and up to 4% in northeastern Cambodia have been reported [28, 29]. So far none of the prevalence studies conducted in Asia addressed the differentiation of the newly introduced *P. ovale wallikeri* and *P. ovale curtisi*, although phylogenetic studies refer to the existence of classic and variant forms in Myanmar and Thailand [11, 26, 29]. In Asia *P. ovale wallikeri* has so far only been described in samples from Thailand, Vietnam and Papua New Guinea in the course of a single study [1]. A study conducted in Congo, Equatorial-Guinea and Uganda confirmed the sympatric distribution of both species in Sub-Saharan Africa with an overall prevalence of 3.1% of all malaria cases [20]. Within our study we were able to prove the sympatric distribution of *P. ovale curtisi* and *P. ovale wallikeri* in South Asia in a relatively confined area of less than 5,000 km^2.

Both *P. ovale* species seemed to evoke similar, mostly mild symptom complexes and more than half of the *P. ovale* infections were asymptomatic, which may have major implications for *P. ovale* control efforts. The parasitaemias seen in monoinfections were between 120 and 6,680 parasites/µl, and are therefore considerably below the maximum parasite densities achievable in experimentally induced infections [3].

The emergence of "new" malaria parasites (e.g. *P. ovale curtisi, P. ovale wallikeri, P. knowlesi*) and new evidence of zoonotic activity of human malaria parasites may further complicate plans to eradicate malaria. Chimpanzees have been used as experimental models for *P. ovale curtisi* [1]. Interestingly the cox1 sequence of a variant type *P. ovale* sample collected from a free-living chimpanzee in Cameroon (FJ409569) was identical to all *P. ovale wallikeri* samples analyzed in our study [14].

P. ovale curtisi and *P. ovale wallikeri* as distinct nonrecombining species showed an estimated divergence between 1-3 MYA [1]. We observed phylogenetic species differences among *cox1*, *SSU rRNA*, and *Porbp2* sequences, as documented previously [1, 14]. Our data suggest that *PoTRA* nested PCR and the combination of the classic primers rOVA1-

rOVA2 and the variant primers rOVA1v-rOVA2v may be suited best for species discrimination.

We report the first evidence of sympatric *P. ovale curtisi* and *P. ovale wallikeri* in South Asia. Further investigations using even larger sample sizes will be required to allow for a differentiation of the clinical and epidemiological characteristics of the two new species.

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Table 1: Demographic, parasitological and symptomatic profiles of PCR confirmed cases with *P. ovale curtisi* (PoC) and *P. ovale wallikeri* (PoW).

Case	Location (Upazilla)	Age (years)	sex [b]	Microscopy [a]	Parasite density Parasites/µl (micros-copy)	Temperature ℃	Symptoms
Po1W	Naikhongchari	4	F	Pv	2240	36.4	dizziness, fatigue, nausea, vomiting, runny nose, cough
Po2W	Bandarban	12	F	Pm	6680	38.9	chills, headache, myalgia, dizziness, fatigue, abdominal pain, nausea, runny nose
Po3W	Bandarban	12	F	Pm	2600	36.3	chills, headache, myalgia, dizziness, fatigue, abdominal pain, arthralgia
Po5W	Rowangchari	38	M	Pv	120	36.9	asymptomatic
Po6W	Lama	10	M	Pf	560	37.0	asymptomatic
Po7W	Naikhongchari	49	M	Pv	440	<36.5	asymptomatic
Po8W	Alikadom	17	M	Pv	320	36.5	asymptomatic
Po9W	Alikadom	5	F	Pf	14520	36.8	asymptomatic
Po11W	Ruma	53	F	negative	-	36.5	asymptomatic
Po13W	Naikhongchari	4	F	Pv	1120	<36.5	asymptomatic
Po19W	Rowangchari	24	F	negative	-	<36.5	asymptomatic
Po21W	Ruma	47	M	negative	-	<36.5	asymptomatic
Po22CW	Ruma	16	F	negative	-	36.7	asymptomatic
Po4C	Bandarban	30	M	Pv	280	37.5	chills, headache, dizziness, fatigue, vomiting
Po10C	Ruma	5	F	Pf + Pv	160 + 320	<36.5	asymptomatic
Po12C	Thanchi	38	M	negative	-	35.9	asymptomatic
Po14C	Naikhongchari	22	F	negative	-	36.7	asymptomatic
Po15C	Bandarban	59	M	negative	-	<36.5	asymptomatic
Po16C	Rowangchari	38	M	negative	-	<36.5	asymptomatic
Po17C	Rowangchari	35	M	Pf	3080	<36.5	asymptomatic
Po18C	Rowangchari	9	M	negative	-	36.9	asymptomatic
Po20C	Ruma	26	M	negative	-	<36.5	asymptomatic
Po23C	Rowangchari	19	F	negative	-	<36.5	asymptomatic

[a] Pf = *P. falciparum*; Pv = *P. vivax*; Pm = *P. malariae;* [b] M = male; F = Female

Table 2: Summary of the PCR results, Genotype analysis and GenBank Accession Numbers for all *P. ovale* isolates examined in this study. (PoC = *P. o. curtisi;* PoW = *P. o. wallikeri;* Pf = *P. falciparum*; Pv = *P. vivax*; Pm = *P. malariae*; nd = not done)

	PCR results [a]					Genotype Sequence Analysis			GenBank Accession Numbers		
Case	**PoR**	**PoC**	**PoV**	**PoTRA**	**including other *Plasmodium* sp.**	***SSU rRNA***	***Porbp2***	***Cox1***	***SSU rRNA***	***Porbp2***	***Cox1***
Po1	+		+	PoW	PoW	PoW	PoW	PoW	JF894407	JF894422	JF894419
Po2	+		+	PoW	PoW	PoW	PoW	PoW	JF894406	JF894423	JF894416
Po3	+		+	PoW	PoW	PoW	PoW	PoW	JF894408	JF894424	JF894417
Po4	+	+		PoC	PoC	PoC	PoC	PoC	JF894403	JF894427	JF894412
Po5	+		+	PoW	PoW	PoW	PoW	PoW	JF894409	JF894425	JF894418
Po6			+	nd	Pf + PoW	mixed infections					
Po7	+		+	nd	Pf + PoW						
Po8	+		+	nd	Pf + Pm + PoW						
Po9	+		+	nd	Pf + PoW						
Po10	+	+		nd	Pf + Pv + PoC						
Po11	+		+	PoW	PoW	PoW	nd	PoW	JF894411	nd	JF894420
Po12	+	+		PoC	PoC	nd	nd	PoC	nd	nd	JF894413
Po13			+	PoW	PoW	PoW	PoW	PoW	JF894410	JF894426	JF894421
Po14	+	+		PoC	PoC	PoC	PoC	PoC	JF894405	JF894428	JF894414
Po15	+	+		PoC	PoC	PoC	PoC	PoC	JF894404	JF894429	JF894415
Po16		+		nd	Pf + PoC	mixed infections					
Po17	+	+		nd	Pf + Pm + PoC						
Po18	+	+		nd	Pf + PoC						
Po19	+		+	nd	Pf + PoW						
Po20	+	+		nd	Pf + Pv + Pm + PoC						
Po21			+	nd	Pf + Pv + Pm + PoW						

Po22	+	+	+	nd	Pf + Pv + Pm + PoC + PoW	
Po23	+	+		nd	Pv + PoC	

[a] PoR = rOVA1-rPLU2 (NP-2002), PoC = rOVA1-rOVA2 (NP-1993), PoV = rOVA1v-rOVA2v (NP-2005)

Table 3: Dimorphic characters in *cox1* nucleotide sequences among 10 *P. ovale* isolates from Bangladesh (*P. ovale curtisi* and *wallikeri*) in comparison with published isolates of *P. ovale* in human and chimpanzee hosts (FJ40569-FJ409571).

Sequences	249	257	449	458	462	473	575	632	657	830	966	1082
***Plasmodium ovale curtisi* (n=6)**	C	C	T	T	C	C	T	G	C	C	A	T
***Plasmodium ovale wallikeri* (n=4)**	T	A	C	C	T	T	G	A	T	T	G	A
***P. ovale* classical type (FJ409571)**			T	T	C	C	T	G	C	C	A	T
***P. ovale* variant type (FJ409570)**			C	C	T	T	G	A	T	T	G	A
***P. ovale* variant type chimpanzee (FJ409569)**			C	C	T	T	G	A	T	T	G	A

Table 4: Dimorphic characterization in *porbp2* and *ssrRNA* nucleotide sequences among *P. ovale* isolates from Bangladesh.

Gene	Nucleotide poly-morphisms	*Plasmodium ovale curtisi*	*Plasmodium ovale wallikeri*
porbp2[a]	39, 85, 121, 145, 193, 249, 294, 310, 323, 375, 431, 459, 516, 538, 561, 563, 581, 602, 628, 741, 745	T.T.G.C.T.A.A.C. A.T.A.T.A.T.A. A.T.A.T.T.A	C.G.A.T.G.G.G.T. G.G.T.C.G.A.G. G.C.G.G.G.T
ssrRNA[b]	201, 202, 205, 206, 207, 234, 236, 244, 284, 287, 675, 682, 684, 697, 714, 731, 775, 779, (782-784), 787, 789, 793, 901, 918	A.T.C.C.G.A.G. T.G.G.A.C.A.A. T.G.T.G. (TAT) T. A.T.G.C	T.G.T.T.A.G.A. C.T.A.G.G.T.G. C.C.C.A. (---) C. T.C.A.T.

[a] Two samples (Po2 and Po3) classified as *P. ovale wallikeri* varied in following position: 293 – A instead of G

[b] Three samples classified as *P. ovale wallikeri* – Po2, Po3, Po13 – shared residues 684 with *P. ovale curtisi*. Furthermore Po13 shared residues 202 with the classic type.

Figure 1:

Plate 1: Summary of *Plasmodium ovale* cases in Bandarban District (PoW = *P. o. wallikeri*, PoC = *P. o. curtisi*)

Plate 2: Summary of mono- and mixed infections of *P. o. curtisi* and *P. o. wallikeri* (Pf = *P. falciparum*, Pv = *P. vivax*, Pm = *P. malariae*)

Plate 3: Visualization of the sympatric distribution of *P. o. curtisi* and *P. o. wallikeri* in Bandarban District

Plate 1:

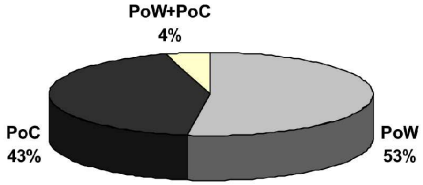


Plate 2:

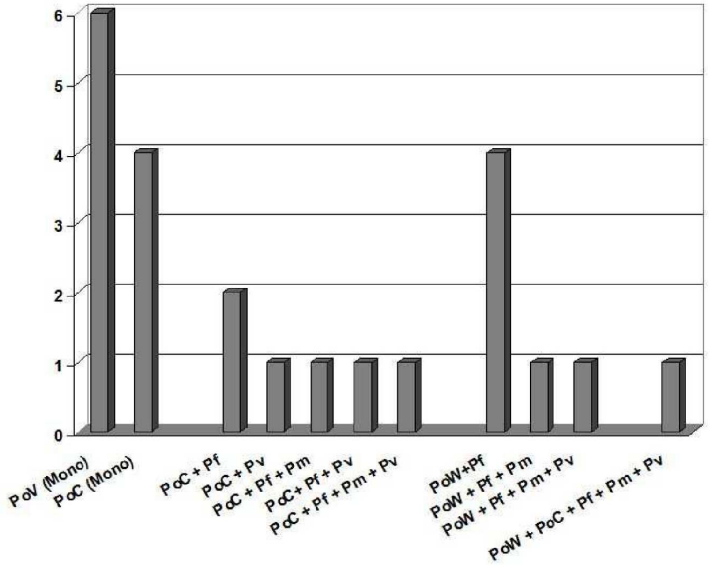


Plate 3:

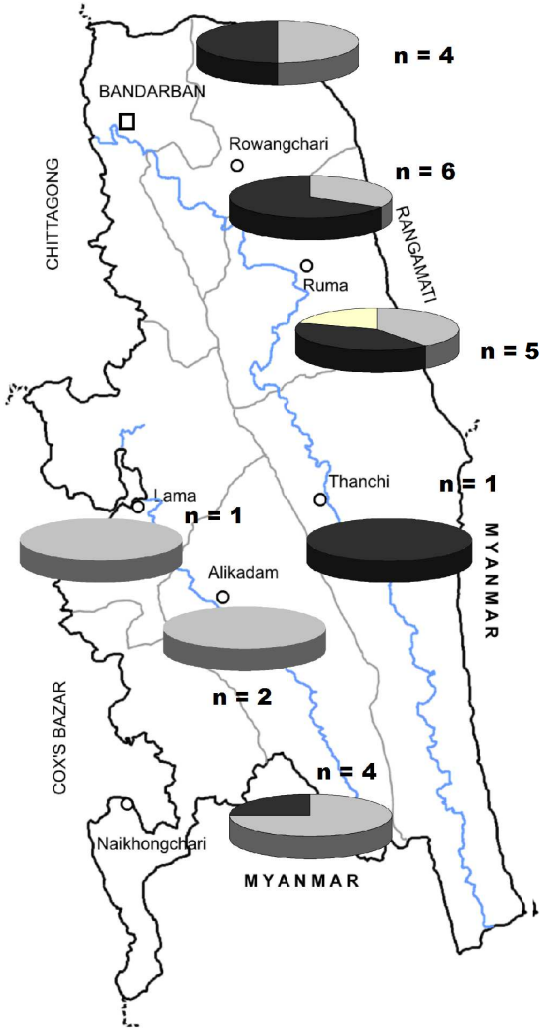
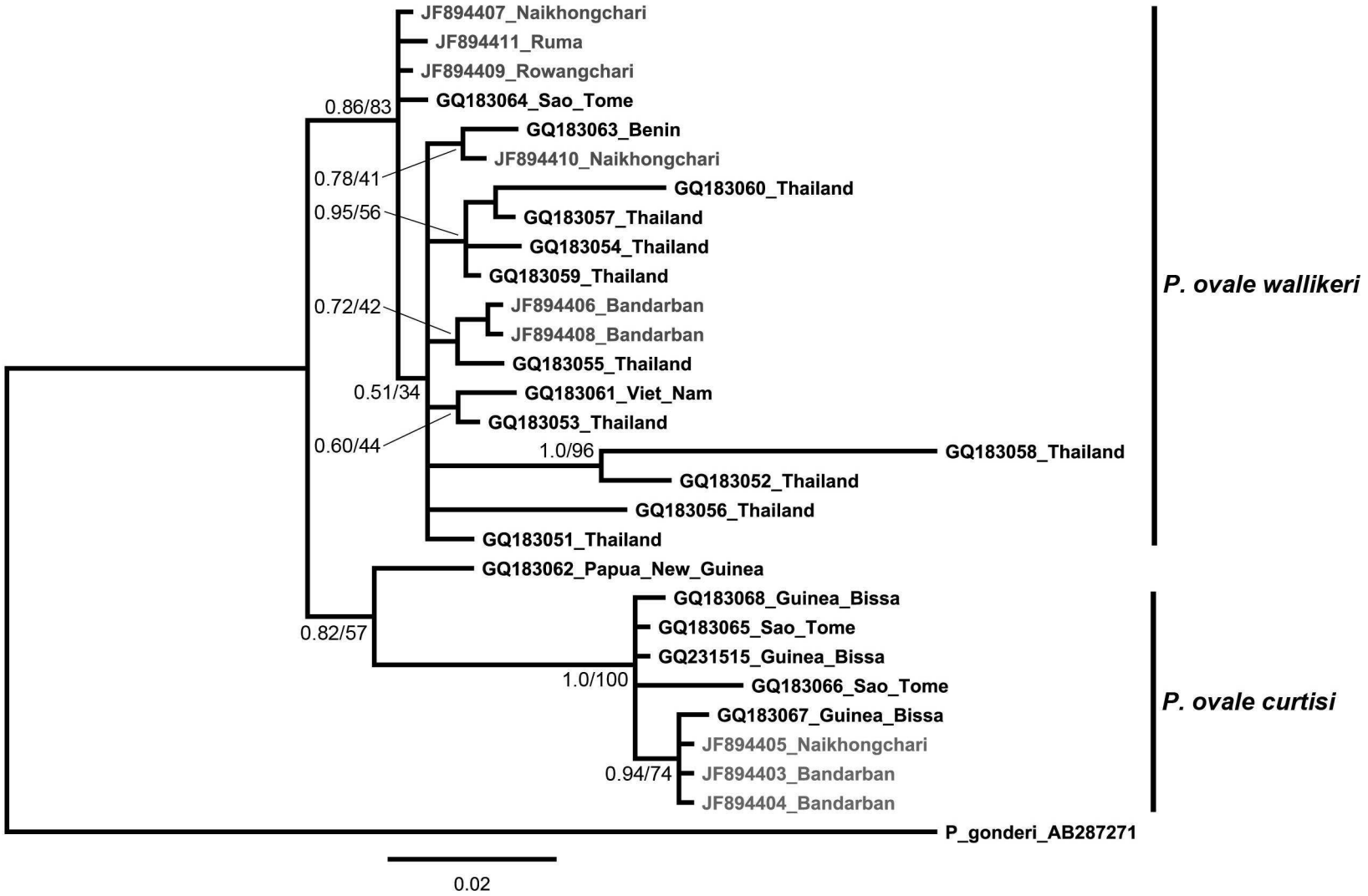


Figure 2:

Bayesian phylogenetic tree inferred from SSU rRNA sequences of Sutherland et al. (2010), and of samples of *P. ovale wallikeri* and *P. ovale curtisi* collected in Bangladesh. *P. gonderi* was used as outgroup. Bayesian posterior probabilities are given left of the slash, respectively bootstrap values of a Neighbor-Joining analyses right of the slash.


JF894407_Naikhongchari
JF894411_Ruma
JF894409_Rowangchari
GQ183064_Sao_Tome
0.86/83
GQ183063_Benin
JF894410_Naikhongchari
0.78/41
GQ183060_Thailand
0.95/56
GQ183057_Thailand
GQ183054_Thailand
GQ183059_Thailand
0.72/42
JF894406_Bandarban
JF894408_Bandarban
GQ183055_Thailand
0.51/34
GQ183061_Viet_Nam
GQ183053_Thailand
0.60/44
1.0/96
GQ183058_Thailand
GQ183052_Thailand
GQ183056_Thailand
GQ183051_Thailand
GQ183062_Papua_New_Guinea
GQ183068_Guinea_Bissa
0.82/57
GQ183065_Sao_Tome
GQ231515_Guinea_Bissa
1.0/100
GQ183066_Sao_Tome
GQ183067_Guinea_Bissa
JF894405_Naikhongchari
0.94/74
JF894403_Bandarban
JF894404_Bandarban
P_gonderi_AB287271
0.02
P. ovale wallikeri
P. ovale curtisi

6.5 Molecular epidemiology of *Plasmodium malariae* and *P. knowlesi* in Bangladesh.

6.5.1 Introduction

Although malaria is known to be hypoendemic in the South-Eastern part of Bangladesh, there is scarce information about 4 of the 6 *Plasmodium* species parasitizing humans (Khan 2011). Beside the two newly described *P. ovale* species, *P. ovale wallikeri* and *P. ovale curtisi*, there is also a lack of knowledge about the distribution of *P. knowlesi* and *P. malariae* in Bangladesh.

P. malariae is known to be endemic in all major malaria endemic areas in the world. This parasite is widespread throughout sub-Saharan Africa, Southeast Asia, the Western Pacific Islands and in many areas of the Amazon Basin of South America (reviewed in Mueller et al. 2007). The prevalence of *P. malariae* has a range from less than 4% to more than 20%, but recent molecular epidemiological studies suggest that the true burden of infections with *P. malariae* is vastly underestimated. In Bangladesh the first published report of this pathogen was filed in 2004, where a prevalence of 1% was determined in a study conducted in the Chittagong Hill Tracts (van den Broek et al. 2004). Furthermore, one case of an extremely rare and severe *P. malariae* infection was documented in 2010 (Rahman 2010).

P. knowlesi was formerly known as a simian parasite occurring in macaques only, but in recent years several human cases of infections with this pathogen were reported until it was recognized as the fifth human malaria species (White 2008). This parasite is known to be endemic in several Southeast Asian countries only (Kantele 2011). The distribution of this parasite is strictly limited to its intermediate hosts (several macaque species with *Macaca fascicularis* as the main host) and its final hosts (mosquitos of the *Anopheles leucosphyrus*-group) (Cox Singh et al. 2008a, Sallum et al. 2005). In Bangladesh the final host as well as the intermediate host are present, although the population of *Macaca fascicularis* is limited to the Southeasternmost part of Bangladesh (Molur 2003).

Within this chapter unpublished data of the distribution of *P. malariae* and *P. knowlesi* and the phylogenetic analysis of *P. malariae* obtained within a hospital-based fever study and a cross-sectional field survey will be summarized (Fuehrer 2010, Fuehrer 2011a).

6.5.2 Materials and Methods

Blood spots on filter papers were collected from 379 patients presenting febrile diseases and 1867 asymptomatic participants as mentioned in chapter xx (Fuehrer 2011a). A nested PCR binding at conserved regions of the mitochondrial SSU rRNA gene was used for the screening of *P. malariae* and *P. knowlesi*. These techniques were mentioned and/or summarized in previous chapters. The species specific primer-pairs rMAL1 and rMAL2 and Pmk8 and Pmk9r were used for the detection of *P. malariae* and *P. knowlesi* respectively.
For the diagnosis of variations of *P. malariae* five positive samples were further analyzed by partial sequence analysis of the SSU rRNA gene. Therefore, five blood samples with *P. malariae* monoinfections were analyzed with the genus-specific primers rPLU2 and rPLU3 and sequenced. The sequences were analyzed as previously described (Fuehrer 2011a).

6.5.3 Results

In asymptomatic participants we found an overall prevalence of *P. malariae* of 2.8%, and 9.6% of those were positive for any kind of malaria. In total 53 participants of the asymptomatic survey and 7 of the fever study were tested positive for *P. malariae* by PCR analysis. The rate of mixed infections was high (Fig. 1 and 2).
We found no evidence for the presence of *P. knowlesi* in those studies.

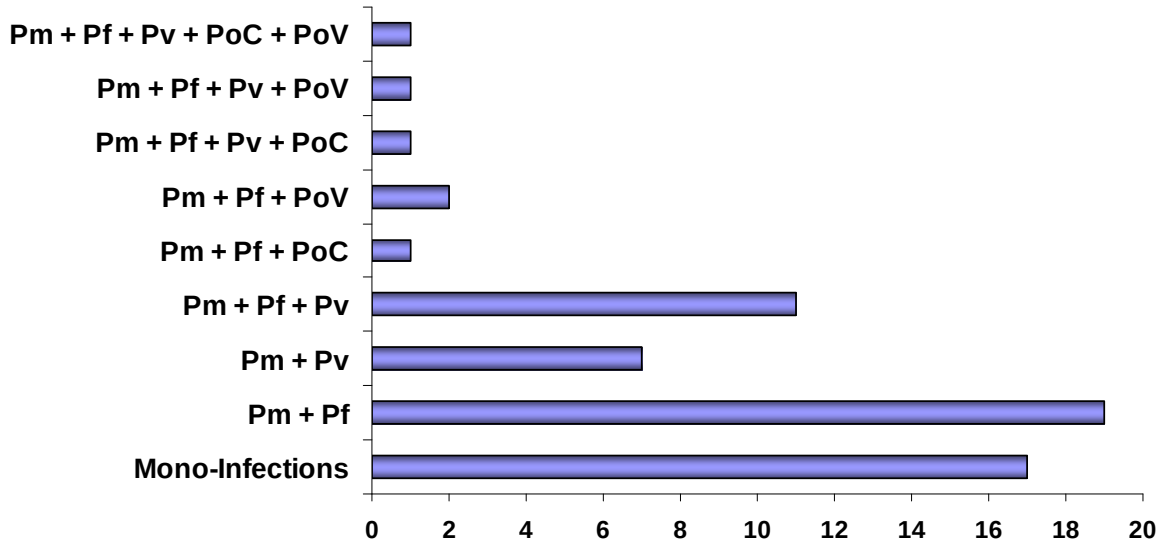


Fig. 1: Comparison of mono- and mixed infections of *P. malariae* in Bangladesh (Pf = *P. falciparum*, Pv = *P. vivax*, Pm = *P. malariae*, PoV = *P. ovale* (variant form), PoC = *P. ovale* (classic form)

Partial sequence analysis of the SSU rRNA gene of 5 isolates of *P. malariae* revealed 5 almost identical partial sequences. These sequences were identical (or at least 99%) to a *P. malariae* isolate collected in a chimpanzee (GenBank Accession Number: AB489195) (Hayakawa et al. 2009).

6.5.4 Discussion

Although *P. knowlesi* was not present in any of the samples examined within this study, the possible distribution of this pathogen in Southeastern Bangladesh should be kept in mind. Human infections with this parasite are often mistaken in microscopy-based diagnosis for infections with *P. falciparum* (early trophozoites) or *P. malariae* (similar morphology) (Singh et al. 2004, Lee et al. 2009). *P. knowlesi* has a daily (quotidian) life cycle, potentially leading to a fatal outcome. Fatal cases have been documented in Malaysia (Cox-Singh et al. 2008b). Hepatorenal dysfunction and hyperparasitemia were reported. However, the nominal distribution of the main intermediate host (*Macaca fascicularis*) to the very Southeastern parts of Bangladesh may be the factor of limitation for the probable absence of *P. knowlesi* in Bangladesh. Due to the fact that this malaria-causing parasite and *Macaca fascicularis* are endemic in nearby Myanmar, and the presence of migration of refugees from Myanmar to Bangladesh, microscopists in Bangladesh should be aware of this to prevent mix-ups with other malaria pathogens (Jiang et al. 2010).

P. malariae possesses a quartan life cycle and its clinical symptoms are known to be less harmful if compared to *P. falciparum.* It is known for its long living blood stage parasites causing recrudescences (Collins and Jeffery 2007). *P. malariae* is responsible for 1-2% of all fever cases in malaria endemic countries and causes up to 50% of all malaria cases in the low-transmission season in areas with seasonal distribution (Mueller et al. 2007). Most epidemiological studies in the past were based on the diagnosis by light microscopy only. However, the microscopical differentiation between *P. malariae* and *P. falciparum* is difficult, and in areas where *P. falciparum* is dominant the true prevalence of *P. malariae* might be mistaken (Mueller et al. 2007). The introduction of molecular diagnostic tools simplifies the accurate diagnosis of parasites like *P. malariae.*

In our study 3.9% of the fever patients presenting with malaria were positive for *P. malariae.* The prevalence of *P. malariae* in asymptomatic malaria positive participants was high with 9.6%. Neighbouring Myanmar is known to be one of the countries with the highest prevalence of *P. malariae* outside Africa, and prevalences between 10 and 20% have been reported in the past (Zhou et al. 1998, Garnham 1966, Haworth 1988). The zoonotic potential of diverse human malaria parasites is an upcoming topic of research. Several authors reported human malaria parasites in monkeys. In 2009 *P. malariae* was found by analysis of the SSU rRNA gene in a chimpanzee from Africa imported to Japan (Hayakawa et al. 2009). Furthermore, *P. malariae* is genetically indistinguishable from *P. brasilianum*, a parasite of platyrrhine new-world monkeys. Recent studies revealed identical sequences within the *Csp*-, *SSU rRNA*-, and *MSP-1* genes and brought up discussions of the host transfer from human to monkeys or vice versa (Escalante et al. 1995, Tazi et al. 2011).

Within this study 5 partially sequenced SSU rRNA genes of *P. malariae* gave almost identical results. However, sequence variations within the SSU rRNA gene are known from China to Southeastern Asia (Liu et al. 1998). We estimate that different forms of *P. malariae* might be present in Southeastern Bangladesh, but limited financial resources allowed the sequence analysis of a few samples only.

Within our study 53 participants were positive for *P. malariae* without showing any kind of symptoms. Such results make it obvious that there is an urgent need for further studies to examine not only the epidemiology, but also the clinical features of *P. malariae* monoinfections and mixed infections in Bangladesh.

Presented in part

HP. FUEHRER, P. STARZENGRUBER, P. SWOBODA, J. MATT, K. THRIEMER, W.A. KHAN, E.B. YUNUS, S.M., HOSSAIN, J. WALOCHNIK, H. NOEDL: PCR-based prevalence screening for *Plasmodium* sp. in the Chittagong Hill Tracts, Bangladesh.- ÖGTP 2009 Vienna/Austria.

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6.6 A highly sensitive in vitro-assay for the detection and quantification of *Plasmodium vivax* parasite biomass after the usage of different cultivation methods.

6.6.1 Introduction

Plasmodium vivax is the most widely distributed and, with the exception of equatorial Africa, the most prevalent causative of malaria infections in humans (Mendis et al. 2001). Annually up to 70 million cases of infections with *P. vivax* are reported from endemic countries from Asia, Africa, Central and South America, Oceania, the Middle East and some countries in Europe. Although the mortality rate of *P. vivax* malaria is lower than the one of *P. falciparum*, this parasite is one of the main factors of socio-economic instability and poverty in affected countries. In comparison to infections with *P. falciparum* the therapy is considerably more complex, because beside its blood forms it can form dormant stages in the liver. First cases of resistances to chloroquine, the drug of choice in the therapy of *P. vivax*, were reported from Papua New Guinea in 1989 (Rieckmann et al. 1989, Schuurkamp et al. 1989). Further Asian countries (e.g. India and Myanmar) reported chloroquine resistances in the upcoming years (WHO 2010). Furthermore, antifolate-resistances of *P. vivax* were documented frequently in Southeast Asia and less commonly in South Asia (WHO 2007). This leads to the urgent need to establish in vitro cultivation techniques to expedite and facilitate the development of drugs against *P. vivax* (Wernsdorfer et al. 2008).

However, the *in-vitro* cultivation of *P. vivax* is confronted with a number of problems and several researchers tried to cultivate this parasite before the 1980s without success (Udomsangpetch et al. 2007). Long-term cultivation of these pathogens require the supplementation and modification of standard culture media (e.g. RPMI) and the addition of reticulocytes obtained from normal or hemochromatoic blood (WHO 2007, Udomsangpetch et al. 2007). In the peripheral blood circulation parasitemias of *P. vivax* are generally low because of the low proportion of reticulocytes (Geaghan 2000). Furthermore, for cultivation only parasite isolates with a predominance of ring forms should be taken (WHO 2007). This circumstance led to the fact that up to now no cultivation technique was employed which allows the cultivation of *P. vivax* under field conditions for 72 hours (e.g. Druilhe et al. 2007).

6.6.2 Materials and Methods

The study was part of a hospital based fever survey which was conducted from 2008 to 2009 at the MARIB field site (Bandarban Sadar Hospital, Bangladesh). From each patient or their legal representatives' written informed consent was obtained before blood collection. The study protocol was reviewed and approved by the Ethical Review Committee of the International Center of for Diarrhoeal Diseases Research, Bangladesh. Heparinized blood samples were collected from patients with microscopically confirmed *P. vivax* monoinfections with a parasitemia of 0.5% or higher. Those samples were cultured on 96-well microtiter plates for 72 hours at 37°C, 5% C, 5% O_2 and 90% N_2 with different techniques to optimize growth.

First, various kinds of standard media were prepared (e.g. RPMI 1640, McCoy 5A) and supplements (e.g. vitamines, hypoxanthine, ascorbic acid) were added to those media in concentrations mentioned by various authors (Tab. 1, Fig. 1). Subsequently tests on the effect of media changes (no change, change every 24 hours, change every 48 hours), the effect of leukocyte filtration, addition of cord blood (retikulocytes), and the influence of different serum concentrations (15-50%) were made.

Table 1: Supplements used for the cultivation of *P. vivax*.

Supplements	used Concentrations
Ascorbic acid	2.84 – 34.1 µM
Dextrose	5.55 – 16.65 mM
Hypoxanthine	73.74 – 370 µM
Thiamine hydrochloride	8.8 µM
Potassium dihydrogene phosphate	120 µM
Calcium chloride	270 µM
Magnesium sulfate	130 µM
HEPES	25 mM
Vitamin B complex	10 µM

In the last trials one cultivation technique with a proven reinvasion of *P. vivax* was chosen to examine the influence of hypoxanthine (no addition, 73 µM, 370 µM, 1,000 µM) on the growth of *P. vivax* and *P. falciparum*.

For the microscopical anlaysis of the parasitemia culture slides (thin smear and thick drop) were made 0, 24, 48 and 72 hours after starting the culture. Parasites were differentiated into growth stages – ring forms, trophozoites and schizonts – as reported previously (Chotivanich et al. 2001). Furthermore, cultured parasite samples were transferred from the culture plate to a second microtiter plate 0, 24, 48 and 72 hours after starting cultivation. A highly sensitive aldolase-double site sandwich ELISA was performed using two polyclonal antialdolase antibodies (RPVA-55A and RPVA-55P, Immunology Consultants Laboratories, Newberg, OR, USA) to detect and quantify aldolase which is produced by all parasites of the genus *Plasmodium*.

Year	Cultivation media	Conditions	Cultivation period (days)
1979	RPMI-1640	Red blood cell (RBC) extracted fraction I	4
1985	SCMI 612	$MgCl_2$ 0.75 mM, ascorbic acid 0.6 μg ml^{-1} 39 °C in candle jar, 15% human AB serum	2
1987	Waymouth:RPMI-1640 (1:2)	$MgCl_2$ 1.8 mM, ascorbic acid 3 μg ml^{-1} 38.5 °C in candle jar, 15% human AB serum	2
1992	RPMI-1640	Ascorbic acid 0.6 μg ml^{-1} 37 °C in flow vessel, 15% human AB serum, 3% CO_2 A mixture of monkey and human erythrocytes (1:1)	22
1997	McCoy's 5A	Ascorbic acid 0.5 μg ml^{-1} 37 °C, 20% human AB serum, 5% CO_2 Reticulocytes from hemochromatotic blood	Continuous
2001	RPMI-1640	Ascorbic acid 6 μg ml^{-1} 37 °C, 50% human AB serum, 5% CO_2 No addition of erythrocytes	12
2007	McCoy's 5A	Ascorbic acid 0.5 μg ml^{-1} 37 °C, 25% human AB serum, 5% CO_2 Cord blood erythrocytes	30–40
2007	McCoy's 5A	Ascorbic acid 0.5 μg ml^{-1} 37 °C, 25% human AB serum, 5% CO_2 Cultivated erythroblast	Continuous

Fig. 1: Udomsangpetch R et al. (2008): Cultivation of *Plasmodium vivax*. Trends Parasitol. 2008 Feb;24(2):85-8.

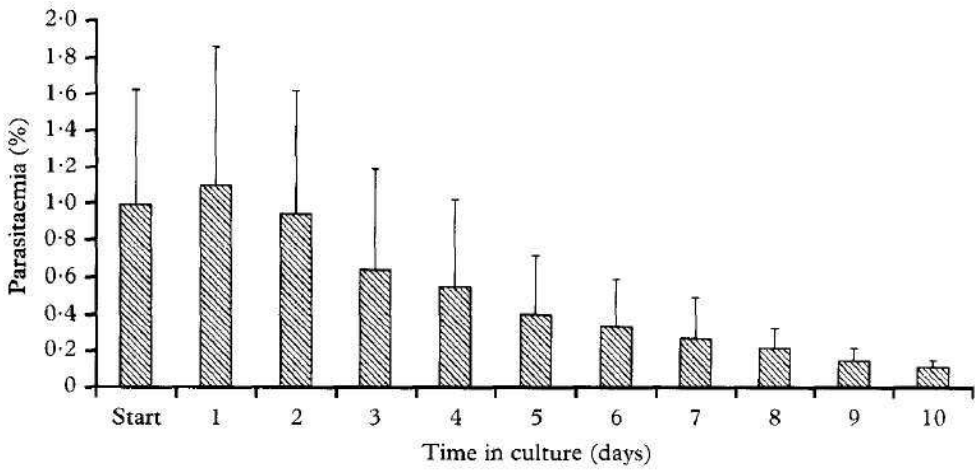
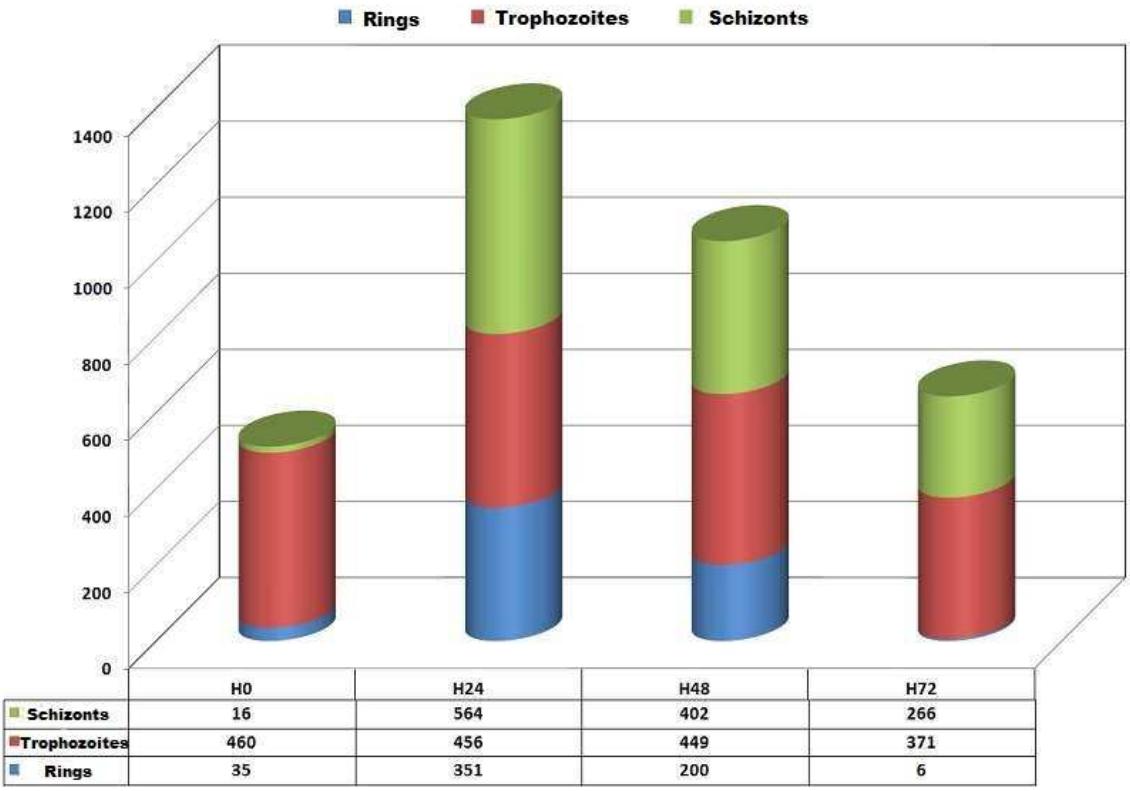


Fig. 2: Chotivanich K et al (2001).: Ex-vivo short-term culture and developmental assessment of *Plasmodium vivax*. Trans R Soc Trop Med Hyg. 2001 Nov-Dec;95(6):677-80

6.6.3 Results and Conclusions

Overall 98 different cultivation techniques were tested with up to 6 different *P. vivax* field isolates to optimize growth of *P. vivax* in *in vitro* culture under field conditions. The addition of supplements (especially ascorbic acid, hypoxanthine, and dextrose) enables an optimized growth for 24 hours with microscopically confirmed reinvasion (Fig. 3). Best parasite growth was obtained with the supplementation according to Chotivanich (2001) consisting of 1 g dextrose, 16 mg $MgSO_4$, 26 mg KH_2PO_4, 30 mg $CaCl_2$, 6 mg ascorbic acid, 3 mg thiamine and 10 mg hypoxanthine to each 1,000 ml of RPMI-1640 medium.



	H0	H24	H48	H72
Schizonts	16	564	402	266
Trophozoites	460	456	449	371
Rings	35	351	200	6

Fig. 3: Cultivation of *P. vivax* with supplemented RPMI-1640 media and 50% serum under field conditions in Bangladesh.

Furthermore, the influence of serum concentrations on parasite growth was confirmed. Within the same media a two-fold increase in growth rate was shown with the use of serum concentrations between 30 and 50% in contrast to serum concentrations between 20 and 25% (Fig. 4).

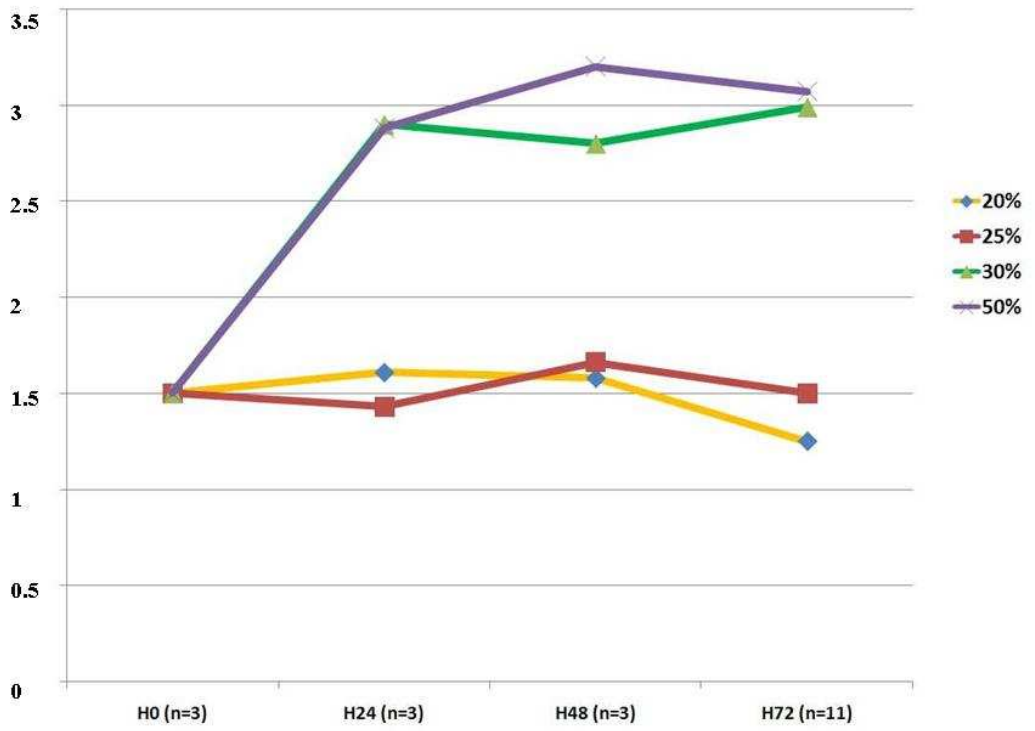


Fig. 4: Influence of serum concentrations on the growth of *P. vivax* at cultivation under field site conditions for 0, 24, 48 and 72 hours (ordinate = ODs 0-3.5 using an ELISA plate reader at 450 nm).

The change of media and leukocyte filtration tests had almost no effects on parasite growth under field site conditions. Furthermore, cultivation of *P. falciparum* in media supplemented with hypoxanthine (73 µM and 370 µM), dextrose and ascorbic acid leads to a 3-5 fold increase of the growth rate in comparison to the cultivation with common hypoxanthine-free RPMI-1640 medium.

The aldolase-ELISA used in this study has proven to be highly sensitive and well suited for quantifying parasite growth and its inhibition. The limit of detection of ELISA reaches 0.0005% of infected red blood cells and is considerably more sensitive in *P. vivax* than in *P. falciparum* (Thriemer et al. 2008).

In 72 h *P. vivax* cultures optimal growth rates and conditions continue to be a problem. The number of samples that actually grow and lead to reinvasion remains small. A short-time cultivation of *P. vivax* for 24 hours with proven reinvasion was confirmed, but after 24 hours parasitemia is declining. Our results hereby confirm those published by the group around Chotivanich in 2001 (Fig 2, Fig 3). However, none of the tested techniques is suitable to reach a growth needed for a 72 hour cultivation technique used at drug activitiy and drug resistance tests at *P. falciparum*. We assume that the main causative is the preference of *P. vivax* for reticulocytes, and tests where highly equipped laboratories are needed turned out not to be suitable for the usage under field site conditions.

There is an urgent need to optimize growth conditions for *P. vivax* to obtain dose-response curves similar to those achieved with the HRP2 drug sensitivity assay standardized for *P. falciparum*.

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K. THRIEMER, H.-P. FUEHRER, R. HAQUE, Harald NOEDL: Ein neuer, hochsensitiver Test für den Nachweis und die Quantifizierung von *Plasmodium vivax* Parasiten.- ÖGTP 2008 Innsbruck/Austria

7 Conclusion

Optimized standards in the diagnosis of *Plasmodium* species are essential to control and adequately treat malaria. Although the limitations of microscopy are known, it still remains the gold standard of malaria diagnosis. Lower sensitivity of microscopy may lead to a vast underestimation of the true malaria burden, especially of less documented species such as *P. malariae*, *P. ovale*, and *P. knowlesi* in South and South-East Asia.

Until very recently only *P. falciparum* and *P. vivax* were known to be endemic in Bangladesh. Most malaria studies had their focus on these parasites only, because these malaria species are the main causatives of symptomatic malaria (e.g. Thriemer et al. 2006; Starzengruber et al. 2009). Less than a handful of cross-sectional malaria surveys were performed in Bangladesh and those were based on the diagnosis by RDTs or microscopy only (Haque et al. 2009; Maude et al. 2008). It took until 2004 before *P. malariae* was first reported, with a prevalence of 1% in patients from the Chittagong Hill Tracts, and since this report only one case of a severe infection with *P. malariae* has been published (van der Broek et al. 2004, Rahman et al. 2010). Neither *P. ovale* nor *P. knowlesi* were considered to be endemic in Bangladesh.

In our initial study we were the first in the region who analyzed blood samples collected in a hospital-based fever survey by nested PCR-corrected microscopy not only to examine the prevalence of *P. falciparum*, *P. vivax*, and *P. malariae* in febrile patients, but with the main purpose of evaluating the endemicity of *P. ovale* and *P. knowlesi* in the Chittagong Hill Tracts. 379 blood samples on filter papers of patients reporting fever within the last 72 hours were analyzed by standardized nested PCR techniques targeting highly conserved regions of the *SSU rRNA* gene (e.g. Snounou and Singh 2002). In total 189 out of 379 (49.9%) febrile participants tested positive for *Plasmodium* sp. at genus-specific Nest 2 PCR. Although *P. falciparum* (81.5%) and *P. vivax* (26.5%) were the dominant species in the district of Bandarban, 3.7% of all patient samples gave positive results for *P. malariae* and 1.6% for *P. ovale*. The rate of mixed infections was high at 13.2%. *P. knowlesi* was not found in any of the analyzed samples.

A second survey conducted in our field site in Bangladesh focused on analyzed blood samples of asymptomatic participants within a cross-sectional survey in the District of Bandar-

ban (Habler et al. 2010). Within this study an asymptomatic malaria prevalence of 22.3% was observed. *P. falciparum* was the dominant species, causing 80.2% of all infections, followed by *P. vivax* (32.4%), *P. malariae* (9.6%), and *P. ovale* (3.44%).

The true malaria burden, especially of the less common malaria parasites and mixed infections, was grossly underestimated before. Our initial results gave a fair warning that molecular tools are not only of higher sensitivity and specificity than microscopy, but also uncover a higher malaria prevalence and number of mixed infections compared to non-PCR-based studies (Steenkeste et al. 2009).

Within the first PCR-corrected study we were able to detect for the first time in Bangladesh *P. ovale* in three (1.6%) patients, who presented febrile, malaria-like symptoms at our MARIB field clinic. The parasites were misdiagnosed by microscopy as *P. malariae* or *P. vivax* because *P. ovale* was not known to be endemic in this region. Moreover, we were able to diagnose the first confirmed case of a relapse caused by this pathogen in Bangladesh, which was further described within a case report by our team (Starzengruber et al. 2010).

Because of the use of the primer pair rPLU2/rOVA1 (a genus-specific primer combined with a classic *P. ovale* specific primer), we decided to sequence these three samples and found that two of these isolates were of the variant type and one of the classic *P. ovale* type, thus leading to the suggestion that both the classic and the variant type of *P. ovale* are present and occur sympatrically in the Chittagong Hill Tracts. As described in the introduction to this thesis, the diagnosis of *P. ovale* by standard nested PCR protocols is problematic (Snounou et al. 1993; Snounou and Singh 2002).

Within the second study we tested all three primer pairs binding in the mitochondrial *SSU rRNA* gene (rOVA1/2; rOVA1/rPLU2; rOVA1var/rOVA2var) in more than 2,200 blood samples of febrile and asymptomatic participants and sequenced those samples presenting monoinfections of *P. ovale* only. Furthermore, the *cox1* gene, which was documented as suitable in the differentiation between variant and classic *P. ovale* parasites, was analyzed after sequencing. However, while the second study was running, the group around Collin Sutherland divided *P. ovale* into the species *P. ovale curtisi* (classic type) and *P. ovale wallikeri* (variant type) because of their dimorphic genetic characteristics in several gene loci. Sympatric distribution from Africa of those pathogens was described. Although samples from Asia (e.g. Thailand) were used in this study, only variant *P. ovale wallikeri* para-

sites were reported (Sutherland et al. 2010). Some previous observations of variations in *P. ovale* in South-East Asia (Myanmar and Thailand) were mentioned (Zhou et al. 1998, Win et al. 2002; Win et al. 2004), but as yet no prevalence study has dealt with the differentiation of both species in Asia.

Within our second study we further performed a nested PCR technique within the *potra* gene to discriminate the two new species and analyzed sequences of the *Porbp2* gene, as described by the group around Sutherland (Sutherland et al. 2010; Oguike et al. 2010). We detected *P. ovale* in 2.12% of *Plasmodium* sp. positive patients presenting febrile illnesses (*P. ovale wallikeri*: 1.59%; *P. o. curtisi* 0.53%) and in 3.44% of all asymptomatic malaria-positive participants (*P. ovale wallikeri* and *P. o. curtisi* 1.63% each, plus one patient presenting both pathogens) by nested PCRs of the *SSU rRNA* gene and the *potra* gene.

Within this study we were able to prove the sympatric distribution of *P. ovale wallikeri* and *P. ovale curtisi* in South Asia for the first time. Furthermore, this is the first analysis of those sympatric distributions within an area of less than 5,000 km^2 worldwide. Phylogenetic analysis within partial sequences of the *SSU rRNA*, *Porbp2*, and *Cox1* genes of blood samples presenting monoinfections with these pathogens confirmed that *P. ovale wallikeri* and *P. ovale curtisi* are two distinct species.

Nested PCR analysis within the *potra* gene is recommendable for the differentiation of both species. For the standard nested PCR binding within conserved regions of the *SSU rRNA*, two primer pairs (= two different PCRs) should be used for the diagnosis of *P. ovale*, namely rOVA1/rOVA2 for *P. ovale curtisi* (classic type) and rOVA1var/rOVA2var for *P. ovale wallikeri* (variant type).

Only mild ovale malaria symptoms like moderate fever, chills, headache, and fatigue were observed in febrile patients. However, most infections with *P. ovale* were asymptomatic in this study. Parasite densities in monoinfections were low at 120–6,680 parasites/µl. Further clinical, epidemiological, and phylogenetic analysis is needed to determine differences between *P. ovale wallikeri* and *P. ovale curtisi*.

Although *P. malariae* was first described in the Chittagong Hill Tracts in 2004 and a severe *Plasmodium malariae* case was reported from Chittagong, information about this parasite is lacking in Bangladesh (van der Broek 2004, Rahman 2010). Overall, 60 patients presented *P. malariae* infections and over 50% of those were mixed infections with other human malaria parasites. Within our studies we examined a prevalence of *P. malariae* in

1.8% of the febrile patients and in 2.8% of the asymptomatic volunteers. The slightly higher prevalence of *P. malariae* in asymptomatic participants can be explained by typically milder clinical manifestations compared to other malaria parasite species, and by the circumstance that this parasite can inhabit its human host for an extended period of time. The prevalence in South-East Asia is normally below 4% (Mueller et al. 2007). However, neighboring Myanmar is known to be one of the countries with the highest prevalence of *P. malariae* outside Africa, and a prevalence of between 10 and 20% has been reported in the past (Zhou et al. 1998, Garnham 1966, Haworth 1988). Within this study five partially sequenced *SSU rRNA* genes of *P. malariae* gave almost identical results. However, sequence variations within the *SSU rRNA* gene are known from China to South-Eastern Asia (Liu et al. 1998). We estimate that different forms of *P. malariae* might be present in South-Eastern Bangladesh, but limited financial resources allowed the sequence analysis of a few samples only.

Overall, our results confirm the presence of *P. malariae* in the Chittagong Hill Tracts with a prevalence of almost 10% in malaria-positive asymptomatic patients. Although *P. malariae* is known to be less severe than *P. falciparum*, care should be taken by practitioners to prevent misdiagnosis.

P. knowlesi, a simian malaria parasite, is now recognized as a species frequently infecting humans in several South-East Asian countries (White 2008). Analysis was performed within the *SSU rRNA* gene using the primers Pmk8 and Pmk9r. Cross-reactivity of these primers with *P. vivax* genomic DNA within the *ssr RNA-S* gene was reported previously and was also confirmed within our study by sequence analysis (Imwong et al. 2009). However, although these primers bind to *P. vivax*, they can be used for the detection of *P. knowlesi*. The amplified sequences only vary minimally in size and can be differentiated with the use of a positive control and, if a large gel is used, for analysis during gel electrophoresis. We observed no case of *P. knowlesi* in any of the samples collected in the Chittagong Hill Tracts. Both natural hosts – *Macaca fascicularis* as well as mosquitoes of the *Anopheles leucosphyrus* group are reported to be endemic in South-Eastern Bangladesh, but the distribution of *M. fascicularis* is limited to the very south-eastern part of the Chittagong Hill Tracts only (Molur et al. 2003). Furthermore, several tribes of the indigenous population hunt monkeys as game, and so the distribution might be scarce. This may have caused the absence of *P. knowlesi* within the studies performed. However, because of the morphological similarities to *P. falciparum* (young rings) and *P. malariae* (other blood

stages), care should be taken to prevent misdiagnosis, not only in epidemiological surveys and local laboratories, but also in the diagnosis of travellers returning from South-East Asia presenting malaria (Lee et al. 2009).

Microscopy and RDTs are the most commonly used diagnostic tools of malaria, and microscopy still remains the gold standard for malaria diagnosis. The limits of detection vary between microscopists and are estimated to range between 50 and 100 parasites/µl (Wongsrichanalai et al. 2007). The introduction of molecular techniques in recent decades allows a far more sensitive method of detection of these pathogens. Within the third study a novel nested Direct PCR technique was established which allows PCR amplifications without prior DNA purification or isolation steps using a Phusion blood polymerase. The biggest advantage of Direct PCR is the saving of time, because the complete step of DNA isolation and purification is not needed. Furthermore, specificity (96%) and sensitivity (99.8%) are very high and comparable to previous protocols used in malaria diagnosis (e.g. Snounou and Singh 2002). Moreover, the limit of detection is only 3 parasites/µl compared to 6 parasites/µl with standard techniques (Singh et al. 1999). Furthermore, Direct PCR is a useful alternative to common PCR protocols, providing a highly sensitive, rapid, and cost-effective method of malaria and other blood pathogen diagnosis. Within our molecular epidemiological studies we used Direct Nested PCR with those samples showing poor positive signals at standard nested PCR (because of low parasitemia) to obtain more evaluable DNA after a Nest 1 reaction for sequence PCRs and sequence analysis.

P. vivax is considered to be the most prevalent and most widely distributed malaria parasite in humans worldwide. However, drug sensitivity tests are hampered by the inability to culture this malaria parasite for at least 72 hours with continuous growth. *P. vivax* has – like *P. ovale* – the preference to invade reticulocytes, but only 1–2% of the red blood cells are of this type in healthy individuals (Kasper et al. 2005). Thus the preference for reticulocytes seems to be the most hampering and aggravating problem in the prolonging cultivation of *P. vivax*.

Following previous published methods we tried to cultivate *P. vivax* parasites for 72 hours under field site conditions. In this experiment, 98 different cultivation techniques were tested. Of all techniques tested, best growth and proven reinvasion was obtained with the supplementation of dextrose (5.55 mM), $MgSO_4$ (0.13 mM), KH_2PO_4 (0.12 mM), $CaCl_2$ (0.27 mM), ascorbic acid (34.1 µM), thiamine (10 µM), and hypoxanthine (73.47 µM) to

RPMI-1640 medium. However, although parasites can be cultivated for 72 hours the parasitemia starts to decline after the first reinvasion, an effect also documented by Chotivanich et al. (2001). We assume that the preference to invade reticulocytes causes this phenomenon. Furthermore, the addition of serum seems to play an important role in cultivating this parasite. We obtained twice-fold growth rates at serum concentrations of 30–50% serum in comparison to 20–25% serum. Overall, we can not recommend any of the tested *P. vivax* cultivation techniques for the usage in drug sensitivity assays under field site conditions, because the decline in parasite densities may cause misinterpretations regarding drug activities in *P. vivax*. Further studies are of urgent need to optimize and simplify growth conditions in *P. vivax*, but also in *P. ovale* and *P. malariae*.

The results of this thesis can be summarized as follows:

1. *P. ovale* was detected in Bangladesh for the first time.
2. Confirmation that *P. ovale* possesses dimorphic characteristics and consists of two species, namely *P. ovale curtisi* and *P. ovale wallikeri.*
3. Both are endemic and occur sympatrically in the Chittagong Hill Tracts in Bangladesh.
4. The sympatric distribution of both *P. ovale curtisi* and *P. ovale wallikeri* was proven in South Asia for the first time.
5. Infections of both types of ovale malaria cause mild syndrome complexes or are asymptomatic.
6. *P. malariae* is a common parasite in the Chittagong Hill Tracts causing mild symptoms or being asymptomatic.
7. There was no evidence of *P. knowlesi*.
8. *P. falciparum* is the dominant species causing human malaria in the Chittagong Hill Tracts, followed by *P. vivax.*
9. A high rate of mixed infections was observed.
10. Efforts to cultivate *P. vivax* for 72 hours under field conditions were of limited success.
11. A novel Direct PCR technique was introduced which allows a cost-effective, sensitive, and rapid molecular diagnosis of malaria parasites.

Several years ago only four different *Plasmodium* species were known to infect humans but now six species have been confirmed as causing malaria in humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi*, *P. ovale wallikeri*, and *P. knowlesi* (Sutherland et al. 2010; White 2008). These verifications were only made possible by the introduction of molecular tools, and so enabled the confirmation of endemicity of five out of the six human malaria parasites in an area with less than 5,000 km^2 in South Asia. Furthermore, our results confirm that the true malaria burden in the Chittagong Hill Tracts in Bangladesh was vastly underestimated.

However, there is still an urgent need to advance (molecular) diagnostic tools to enable exact diagnosis of the parasites causing malaria. Progress in the cultivation of different *Plasmodium* species is also needed to allow accurate examinations to confirm resistances and to develop new antimalarial drugs.

8 Abstract

Malaria infections are still one of the major global health burdens, and recent reports of emerging artemisinin resistance in Southeast Asia will add another challenge to malaria control efforts. High standards in the diagnosis of the malaria-causing *Plasmodium* species are essential in order to control and adequately treat malaria. In spite of its known limitations, microscopy remains the gold standard of malaria diagnosis which may lead to a significant underestimation of the true malaria burden, especially of less prevalent and less documented species such as *P. ovale*, *P. malariae* and *P. knowlesi* in Asia. Also the phylogeny of *P. ovale* is pending and recent studies established that *P. ovale* comprises 2 sympatric occurring non-recombining species; *P. ovale curtisi* and *P. ovale wallikeri*.
This project aimed to detect the distribution and prevalence of *P. ovale* (*P. o. curtisi* and *P. o. wallikeri*), *P. malariae* and *P. knowlesi* in the Chittagong Hill Tracts in Bangladesh. Therefore 2,246 blood spots of asymptomatic and febrile participants were analyzed by nested PCR targeting highly conserved regions of the small subunit ribosomal RNA (SSU rRNA) gene. *P. ovale* was detected in 1.02% of the study participants; 0.45% thereof were infected with *P. o. curtisi*, 0.53% with *P. o. wallikeri* and 0.04% presented infections with both parasites. Both parasite species occur sympatric in the District of Bandarban and demonstrate none or mild symptoms. Furthermore dimorphism between both species was confirmed by different PCR techniques (e.g. comparison of different protocols targeting the SSU rRNA gene; PoTRA gene) and multilocus sequence analysis (SSU rRNA, Cox 1, PoRBP2). Not only the first description of *P. ovale* in Bangladesh was documented in the course of this thesis, but also the sympatric distribution of *P. o. curtisi* and *P. o. wallikeri* was confirmed in (South) Asia for the first time.

A high prevalence of 9.6% of *P. malariae* was found in those asymptomatic patients positive for *Plasmodium* sp. by nested PCR. In comparison, 3.7% of those fever patients presenting malaria were diagnosed as *P. malariae* infections. There was no evidence of *P. knowlesi* within those studies.

Furthermore we demonstrated that the newly employed use of direct nested PCR enabled the detection of *Plasmodium* sp. from blood spots on filter papers without the time-consuming use of DNA isolation and compared it to a standardized nested PCR technique.

Direct nested PCR is highly sensitive and the limit of detection is as low as 3 malaria parasites/µl.

However, attempts to cultivate *P. vivax* under field conditions for 72 hours failed. Although reinvasion was achieved with some media, none of the more than 90 cultivation techniques resulted in an exponential growth rate comparable to the growth of *P. falciparum* because of a decline of parasitemia after 48h.

9 Zusammenfassung

Malaria-Infektionen sind auch heutzutage noch eine der größten Bürden für das Gesundheitssystem weltweit. Kürzlich nachgewiesene Artemisinin-Resistenzen in Südostasien erschweren Bemühungen der Malaria Herr zu werden. Daher sind hohe Standards in der Diagnose der Malariaerreger aus der Gattung *Plasmodium* notwendig um Malaria zu kontrollieren und adäquat zu therapieren. Obwohl die Nachteile der Malariadiagnose mittels Mikroskopie bekannt sind, ist jene immer noch der Goldstandard, das wiederum zu einer Unterschätzung der wahren Malariazahlen führen kann und speziell den Nachweis weniger häufiger und dokumentierter Arten wie *P. ovale*, *P. malariae* und *P. knowlesi* in Asien betrifft. Die Phylogenie von *P. ovale* wird derzeit neu bearbeitet und eine kürzlich veröffentlichte Studie teilte die ursprüngliche Art *P. ovale* in zwei sympatrisch vorkommende Arten auf: *P. ovale curtisi* und *P. ovale wallikeri*.

Ein Hauptziel dieser Studie war die Untersuchung des Vorkommens sowie der Prävalenz von *P. ovale* (*P. o. curtisi* und *P.o. wallikeri*), sowie von *P. malariae* und *P. knowlesi* in den Chittagong Hill Tracts in Bangladesh. Dafür wurden 2.246 Blutproben auf Filterpapier von asymptomatischen, sowie febrilen Studienteilnehmern mittels einer nested PCR die an höchst konservierten Regionen der kleinen ribosomalen Untereinheit des SSU rRNA Gens bindet, untersucht. 1,02% aller Studienteilnehmer waren *P. ovale* positiv. Bei 0,45% konnte eine Infektion mit *P. o. curtisi*, bei 0,53% mit *P. o. wallikeri*, sowie bei 0,05% eine Mischinfektion mit beiden Arten diagnostiziert werden. Der Nachweis von sympatrischen Vorkommen beider Erreger konnte im Distrikt Bandarban erbracht werden, wobei die betroffenen Patienten keine oder milde Symptome aufwiesen. Außerdem konnte ein genetischer Dimorphismus jener zwei Arten anhand verschiedener PCR-Techniken (z.B. Vergleich verschiedener Protokolle mit SSU rRNA Gen als Zielsequenz; PoTRA) sowie von Sequenzanalysen (SSU rRNA, Cox1, PoRBP2) bestätigt werden. Im Rahmen dieser Studie konnte nicht nur *P. ovale* erstmals in Bangladesh beschrieben werden, sondern auch die erstmalige Beschreibung von sympatrischen Vorkommen von *P. o. wallikeri* und *P. o. curtisi* in (Süd) Asien erbracht werden.

Eine hohe *P. malariae*-prävalenz von 9,6% konnte bei asymptomatischen Patienten mit PCR-bestätigter Malaria nachgewiesen werden. Hingegen wurden Infektionen mit diesem Erreger nur in 3,7% aller Malaria-positivien Fieberpatienten gefunden. In keiner dieser Studien konnte *P. knowlesi* nachgewiesen werden.

Mittels einer neuen direkten nested PCR Technik wurde die Diagnose von *Plasmodium* sp. von auf Filterpapier gesammelten Blutproben ohne die zeitaufwendige Anwendung diverser DNA-Isolierungstechniken etabliert und mit standardisierten nested PCR Protokollen verglichen. Hierbei wies die direkte PCR sowohl eine höhere Sensitivität als auch ein geringeres Parasitennachweisbarkeitsgrenze von 3 Parasiten/µl auf.

Versuche *P. vivax* unter Feldbedingungen für 72 Stunden zu kultivieren schlugen fehl. Es konnte zwar bei einigen Medien eine erfolgreiche Reinvasion nachgewiesen werden, jedoch wurde bei keinem der über 90 verschiedenen untersuchten Kultivierungstechniken ein mit *P. falciparum* vergleichbares exponentielles Wachstum beobachtet werden, da es nach einer Kultivierungszeit von 48 Studien zu einem Abfall der Parasitämie kam.

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11 Curriculum Vitae

PERSONAL DATA

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FORMAL AND UNIVERSITY EDUCATION

2008- current	Doctoral Thesis: Zoology, University of Vienna
2008- current	Institute of Specific Prophylaxis and Tropical Medicine (Med Univ Vienna)
2005 - 2007	Diploma Thesis: Department of Medical Parasitology; Clinical Institute of Hygiene and Medical Microbiology; Medical University of Vienna/Austria
2004 - 2007	Study Zoology (University of Vienna)
2004	1. Diploma Examination
2004 – current	Studium Irregulare – Zooparasitology (University of Vienna)
1999 - 2007	Study: Biology (University of Vienna)
1998 - 1999	Study: Veterinary Medicine
1994 - 1998	BORG Hegelgasse 14; 1010 Vienna
1992 - 1994	Hauptschule 1., 2230 Gaenserndorf
1989 - 1992	Sigmund Freud-Gymnasium, 1020 Vienna
1985 - 1989	Volksschule Vorgartenstraße 191, 1020 Vienna

WORK EXPERIENCE

since *March 2008*	MARIB – Malaria Research Initiative Bandarban, Bangladesh
August 2009	Certified Quality Control Training – Ranbaxy New Delhi/India
Jan. and Feb. 2008	Medical Parasitology (routine diagnostics) – Contract for Work and Labour
March 2007	Medical Parasitology (routine diagnostics) – Contract for Work and Labour
2008 - 2010	Lab/Practicals Tutor: Medical Microbiology (Bacteriology) in Hygiene for medical students
2005 - 2010	Lab/Practicals Tutor: Parasitology in Hygiene for medical students
2006 - 2008	Vacation Job – Medical Parasitology (routine diagnostics)
2000 - 2002	Co-Founder and freelancer at X-Over-Cards (Import business)
1995 - 2005	Diverse job activities (part-time; vacation jobs; freelancer)

PUBLICATIONS:

STARZENGRUBER P, THRIEMER K, HAQUE R, KHAN WA, FUEHRER HP, SIEDL A, HOFECKER V, LEY B, WERNSDORFER WH, NOEDL H. Antimalarial activity of tigecycline, a novel glycylcycline antibiotic. Antimicrob Agents Chemother. 2009 Sep;53(9):4040-2.

FUEHRER HP, SCHNEIDER R, WALOCHNIK J, AUER H. Extraintestinal helminths of the common vole (Microtus arvalis) and the water vole (Arvicola terrestris) in Western Austria (Vorarlberg). Parasitol Res. 2010 Mar;106(4):1001-4.

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FUEHRER HP, HABLER VE, FALLY MA, STARZENGRUBER P, SWOBODA P, KHAN WA, BLÖSCHL I, NOEDL H. Variations of *Plasmodium ovale* in Bangladesh: First evidence of the sympatric distribution of *P. ovale curtisi* and *P. ovale wallikeri* in South Asia. J Infect Dis. – in Review

FUEHRER HP, IGEL P, AUER H. *Capillaria hepatica* in man - An overview of hepatic capillariosis and spurious infections in human. Parasitol Res. 2011 Jun 30. [Epub ahead of print]

ORAL PRESENTATIONS:

HP FUEHRER, VE HABLER, K BUCZOLICH, J HARL, MA FALLY, I BLÖSCHL, P STARZENGRUBER, P SWOBODA, J MATT, R HAQUE, WA KHAN, J WALOCHNIK, H NOEDL: Molecular epidemiology of Malaria in South-Eastern Bangladesh with the main focus on the diagnosis and distribution of P. ovale curtisi and P. ovale wallikeri. ÖGTP 2011 Vienna/Austria

HP FUEHRER, WA KHAN, VE HABLER, P STARZENGRUBER, P SWOBODA, MA FALLY, R HAQUE, H NOEDL: Molecular Epidemiology of Plasmodium ovale and P. malariae in Bangladesh. 13th Annual Scientific Conference of ICDDR,B. (14-17 March 2011) ASCON 2011 Dhaka/Bangladesh

HP FUEHRER, VE HABLER, P STARZENGRUBER, P SWOBODA,
MA FALLY, J WALOCHNIK, H NOEDL: Molecular epidemiology of Plasmodium ovale and Plasmodium malariae in Bangladesh. ÖGTP 2010 Graz/Austria

HP FUEHRER, P STARZENGRUBER, P SWOBODA, J MATT, K THRIEMER, W. KHAN, EB YUNUS, SM HOSSAIN, J WALOCHNIK, H NOEDL: PCR-based prevalence screening for Plasmodium sp. in the Chittagong Hill Tracts, Bangladesh.- ÖGTP 2009 Vienna/Austria

HP FUEHRER, J WALOCHNIK, H AUER: Extraintestinale Parasiten der Schermaus (Arvicola terrestris) und der Feldmaus (Microtus arvalis) in Westösterreich (Vorarlberg). – ÖGTP 2006 Linz/Austria

POSTER PRESENTATIONS:

HP FUEHRER, VE HABLER, MA FALLY, P STARZENGRUBER, P SWOBODA, R. HAQUE, WA KHAN, H NOEDL: Molecular epidemiology of malaria in Southeastern Bangladesh, with the main focus on the sympatric distribution and diagnosis of P. ovale wallikeri and P. ovale curtisi. – ASTMH 2011 Philadelphia/USA

HP FUEHRER, MA FALLY, VE HABLER, P STARZENGRUBER, P SWOBODA, H NOEDL – A novel Direct PCR technique for the diagnosis of Plasmodium spp. from blood spots collected on filter paper. ASTMH 2010 Atlanta/USA

HP FUEHRER, K THRIEMER, HAQUE R, MATT J, STARZENGRUBER P, SWOBODA P, FALLY M, NOEDL H: A high sensitive in vitro-assay for the detection and quantification of Plasmodium vivax parasite biomass in cultures using different cultivation techniques.- DTG 2009 Munich/Germany

Member of Scientific Societies:

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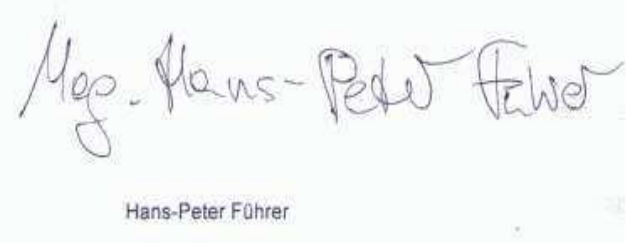
- Österreichische Gesellschaft für Tropenmedizin und Parasitologie (ÖGTP) (Austrian Society of Tropical Medicine and Parasitology) – since 2005
- American Society of Tropical Medicine and Hygiene (ASTMH) – since 2011

Further Certifications:

TOEFL

CITI - Basic Course passed on 04/18/11 (Ref # 5909856)



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