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Non-malaria febrile illness - a cross-sectional, observational study in rural areas of Cambodia

Dissertation

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To the Kingdom of Cambodia

ព្រះរាជាណាចក្រកម្ពុជា

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***Plasmodium knowlesi* infection in humans, Cambodia, 2007-2010.**

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Table of content

1 Introduction

Fever is a clinical symptom of various tropical infectious diseases. In developing countries, where diagnostic facilities are limited, etiologies of acute febrile illness remain largely unknown, whereas case fatality rates are often high and the range of potential causes is broad [1].

In Southeast Asia, malaria has long been considered one of the infectious diseases with the highest impact on public health, and has become a focus of international attention with the emergence of multi drug resistant parasite strains at the Thai-Cambodian border. Thus, control and prevention of the disease has intensified over the past decades, and as a result malaria incidence has dropped significantly [2-3]. Meanwhile acute febrile illness remains a major cause of morbidity in the region. Surveys in Thailand, Vietnam, Lao People Democratic Republic (Lao PDR) and Cambodia suggest that diseases such as leptospirosis, rickettsiosis, scrub typhus and dengue fever are important causes of fever in the region, all of which are characterized by unspecific malaria-like symptoms and thus are difficult to distinguish clinically. Although by the distribution of rapid diagnostic tests, parasite-based malaria diagnosis has become more feasible at peripheral health services, presumptive treatment of acute febrile illness with anti-malarial drugs continues to be a widespread practice. Malaria-negative fever cases are currently treated with antibiotics, most of which are ineffective against the above mentioned, potentially life-threatening diseases. This indiscriminate treatment of fever cases is not only posing a risk to the health of patients but is resulting in the unwarranted use of expensive anti-malarial drugs and the reinforcement of drug-resistance in the area [4].

For these reasons the World Health Organization Western Pacific Regional Office (WHO/WPRO), in cooperation with the Cambodian National Centre of Parasitology, Entomology and Malaria Control (CNM), the Cambodian Pasteur Institute (IPC), the Wellcome Trust Oxford University Tropical Medicine Research Collaboration in Mahosot Hospital, Vientiane, Lao PDR, and the Institute for Tropical Medicine at the University of Munich initiated a cross-sectional, observational study to investigate the causes of non-

malaria febrile illness in rural areas of Cambodia and Lao PDR. Findings of the Cambodian arm of the study are presented in this work.

1.1 Background information on Cambodia

1.1.1 Country profile

The Kingdom of Cambodia is an agricultural country, 181,035 square kilometers in size and located in the heart of Southeast Asia. Cambodia is bordered by Thailand in the west and north, by Lao PDR in the north and by Vietnam in the east, as shown in figure 1. The southeast of the country adjoins the gulf of Thailand, forming the 443 kilometers of coastline. The country's central region is characterized by the Tonle Sap Lake and expansive green rice paddies, whereas the borders in the north and west of the country are lined with mountainous ridges, of which the highest peak reaches 1,771 meters of altitude. Despite significant developments in infrastructure in recent years, some of the hilly and thickly forested areas along the borders remain very isolated [5].

Figure 1: Location of Cambodia (map created with stepmap.de®)

Cambodia's climate is tropical and characterized by the monsoon seasons. The rainy season starts in late April and lasts until early October, with the heaviest rainfall in the months of August and September.

Figure 2: Cambodian landscape impression (Tara Müller, 2009).

According to the Demographic Health Survey 2010 and Cambodia's Ministry of Health (MoH), there are currently 13.4 million people living in Cambodia, the vast majority (80.5 %) of which live in rural areas [5-6]. The annual population growth rate was 1.5 % in the year 2008 [6]. Appendix 9.1 summarizes important socio-demographic data of Cambodia. Cambodia has a relatively broad-based population-pyramid structure because 45.0 % of the population is less than 20 years old [6]. However, the percentage of people age 30-39 is less than would be expected. On the one hand this can be explained by the recent history of the country, marred by 30 years of civil war and the brutal regime of Pol Pot and the Khmer Rouge from 1975 to 1979, during which around one third of the Cambodian people lost their lives. On the other hand the birth rate during this time was very low while infant mortality was extremely high. Nowadays, the life expectancy at birth is 60 years for male and 64 years for female individuals, and the infant mortality is 45/1,000 live births [6].

Since the early 1990s the political and economical situation of the country has stabilized, due to the Paris Peace Accord signed in 1991 and the promulgation of the Constitution of the Kingdom of Cambodia in 1993. Still, Cambodia remains one of the poorest countries in the world, with around 28.0 % of the population living below the poverty line [5-6]. The main economic sector is agriculture, especially cultivation of rice, but economic activity is rising in new sectors, such as garment production and tourism. However, in the year 2010, the gross domestic product (GDP) per capita was approximately US$ 800 [5-6].

1.1.2 Health situation and health system

Despite the progress made over the last years, the health status of Cambodia's population is still among the lowest in Southeast Asia [5, 7]. In 2005, the World Bank performed a detailed analysis of health status and health care utilization related to poverty level and geographical factors in Cambodia. The report showed that, according to the village leaders, the major problems with health services on village level are insufficiency of drugs, long distances to quality health care as well as the relative high costs of healthcare [7].

In 2006 the MoH subdivided the 24 Cambodian provinces into 77 "Operational Districts" (OD). Appendix 9.2 shows a flowchart of Cambodia's health system structure. Each OD comprises 10 to 20 health centers and at least 1 referral hospital and thus should be able to provide equal quality health services to all its inhabitants. In practice, however, only 16.6 % of the population with health issues are consulting a provider in the public sector, whereas the vast majority (80.6 %) makes use of services in the private sector (private doctors, traditional healers) or rely on self treatment [7-8]. The reasons for the underutilization of the public health system are various. To begin with, there is a serious shortage of qualified health professionals due to the years of war and genocide, in which educated people, especially with an urban background, were either killed or fled the country. This severe loss of human resources and the resulting truncated education of health staff had a disproportionate and regressive impact on the health sector. Nowadays, the new generation of health professionals prefers to stay in more developed urban areas and most of them work in the better paid private sector, which adds to the public-staff shortage in the rural and remote areas of Cambodia. Inadequate financing is also a major strain on the

public health sector. Since governmental salaries for health centre staff is insufficient, many staff subsidize their living by side-practicing in the private sector. This again leads to irregular opening hours and possibly further loss of quality service provision in the public health care sector [6, 8].

Like in many other developing countries, major health threats in Cambodia emanate from unsafe water, unsafe food supplies and vector-transmitted diseases. Only 56.0 % of the rural Cambodian population have access to an improved water source [6]. In addition the modernization of the country leads to an increasing number of road and mining accidents, indoor and outdoor pollution and an increasing use of solid fuel and chemicals, all of which pose a further threat on public health. Environmental hazards like floods and storms present another serious danger [6]. Furthermore the incidence of non-communicable diseases continues to increase, and the level of diabetes, hypertension and cardiovascular disease is rising steadily, all of which puts a further strain on the health system [9]. Moreover, Cambodia is classified as one of the 22 countries worldwide with a high burden of tuberculosis (639 cases/100,000 population/year), and the prevalence of human immunodeficiency virus (HIV) in adults is estimated to be around 0.7 % [6]. According to WHO-data from 2010 the leading infectious cause of both morbidity and mortality in Cambodia are acute respiratory infections (ARI), malaria and gastro-enteric infections. Outbreaks of dengue fever also contribute substantially to the leading causes of morbidity (see appendix 9.3) [6]. The HIS-report (health information system, MoH) from 2007 however, lists diarrhea, dysentery and cholera as the top 3 health problems among inpatients in national and referral hospitals, closely followed by ARI, malaria and dengue fever (see appendix 9.4) [9]. As shown in table 1, on the health center (outpatients) level the most commonly diagnosed diseases were ARI followed by diarrhea and dysentery. In 42.0 % (“others” in table 1) of the health center cases there was either no diagnosis established or it was not possible to report the diagnosis on the designated form [9]. Unfortunately only the presumptive diagnosis as well as age and gender of the patients are reported to the HIS, whereas information on symptoms that the patients present themselves with in the health center is not retrievable, which makes it difficult to estimate the actual incidence of acute febrile illness. In addition, a large proportion of febrile illness patients are consulting in the non-reported private health sector. Furthermore, febrile

illnesses pose a financial burden to households in rural Cambodia. A study on the cost of dengue fever and febrile Illness in Cambodian children conducted in 2006 found that to finance the febrile illnesses, 67.0 % of the included households incurred an average debt of US$ 23.5 (range: US$ 0.5-50.0). This was more than double the average amount households spent on food in 2 weeks, which was average US$ 9.5 per week prior to interview. Hospitalization significantly increased incurred debt from US$ 4.5 for an out-patient, to US$ 23.1 ($p < 0.01$), which is why children from poor families often did not get hospitalized [10].

Table 1: Health problems and number of cases registered at all Cambodian health centers in 2007 [9]

Health problem	Number of cases	Health problem	Number of cases
Upper ARI	1,521,265	Dengue fever	9,061
Lower ARI	835,085	Malnutrition	9,022
Simple diarrhea	285,736	Genital ulcer	3,332
Dysentery	269,436	Goiter problem	1,394
Skin infection	200,781	Genital warts	853
Vaginal discharge	153,441	Substance abuse	669
Eyes diseases	79,549	Mine accidents	469
High blood pressure	72,877	Other tetanus	152
Malaria	46,187	Pertussis	60
Severe diarrhea	30,759	Diphtheria	31
Other mental health	28,564	Measles	29
Traffic accident	25,711	Neonatal tetanus	21
Urethral discharge	18,164	Acute flaccid paralysis	4
Cough more than 21 d	11,966	Others	2,670,558
		Total	6,382,870

1.2 Malaria and fever management in Cambodia

1.2.1 Malaria: a short introduction

Malaria is a life-threatening disease caused by protozoan parasites of the genus *Plasmodium*, which are transmitted to humans through the bites of infected, female Anopheles mosquitoes. In 2008 there were 247 million estimated cases of malaria and nearly 1 million deaths worldwide [2]. The disease is endemic in tropical and many subtropical regions of the world. Countries of sub-Saharan Africa account for the majority

of all malaria cases, with the remainder mostly clustered in India, Brazil, Afghanistan, Sri Lanka, Thailand, Myanmar, Indonesia, Vietnam, Cambodia, and China. Of over 100 different *Plasmodium* species (spp.), only 5 can infect humans, namely *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* [11]. Each of the 5 species has a distinct morphology, causes a distinct immune response in the host and differs in its life-cycle and geographical distribution. Whereas *P. vivax* is the most frequent species worldwide, *P. falciparum* is the most lethal. *P. malariae* and *P. ovale*, which is found mostly in West-Africa, are less frequent and generally cause a milder form of the disease [2]. *P. knowlesi*, which is originally responsible for the simian malaria is increasingly reported to infect humans, especially in Asia [12].

The complex life cycle of the *Plasmodium* parasites, illustrated in figure 3, starts with the *Anopheles* mosquito biting an infected host and ingesting gametocytes of the parasite into its gut. These gametocytes develop into oocysts, which burst after 1 to 2 weeks of incubation, and release sporozoites into the mosquito's hemo-lymph, through which they gain access to its salivary glands. If this mosquito then feeds on a human, the sporozoites are transmitted to the human's bloodstream. Via the bloodstream they migrate into the human liver, where they grow into merozoites, using hepatocytes as their host cells. This transformation takes about 1 week and corresponds to the clinical incubation period. The grown merozoites burst the liver-cells and are released into the blood stream where they infect the red blood cells (RBCs). In the RBCs they replicate until the cells burst and more merozoites are released into the bloodstream, where they infect more and more RBCs. Each new release of merozoites into the bloodstream is leading to fever-paroxysms with intense chills and sweating in the infected human. When the merozoites mature into gametocytes outside of the RBCs, they can be taken up by another mosquito and restart the whole cycle again [11, 13]. There are some differences in the life cycle of the different *Plasmodium* species, for example the frequency of fever-paroxysms varies depending on the speed of RBC-bursting and replication of the merozoites which is every 2 days for *P. vivax* and *P. ovale*, and every 3 days for *P. malariae*. *P. falciparum* is the only species which can infect erythrocytes in all stages of their development and thus causes the highest frequency of paroxysms and the most severe anemia in patients. *P. vivax*, *P. malariae*, and *P. ovale* can all cause relapses, due to their feature of dormant merozoites

in the liver, like this *P. malariae* can persist in the host for decades before manifesting any symptoms. The classical symptoms of uncomplicated malaria are fever, chills and sweating. Attendant symptoms can be headache, malaise, fatigue, muscular pains, occasional nausea, vomiting or diarrhea. Severe or complicated malaria, mostly caused by *P. falciparum*, is usually complex and key processes such as jaundice, kidney failure and severe anemia can cause serious and even fatal course of disease [11]. The diagnosis of malaria is usually established with the combination of clinical features and the microscopic evaluation of an eosin-methylene-blue-stained blood film (May-Grünwald-Giemsa stain). However, the accuracy of this technique depends largely on the quality of supplies and reagents, the presence and maintenance of satisfactory microscopes, and the technical competence of the microscopist [14].

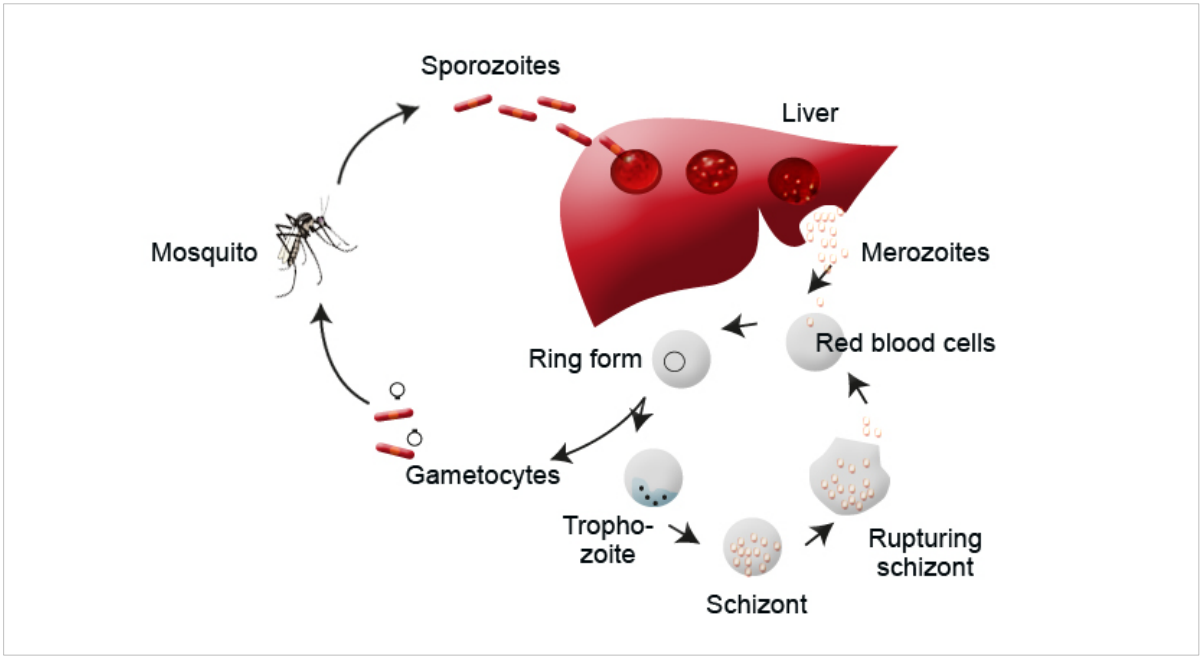


Figure 3: Schematic life cycle of *Plasmodium* parasites in the human body (Tara Müller, 2010).

New techniques like rapid diagnostic tests (RDTs) are based on specific malarial antigens, such as histidine-rich protein 2 (HRP2) and *Plasmodium* lactate dehydrogenase (pLDH), that react with antibodies on a plate or pad and show a visible band if positive. Both RDTs and microscopy can be used to determine the specific *Plasmodium* species the patient harbors [14]. More advanced techniques with high levels of sensitivity and specificity are

enzyme-linked immunosorbent essay (ELISA), polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) assays [11].

Currently, the recommended first-line treatment for malaria is artemisinin-combination therapy (ACT). ACT consists of artemisinin combined with quinolones or antifolates. The combination of quinolones, such as quinine, chloroquine, mefloquine, and amodiaquine, and antifolates, such as sulfadoxine and proguanil, is recommended to ensure the complete elimination of residual parasites and to prevent that drug-resistance can be contained [2, 11]. The spread of drug resistance is a major problem in malaria control, especially as there are no clinically approved malaria vaccines available to date, even though some already are in development and testing [13].

1.2.2 Current malaria situation in Cambodia

Despite all efforts and improvements, malaria remains a major health concern and a big challenge for the public health system of Cambodia. According to the national report system in the year 2010, the total number of treated malaria cases in the public sector was 56,217 [3]. Overall the number of treated malaria cases is decreasing over the past 10 years, as shown in figure 4 [3, 15]. In the year 2009, a sudden increase of case numbers occurred in some provinces. The reasons for this increase are suggested to be due to heavy rain and unusual climate, as well as migration of the population from non-endemic to endemic malaria areas (especially in the border provinces with Thailand and Vietnam) [16].

According to the official data the majority of malaria cases in Cambodia are caused by the species *P. falciparum*. As shown in figure 5, *P. falciparum* was responsible for 59.0 % of 13,345 microscopy confirmed cases in Cambodia in the year 2010, whereas *P. vivax* was found in 33.0 % and mixed infections with both species accounted for the remaining 8.0 %. Infections with the other 3 species, *P. ovale*, *P. malariae* and *P. knowlesi* have not reported by the CNM to date [3].

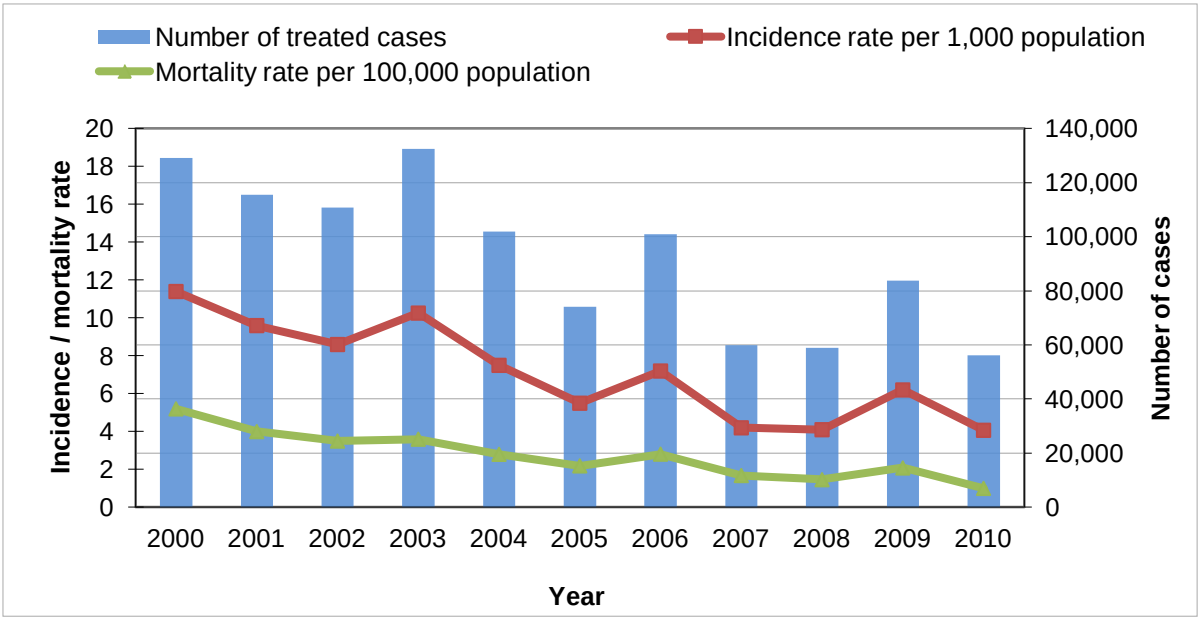


Figure 4*:* Number of treated malaria cases (clinically suspected cases) as well as incidence rate and mortality of malaria in Cambodia from 2000 to 2010 (CNM annual report 2010).

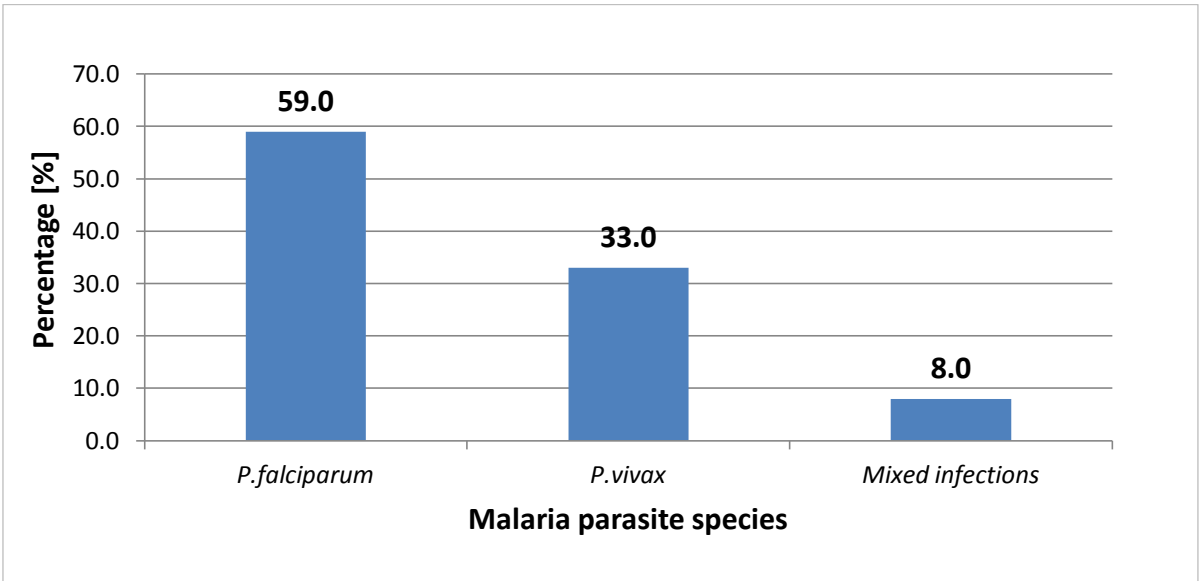


Figure 5: Malaria parasite species distribution of 13,345 malaria-cases confirmed by microscopy, in Cambodia 2010 (CNM annual report 2010).

However, a large scale malaria survey conducted by Incardona *et al.* in 2005 showed that the malaria situation is more complex than it appears regarding the national data [17]. In this study of 11,652 individuals, one major observation was that a large asymptomatic reservoir was present with only 23.0-33.0 % of the enrolled parasite carriers being febrile. It was also noticed that in some areas there was a larger than expected proportion of

P. vivax infections (up to 50.0 %), especially in areas of low transmission. *P. malariae* infections are not reported by the CNM, whereas in the malaria survey by Incardona *et al.* 8 cases of *P. malariae* were detected (4 of them mixed infection with *P. falciparum*) [17]. Furthermore, the malaria incidence varies in different areas of the country, due to the nature of mosquito-borne transmission of the disease. Border regions in the north-west and north–east are considered hyper-endemic, whereas in the central plain fields there is little or no transmission. Moreover, the level of transmission is seasonal, and generally there are more cases reported during the wet season (April-October), though in the highly endemic areas this variety is of little relevance [3, 17-18]. In the more elevated and forested areas transmission is perennial, given that the 2 major mosquito vectors are *Anopheles dirus*, which predominates in the rainy season, and *Anopheles minimus*, which predominates in the dry season [3, 19]. *A. dirus* is a vector which is very difficult to control, due to its exophilic[i] behavior and its ability to adapt to environmental changes. In Cambodia, natural forests are being increasingly replaced with coffee, tea and rubber plantations. However, thick forests still cover around 37.0 % of the countries landmass, providing sufficient breeding places for the mosquitoes [19].

It is estimated that around 1.6 million people in Cambodia are exposed to a high risk of malaria infection [3, 18]. Analysis of the risk factors showed that in particular the male population between the ages of 15-49 years is affected, contributing to about 51.0 % of confirmed malaria cases in 2009. Females of the same age group only accounted for 18.0 %, children from 5-14 years for 17.0 %, children from 0-4 years for 7.0 %, and adults above the age of 50 years for 7.0 %. Other risk factors include the “distance-to-forest”, “distance-to-healthcare” and the use of insecticide treated bed nets (ITN) [16-17]. The malaria situation in Cambodia became of global interest since the area close to the Thai-Cambodian border is considered an epicenter of multi-drug resistant (MDR) *Plasmodium falciparum* [3, 15, 20]. Reports on drug-resistant malaria in this area have emerged as early as the late 1950’s. The situation has progressively worsened and today the region is considered one of the worlds established MDR-malaria areas [15, 21]. In recent years the Cambodian Government, and several international organizations which are active in the country, have focused their efforts on improving Cambodia’s strategy in the fight against

[i] Exophilic/endophilic: the tendency of mosquitoes to rest outdoors or indoors in between blood meals.

malaria. The National Malaria Control Program (NMCP), is an exemplary, evolving initiative of the Government of Cambodia, supported by the GFATM, the United States Agency for International Development (USAID), the WHO, the World Bank, and several other donors [16]. The NMCP's key functions are strengthening clinical management of malaria cases, improving access to treatment and good quality drugs, providing surveillance and health education, promoting preventive behavior, and finally the containment of artemisinin- and mefloquine-resistance. Other important achievements include the creation of "The Society for Malaria Control in Cambodia" (SMCC) in 2003, as well as the introduction of Village Malaria Workers (VMW) in 2001, to reach out to people even in the most remote areas [16, 18]. These combined efforts resulted in Cambodia being cited as "a shining example for malaria control" in the year 2005 [8]. Nevertheless, current estimates of the malaria burden in Cambodia rely on the data collected by the public HIS, which includes only symptomatic patients that consult in public health sector facilities [15, 17-18]. As mentioned earlier, only a small proportion of people, especially in remote areas, are seeking consultation in the public facilities. Cases treated in the unregulated private sector or at home, as well as asymptomatic carriers are not detected. Therefore, actual malaria figures are a lot higher than reported and malaria prevalence is still high in Cambodia [3, 8, 15, 17-18].

1.2.3 Malaria diagnosis and treatment at village level in Cambodia

According to the "National Treatment Guidelines for malaria in the Kingdom of Cambodia" established by the CNM in 2004 [22] the diagnosis of malaria should be obtained through taking the patients history, performing a clinical examination and an additional diagnostic test for confirmation. A patient is suspected of having malaria if showing clinical signs, like fever, chills and sweating, especially if they are combined with the following risk factors:

- The patient is living or working in or close to the forest.
- The patient recently moved to an area of high transmission.
- The patient is living in an area of high transmission and is not using a bed net.
- The patient is pregnant.
- The patient is a child.

In either of these cases, the patient should be tested for malaria by microscopy or RDT. RDTs were introduced nationwide in Cambodia in the year 2001 and ever since have played a very important role in diagnostics at village level (VMW, health centers), since they are very easy to perform, to transport and to store, and there is no additional equipment needed. RDTs used in Cambodia are manly HRP2[ii] based, which can detect *P. falciparum* only, and are widely available at pharmacies, drug stores, private practitioners, hospitals and health centers. These tests show sensitivity and specificity both over 90.0 % and currently cost less than US$ 1 per piece [18]. RDTs based on pLDH, which can additionally detect non-*falciparum Plasmodium* spp., are utilized mainly in the private sector [personal communication with CNM- and health centre staff]. The disadvantage of these tests is their lack of stability in hot and humid conditions, and the decrease of their sensitivity for concentrations of less than 200 parasites per µl blood, which is why additional quality assurance laboratories are needed to ensure the performance of RDTs [14]. However, currently microscopy remains the gold standard for malaria diagnosis in Cambodia, and tools are provided in most of the health centers. The CNM regularly trains health center staff and VMWs in laboratory and clinical diagnosis and cross-checks slides from randomly chosen health centers in its own laboratory for quality assurance. All confirmed malaria cases that occur in public health centers are reported to the HIS/MoH and the CNM [16]. The algorithm shown in figure 6 is used as guideline for correct treatment of malaria in health centers since 2004. In 2011 a reviewed recommendations for malaria treatment will be released, which will no longer contain a recommendation for treatment with chloroquine [3]. If a patient's test is positive for malaria, treatment depends on the severity of the clinical conditions. Uncomplicated malaria is defined by the symptoms fever, chills and sweating. Other common clinical signs include headache, back or muscle pain, joint pain, pallor, jaundice, abdominal pain, nausea, loss of appetite, diarrhea, abdominal swelling and enlarged spleen or liver. In this case, the recommended treatment is a combination of artesunate and mefloquine as first-line treatment, or alternatively quinine and tetracycline as second-line treatment [22]. Severe or complicated malaria is defined by the classic malaria symptoms, as mentioned above, plus 1 or more of the complications listed in table 2.

[ii] HRP-2 is a water soluble protein that is released from parasited erythrocytes of infected individuals and is specific for *P. falciparum*. The test is an immunoassay; anti-HRP-2 monoclonal antibodies on the test's membrane build a coloured complex with the *Plasmodium*-HRP-2.

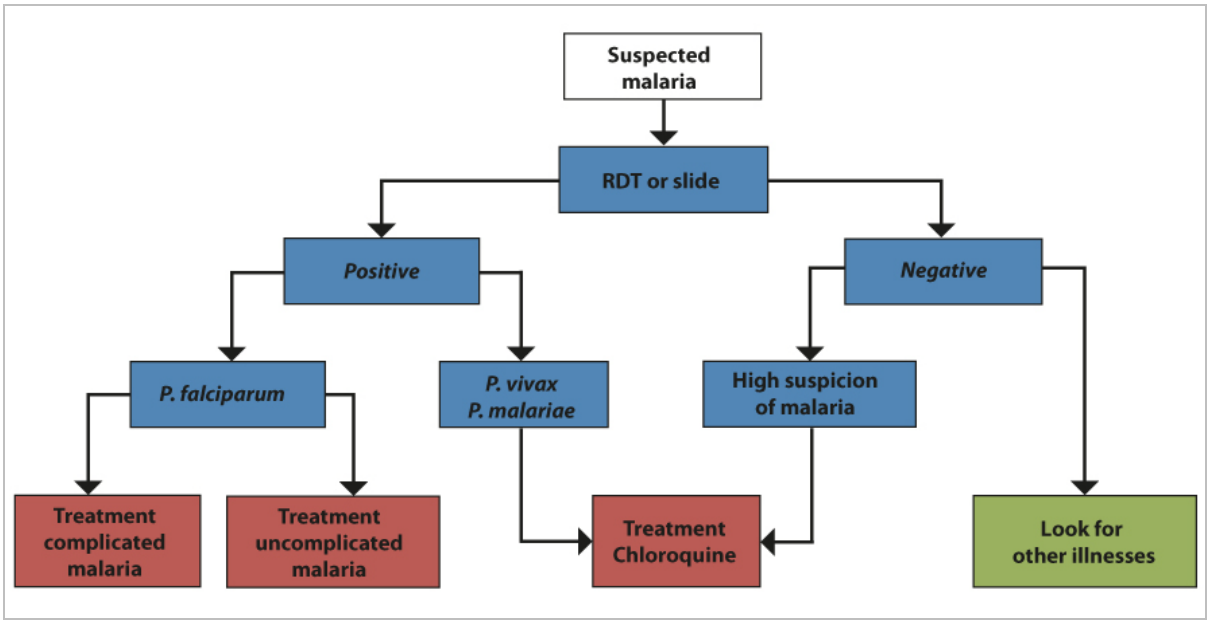


Figure 6: Malaria diagnosis and treatment algorithm for health center level, Cambodia 2004 to 2011 (Ministry of Health, Treatment guidelines for Malaria, 2004).

Table 2: Defining symptoms of severe malaria according to the national malaria treatment guidelines [22]

- Prostration
- Impaired consciousness
- Respiratory distress
- Hyperlactatemia or metabolic acidosis
- Renal failure or scanty urine, oliguria
- Frequent vomiting
- Hypoglycemia (< 0,4 g/l)
- Circulatory collapse
- Pulmonary edema (radiological features)
- Abnormal bleeding
- Severe anemia or very pale color
- Macroscopic hemoglobinuria
- Jaundice
- Multiple convulsions

The recommended treatment for severe malaria is immediate intramuscularly injection of artemether, which should be continued once daily for up to 5 days. The therapy should then be continued by mefloquine tablets. If this therapy is not available, the patient should be immediately forwarded to the next referral hospital. If the test is negative but clinical conditions are still highly suspicious of malaria, as well as in case of *P. vivax* or *P. malariae* infection, chloroquine is the recommended medication. Furthermore, it is recommended to “look for other illness” if RDT and slide results are malaria-negative [22].

The overall surveillance and control of malaria treatment in Cambodia remains an enormous challenge, as it is unknown how faithfully diagnosis and treatment algorithms are adhered to in the unregulated private sector, even though up to 75.0 % of patients are

believed to seek malaria diagnosis and treatment in the latter [18]. Anti-malarial drugs and rapid diagnostic tests of unproven quality are distributed in pharmacies without any prescription needed. However, the CNM annual report of 2010 notes that the percentage of patients, who receive anti-malarial treatment without diagnostic confirmation at public facilities, has reduced from almost 50.0 % in the year 2000, to under 20.0 % in the year 2010, as shown in figure 7 [3]. Moreover, figure 7 shows that in 2010 of 178,364 people with suspected malaria (tested cases), only 25.8 % were confirmed by microscopy or RDT [3], suggesting that the symptoms (fever) occurred due to another infection than malaria, and patients are frequently being subjected to inappropriate treatment regimes. Notice of this problem gave the first motivation to investigate non-malarial causes of fever within this observational study.

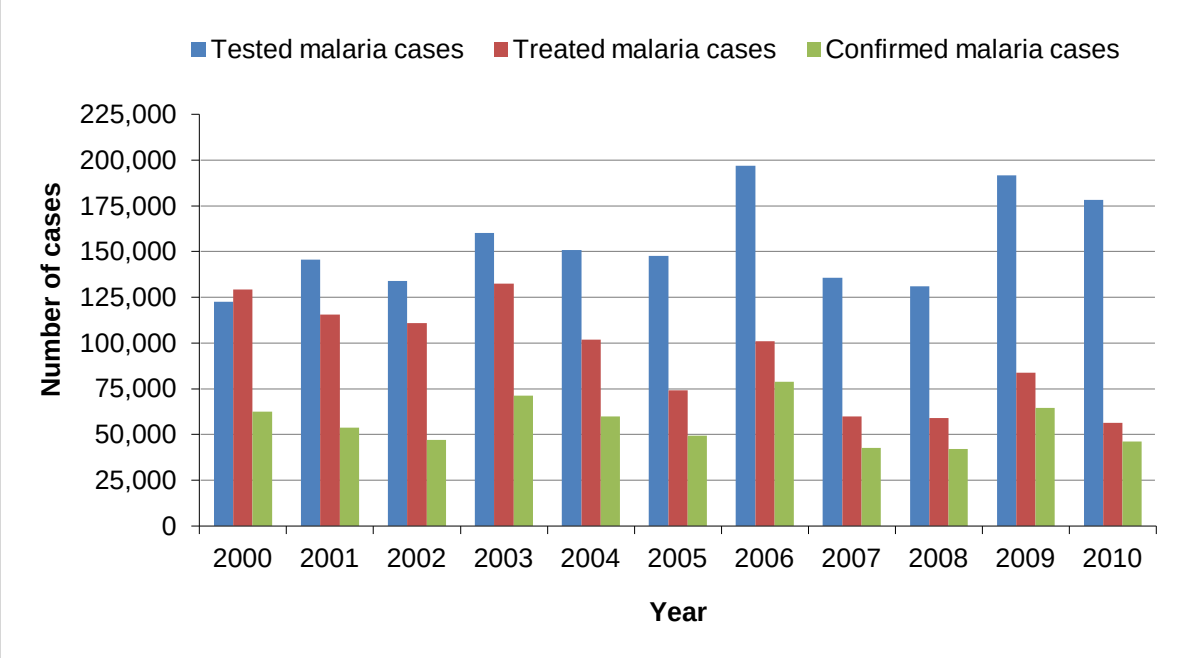


Figure 7: Number of tested, treated and confirmed malaria cases in Cambodia 2000 to 2010 (CNM annual report 2010).

1.2.4 Existing diagnostic tools for non-malaria febrile illness in Cambodia

Diagnostic facilities for infectious diseases at peripheral public health posts in Cambodia remain very limited. In many health centers, malaria is the only disease that can be diagnosed by laboratory, using microscopy or RDTs. In the national treatment guidelines

for malaria it is mentioned that symptoms of malaria can be very similar to those of other diseases and that it is possible to have malaria and another infection at the same time. Diseases that are taken into account for differential diagnosis of uncomplicated malaria are viral infections like influenza, measles, and dengue fever, and bacterial infections of ears, throat or chest. Meningitis and encephalitis, typhoid fever, septicemia, pneumonia, hemorrhagic dengue fever in children and eclampsia in pregnant women are listed as differential diagnoses for complicated malaria [3, 22]. Nevertheless, there are currently no consistent guidelines on how to diagnose and treat those diseases in peripheral Cambodian health centers.

Currently, serological antibody tests and antigen-based RDTs for febrile illnesses like dengue fever, typhoid fever, leptospirosis or melioidosis are only available in specialized laboratories in Cambodia. Even though rapid tests for these illnesses do exist, their large-scale production and distribution in developing countries are not profitable for international firms, even thought they would be urgently needed [23]. Furthermore, there are limitations these tests regarding their sensitivity, specificity and feasibility in peripheral health posts. The diagnosis of typhoid fever and melioidosis can also be established by blood culture bottles, but since they require immediate laboratory processing and multiple blood withdrawal to improve the level of sensitivity [24], they are not suitable for peripheral routine settings. Molecular diagnostic tools like PCR are currently only available in specialized laboratories in the capital city Phnom Penh. Subsequently, acute febrile illnesses in peripheral health services are generally diagnosed and treated based on the presumptive clinical diagnosis. In general, malaria-negative fever cases are treated indiscriminately with antibiotics, most commonly cotrimoxazol, if clinical signs include intestinal disorders or amoxicillin, if clinical signs point towards a respiratory infection [22]. In the private sector and most pharmacies, little plastic bags with “drug cocktails” (containing anti-malarials, antibiotics and painkillers) are sold to treat undifferentiated fever [18].

1.3 Suspected etiologies of non-malaria febrile illness in Cambodia

Little is known about the etiologies of acute undifferentiated fever in Cambodia. Based on the results from several studies on the etiologies of febrile illness in the neighboring countries (Thailand, Lao PDR and Vietnam), the most frequently diagnosed diseases have been further investigated in this study. A detailed overview of these studies and their findings is presented in appendix 9.8. Even though most of these studies have been performed on specific populations and regions and their findings vary, it can be observed that leptospirosis, rickettsiosis, scrub typhus, dengue fever, influenza and typhoid fever are the most common non-malarial febrile illnesses in the Southeast Asian region [25-32]. In the following chapter details about these diseases and their incidence in Cambodia will be outlined. HIV-infections, viral hepatitis and tuberculosis can also cause acute undifferentiated fever but were not evaluated in this study due to ethical reasons.

1.3.1 Leptospirosis

Leptospirosis is a zoonotic disease worldwide distribution with an annual incidence around 10-100/100,000 in the tropics, caused by pathogenic spirochetes of the genus *Leptospira* [33]. Currently, this genus compromises 20 species [34], which are listed in figure 8, and can been further grouped into “pathogenic”, “intermediate/opportunistic” and “non-pathogenic/saprophytic” *Leptospira* spp., based on their phylogenetic relatedness. Simultaneously, a serologically based taxonomy is used grouping strains to serovars, based on their differences in the reaction to hyper-immune rabbit sera. To date, 300 different serovars have been described. For epidemiological understanding serovars are sometimes merged into serogroups [33-34].

Leptospirosis is transmitted to humans by direct contact with infected animal body fluids, or indirect contact with contaminated water, vegetation or soil. The natural reservoir is maintained by chronic renal infection of feral and domestic animals, which excrete the organism with their urine. Rats and rodents are currently recognized as the most important reservoir. Therefore, the disease especially poses a threat to the health of the rural population living or working in endemic areas, and not having access to a safe water source

or sanitation system [33, 35]. Clinical manifestations of leptospirosis are highly variable. The bacteria cause damage to the endothelial lining of small blood vessels that can result in vasculitis, which may affect all organs. Differences in virulence factors and pathogenesis between serovars are still poorly understood. Even though some serovars tend to cause a milder form of the disease, in principle any serovar may cause severe disease in different hosts [35]. First symptoms generally occur abruptly after an incubation period of 5 to 14 days, range from 2 to 30 days, and often resemble the flu including high fevers, severe headache and generalized myalgia. Other common symptoms are conjunctival suffusion, prostration, nausea, vomiting, diarrhea, abdominal pain and skin rash [35-36]. This wide range of unspecific symptoms makes the clinical diagnosis of this disease extremely difficult especially in its early stage [33, 37].

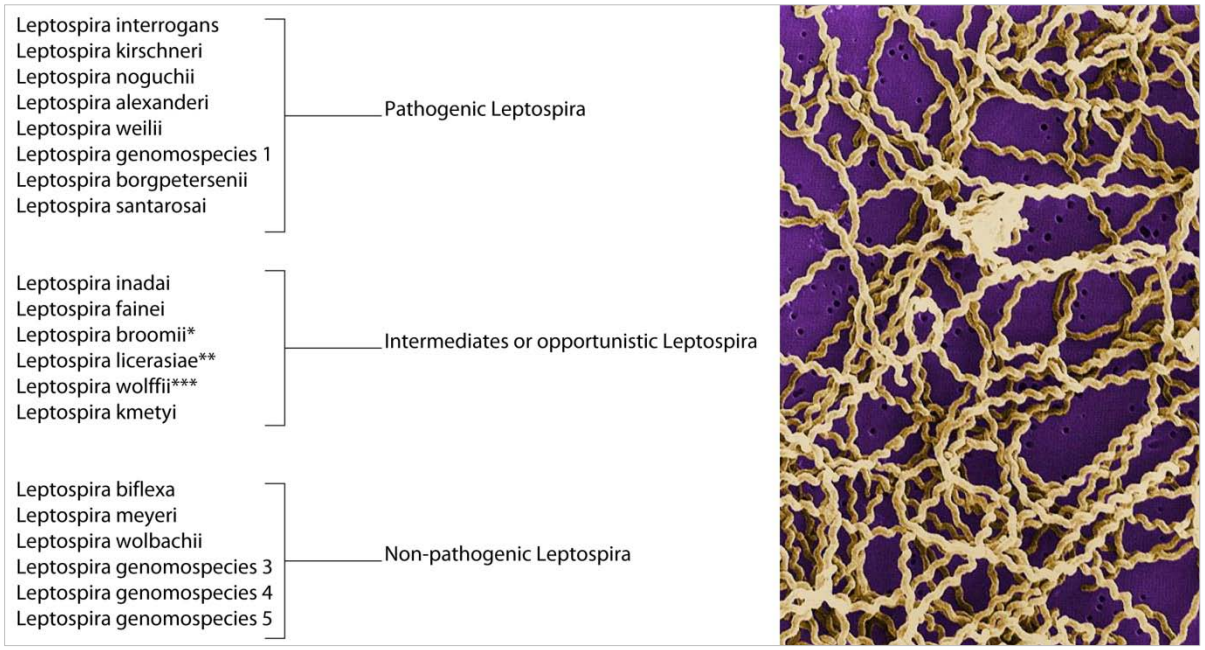


Figure 8: Left: Grouping of currently recognized Leptospira species (Tara Müller, 2009).Right: Electron micrograph scan of *Leptospira* on a 0.1 µm polycarbonate filter (Centers for Disease Control and Prevention, Public Health Image Library, 2008).

Severe complications of the disease, like Weil's disease, defined by jaundice combined with oligo- or anuria and bleeding, are believed to be secondary and thus can appear subsequently to the acute infection. They can affect different organs and appear in various combinations, which complicate the differential diagnosis even more, and can result in fatal renal failure, cardiopulmonary failure, widespread hemorrhage, and shock. Case

fatality rates of leptospirosis range from 5.0-50.0 %. Late sequels of leptospirosis include neuropsychiatric symptoms such as chronic fatigue, chronic headache, paresis, paralysis, mood swings, or depression, as well as ocular symptoms like chronic uveitis or iridocyclitis [34-37]. After the *Leptospira* have gained access to the patient's bloodstream, usually trough the lymphatic system, they disperse rapidly and can potentially invade all organs and tissues. Causing an immune response of the host, they are cleared from the blood after 7 to 10 days, but can remain settled in the convoluted tubules of the kidneys or other immunologically privileged sites like the eyes or the central nervous system, where they can cause sequels. Thus *Leptospira* may be detected in the patient's urine or cerebrospinal fluid (CSF) for up to 60 days after infection. Hence, the clinical samples for the diagnosis of leptospirosis have to be chosen according to the stage of disease [35]. There are several different approaches for the laboratory based diagnosis of leptospirosis, but a rapid and reliable standard method is still lacking. Table 3 gives an overview of the most frequently used methods and their characteristics. Figure 9 shows an image of a positive MAT under a darkfield-microscope. Currently the microscopic agglutination test (MAT) is considered as the golden standard. However, the MAT is usually only positive 10-12 days after the appearance of the first clinical symptoms and signs, therefore it is not suitable for early diagnosis, similar to all other currently evaluated serological tests [35, 37-38].

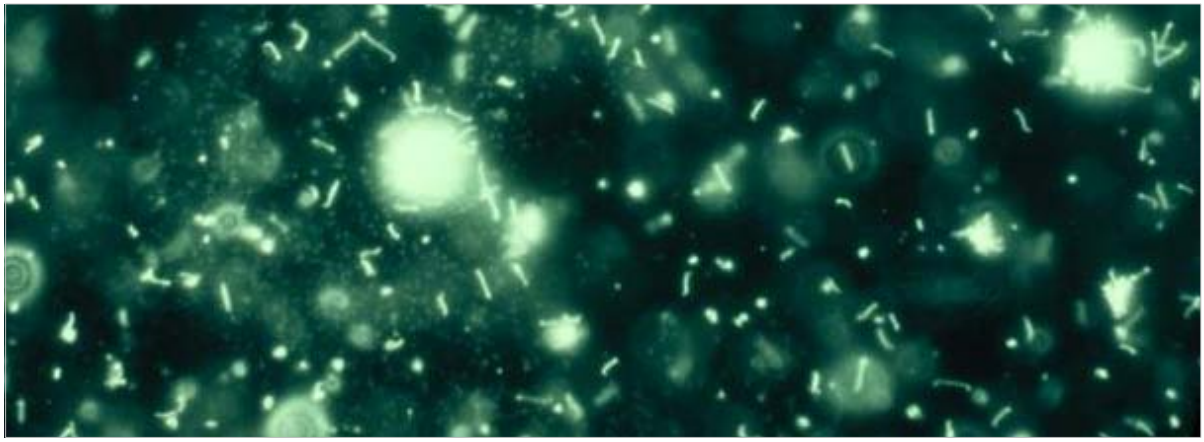


Figure 9: Photomicrograph of leptospiral MAT with live antigen using darkfield microscopy technique (Centers for Disease Control and Prevention, Public Health Image Library, 2008).

Table 3: Overview of currently available laboratory tests for Leptospira spp., their test principle as well as their advantages and disadvantages

Method	Principle	Advantage	Disadvantage
Culture	Growth of live *Leptospira* on special media (Ellinghausen & McCullough modification by Johnson & Harris)	Possible from any kind of clinical sample	Very slow growth (up to 8 weeks), requires special media, low sensitivity (14.0-50.0 %)
Darkfield-Microscopy	Oblique light is thrown on to *Leptospira* in serum, by the use of a special condenser, while central light is interrupted. *Leptospira* stand out as silvery threads against a dark background (see figure 9)	Easy access, useful for observing cultures or agglutination level in MAT. Can be used in combination with other tests like ELISA.	Technically demanding, high rates of false positives due to artifacts, thus not suitable for definite diagnosis. Sensitivity and specificity around 60.0 %.
Staining	Methods include: Silver- staining, direct immuno-fluorescence staining, immuno-peroxidase staining	Useful for observing cultures or agglutination level in MAT.	Difficult preparation, high rates of false positives, due to artifacts, thus not suitable for accurate diagnosis
PCR	Amplification of specific DNA segments	Highly specific, rapid testing in the early stage of the disease, when there are no antibodies yet.	Requires designated lab space and skilled staff. Various techniques haven't been broadly evaluated yet.
MAT	Determines antibodies by mixing it in various dilutions with live or killed, formolized *Leptospira*. Antibodies present in the serum cause *Leptospira* to stick together to form clumps, that can be observed in darkfield microscope.	Currently the golden standard because of its high specificity for different serovars.	Live *Leptospira* have to be kept in culture to provide antigens. Time-consuming and laborious, antibodies might not be detectable if the causative strain is not included in the panel.
ELISA and other commercial tests*	A broadly reactive so-called genus-specific antigen is generally used to detect IgM-, and sometimes also IgG-antibodies.	Sensitivity around 80.0 % easy to perform, useful for genus-specific screening. Can be standardized. Can detect antibodies 24-48hours earlier then MAT.	Convalescence sample needed. Also detects presence of saprophytic *Leptospira*, gives no information about the serovar. Difficult to distinguish acute infection.

**Other commercial serological test: Macroscopic slide agglutination test (SAT), Indirect fluorescent antibody test (IFAT), Latex agglutination test (LA), Dipstick tests (LEPTO Dip-S-Tick, LeptoTek Lateral Flow), and many more[35].*

Due to this lack of feasible diagnostic tools especially in peripheral settings, it is currently recommended to initiate effective chemotherapy as soon as the diagnosis is clinically suspected [35, 39]. Antibiotic treatment of the disease should be initiated as soon as possible, preferably in the first 5 days after infection. Depending on the severity, intravenous or oral application of ß-lactam antibiotics is indicated. Severe cases should be treated with intravenous penicillin, mild forms can be treated with oral amoxicillin, ampicillin, erythromycin or doxycycline, alternatively third-generation cephalosporins can be used [35-36]. The benefit of antibiotics after the fifth day of the disease is controversial. However, most clinicians treat with antibiotics regardless of the date of onset of the illness [35].

Leptospirosis is endemic in the tropical and sub-tropical regions of Southeast Asia [33]. Reports on human infections in the region are numerous and several studies conducted in recent years have increased the awareness of the disease. Nevertheless, it is believed that due to the non-specificity of symptoms and the lack of simple diagnostic tools and facilities, the disease is been widely underreported [33, 40-45]. In Cambodia the first study on leptospirosis was conducted from 1999 to 2001 in the National Pediatric Hospital, Phnom Penh, and found 12.0 % of 202 patients with a positive immunoglobulin M (IgM) titer and 2.5 % with a positive PCR-result [41]. The second study, dating from 2003, was conducted in the provincial Takeo Hospital and found a prevalence of 9.1 % of 121 patients by MAT and PCR [42]. From 2006 to 2007 the biggest surveillance study on leptospirosis in Cambodia to date was conducted and showed that almost 30.0 % of the 612 recruited patients had at least 1 biological test (ELISA, MAT or PCR) positive for leptospirosis. Thirteen different serogroups had been identified, predominately *L. panama*, *L. pyrogenes* and *L. australi*s, which indicates a big variety of reservoir hosts in Cambodia. Furthermore, the findings of this study show that there was no significant linkage of the infection to risk factors like gender, age or occupation, suggesting that the population is rather permanently exposed to the risk of contact or infection with *Leptospira* [44]. Studies in the neighboring countries found high sero-prevalence rates (18.8 % to 23.9 %) in their subject populations, too [40, 43, 45]. Especially in Thailand, the incidence of leptospirosis has dramatically increased over the last 10 years, with a peak of 14,285 cases in the year 2000 [43, 46-47]. One reason for this increase may be the rising awareness and consequently

more accurate reporting of the disease. Findings in Thailand furthermore indicate that the incidence of leptospirosis is seasonal, being highest in the wet season [36, 43, 46-47].

1.3.2 Rickettsiosis

Rickettsiosis is the general term for diseases caused by infection with intracellular bacteria of the genus *Rickettsia*, which further comprises 2 subdivisions, the spotted fever group (SFG) and the typhus group (TG). In recent years, numerous novel species of *Rickettsia* have been isolated around the world and the improvement in molecular technologies has helped to clarify the genetic relationships within the order of *Rickettsiales* [48-49]. To date, there are 25 recognized species of *Rickettsia*, of which 16 are considered as human pathogens. All currently known species are listed in appendix 9.5. Figure 10 gives an overview of the species which are endemic in Southeast Asia. Common characteristics of all *Rickettsia* are that they are obligate intracellular small rods and that they are associated with arthropods, such as ticks, fleas and lice that may act as vectors or as reservoir. Rats, rodents or other small mammals act as maintenance hosts. The majority of SFG-*Rickettsia* are tick-borne, except for *R. felis* which is associated to fleas, whereas TG-*Rickettsia* can be flea (*R. typhii*) or lice borne (*R. prowazekii*). The infection can result from direct bites of the vector as well as contamination of disrupted skin, for example with flea feces [48-49].

Clinical signs of rickettsiosis usually appear 6-10 days after the vector bite and include various unspecific symptoms like fever, headache, myalgia, night-sweats, local or generalized lymphadenopathy, conjunctival suffusion and gastrointestinal disorders like nausea, vomiting, diarrhea and abdominal pain. More specific signs that should raise attention are a macular, sometimes petechial skin rash (“spotted-fever”) and the presence of a so called “eschar”, a small epidermal necrosis with surrounding erythema, at the arthropod bite site. Figure 11 shows a picture of an eschar after a tick-bite on the hip of a patient at Calmette Hospital in Phnom Penh. However, none of these symptoms are present in every infection. SFG-rickettsiosis can manifest as a mild, severe and sometimes even fatal disease, depending on the involved species and the general condition of the host [49-50]. The bacteria target endothelial cells in humans, where they cause disruption of cell-to-cell adherence which can lead to increased micro-vascular permeability and

vasculitis. As a result, severe complications such as gangrenous extremities, bowel perforation, liver dysfunction, renal failure, meningo-encephalitis, pneumonia and disseminated intravascular coagulation (DIC), can occur [50-51]. The flea-borne *R. typhii* is causing murine typhus in humans, which is a relatively mild, often self-limiting disease with non-specific symptoms and is therefore believed to be frequently under-diagnosed [49, 52].

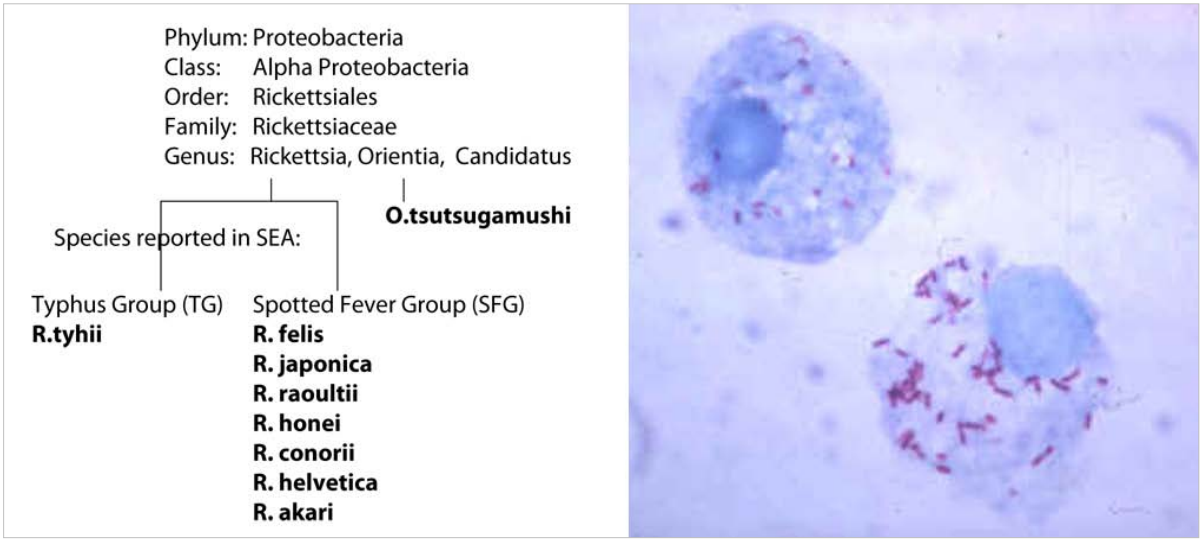


Figure 10: Left: Overview of *Rickettsia* species described in Southeast Asia (SEA) and their classification (Tara Müller, 2009). Right: Gimenez stain of tick hemolymph cells infected with *Rickettsia rickettsii* (Centers for Disease Control and Prevention, Public Health Image Library, 2008).

Since *Rickettsia* are intracellular organisms, staining after Gram is not applicable, but other methods such as staining after Giemsa or Gimenez [53] can be used for microscopic observation (see figure 10). The current gold standard in diagnostics is serological detection of rickettsial IgG- or IgM- antibodies, most frequently by specific micro-immunofluorescence assays (IFA) or ELISA [48-49]. However, a large panel of antigens can only be tested in reference centers with the facilities for further testing such as cross-absorption-studies and western-blotting for the exact determination of the species. Moreover, convalescence sera samples are needed, so these methods are inappropriate for rapid diagnostics. In remote settings the Weil-Felix agglutination test is still used, though in recent years it has been labeled unsuitable for correct diagnosis [23]. Molecular tools like PCR-based methods, which are highly sensitive and specific, are very effective but likewise

require designated facilities and skilled personnel. Culture of *Rickettsia* from blood or tissue samples is possible but very laborious and challenging, since they are obligate intracellular organisms and thus depend on living host-cell-cultures [48-50].

Treatment of choice for SFG-rickettsiosis is antibiotic therapy with doxycycline. Fluorochinolones and some macrolides are considered as alternatives. For TG-rickettsiosis such as murine typhus, the same drugs can be used whereas a single oral application is usually sufficient. Penicillin and other ß-lactam antibiotics as well as cotrimoxazol, which are frequently used in the presumptive treatment of acute febrile illnesses, are not efficient against *Rickettsia* [48-49].

No data in regard to rickettsial infections in Cambodia has been published recently. In the 1990s, 2 prevalence studies were carried out and revealed serological evidence of rickettsial antibodies in their study population. Both studies included a very specific population and therefore they are not appropriate to estimate the incidence of rickettsiosis in the whole country. The first study recruited 40 patients with undifferentiated febrile illness among displaced Cambodians at the Thai-border and found 26 (70.0 %) of them serologically positive for murine typhus [54]. The second study observed a group of 248 Indonesian peacekeeping soldiers that had been stationed in Cambodia from 1992 to 1993 and found a sero-conversion rate of 24 per 1,000 for *R*. *typhii* [55]. However, numerous studies that have been conducted in the neighboring countries outline the importance of rickettsiosis in the region (see appendix 9.8). Furthermore, reports on new SFG-rickettsial infections in humans are becoming more and more frequent from this region, especially from Thailand [56]. Hence, it is supposable that rickettsiosis plays an equally important role in Cambodia. However, given that diagnostic facilities are lacking the disease is largely unrecognized at present.

1.3.3 Scrub typhus

Scrub typhus or the *Tsutsugamushi*-fever is a common zoonosis in the Asian and Pacific region, with approximately 1 million reported cases per year [57]. The causative agent, *Orientia tsutsugamushi* belongs to the order of *Rickettsiales*. For a long time it had been

classified in the genus of *Rickettsia*, since it shows many similarities like being an obligate intracellular, gram-negative rod associated with arthropods. However, improvements in the genetic analysis of the organism have distinguished it as belonging to the reformative genus of *Orientia,* as is shown in figure 10 [48-50]. The disease is transmitted to humans by the bite of larval trombiculid mites (chiggers), primarily of the genus *Leptotrombidium*, which usually feed on rats or field rodents [57]. Risk-factors for scrub typhus include occupational activities in rural endemic areas, such as working in rice fields or other fields that can serve as a biotope for the mite, clearing of land, road building or military operations. Risk of transmission increases during the rainy season when the general number of rodents is higher, as well as the number of rodents with attached mites [50, 56].

As the bite of the chiggers is often painless and located in areas hard to examine, like skin folds, axillaries or the genital region, it usually remains undetected. Sometimes a papule forms at the bite site that later ulcerates to a black crust or even an eschar similar to the rickettsial infections, shown in figure 11. As for rickettsiosis, the eschar is the most helpful clue in the clinical diagnosis if it is present [56]. General symptoms occur after a variable incubation period from 1-2 weeks and typically include fever, headache, myalgia, generalized lymphadenopathy, and a transient macular or maculopapular skin rash dominating the trunk. If no complications occur, the disease is usually self-limiting, but gastrointestinal, hepatic and respiratory involvement is frequent, the latter eventually causing serious to fatal complications such as interstitial pneumonia, interstitial edema, hemorrhage and acute respiratory distress syndrome. Infection of the central nervous system has been reported as well, ranging from aseptic meningitis to severe meningo-encephalitis [50, 56]. This variability in the severity of the course of disease might be linked to the high genetic diversity and the high plasticity of the *O. tsutsugamushi* genome [58]. The pathological mechanisms of the infection are still poorly understood and even the target cells are not known with certainty. It is believed that the main pathologic change is caused by the destruction of endothelial cells, leading to local or generalized vasculitis. Furthermore, the infiltration of macrophages [59] and peripheral leukocytes during acute infection [60] has been demonstrated. The interesting fact that scrub typhus infection inhibits the viral replication of HIV will enhance further research that will hopefully reveal more about its patho-mechanisms [50, 59-60].

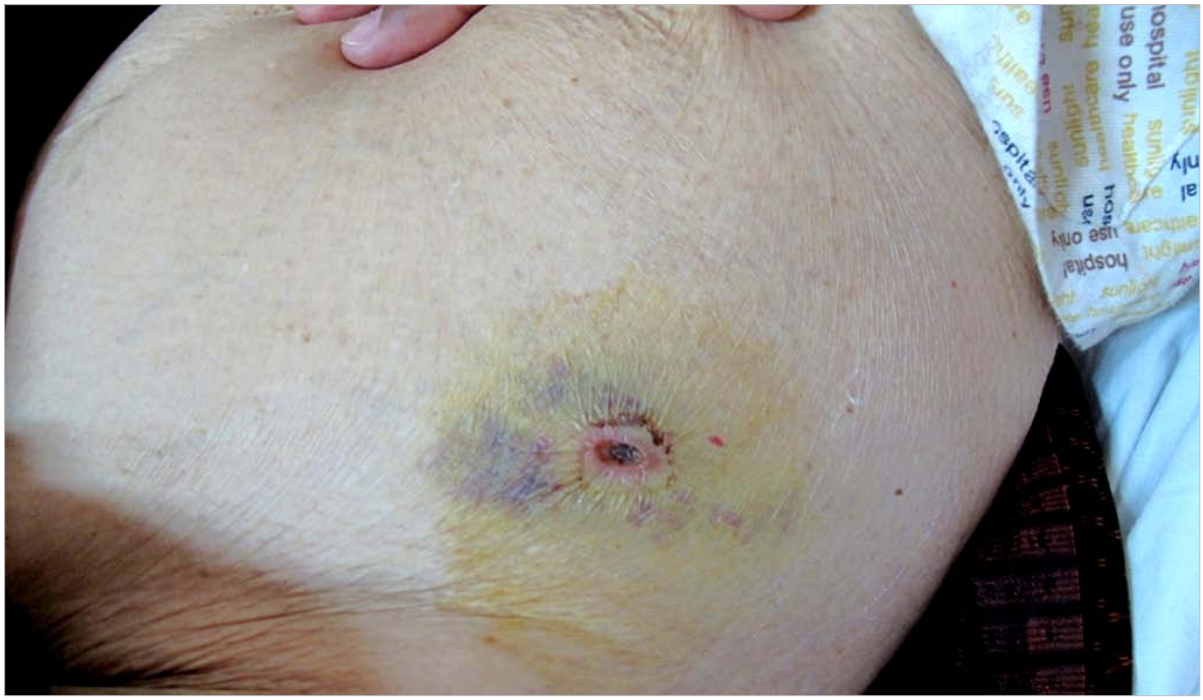


Figure 11: Large eschar on the hip of a patient with confirmed scrub typhus at Calmette Hospital, Phnom Penh (Institut Pasteur du Cambodge, 2010).

Representing yet another disease with notoriously unspecific clinical signs and symptoms and no rapid diagnostic test available, scrub typhus goes mostly undiagnosed in countries like Cambodia. Therefore, treatment has to be presumptive, with the diagnosis relying on clinical clues combined with exposure to risk factors. The confirmatory tests of choice currently are serologic methods such as IFA or indirect fluorescent antibody test (IFAT), which are only applicable in comprehensive laboratory facilities and are not appropriate for rapid diagnostics, since they require the collection of a convalescence sample. Several promising molecular methods have been published, but again these are limited to reference laboratories [61-62].

Antibiotic treatment of choice is a 1 week course of doxycycline as oral application in mild cases or parenteral application in severe cases. Azithromycine or chloramphenicol can be used as second line treatment [49-50]. In northern Thailand, scrub typhus cases that showed a poor response to these drugs have been reported [63], however, no mechanisms of drug-resistance or its geographic distribution have been described so far.

A study conducted in 2010 by Duong *et al.* showed that all of the recruited, scrub typhus positive patients originated from and were infected in southeastern provinces of Cambodia, but were all infected with different strains, implicating a high genetic diversity of *O. tsutsugamushi* in Cambodia [58]. Unpublished recent data of the Department of Virology at IPC on dengue virus negative samples, suggests an approximate incidence rate of scrub typhus around 2.0 % in Cambodia. In neighboring Thailand, several studies of the clinical features, epidemiology [25-26, 28-31, 52, 56] and drug sensitivity [63] have been conducted in the past decade. Since 2001, 3,000-5,000 cases per year have been reported to the Public Health Ministry of Thailand [56]. Studies from Lao PDR [45, 51] found similar results and underline the importance of the disease in the Indochinese area. Hence, there is need to determine its impact in Cambodia, where the awareness of scrub typhus is still extremely low at all levels of the health system.

1.3.4 Dengue fever

Dengue fever is an acute febrile viral illness of global importance, known to be endemic in over 100 countries, with 2.5 billion people living in areas of risk, worldwide [64-65]. Around 75.0 % of the current global disease burden is carried by countries of Southeast Asia and the Western Pacific region [65]. The plus strand ribonucleic acid (RNA)-virus belongs to the genus of *Flavivirus*, in the family of *Flaviviridae*, and presents in 4 different serotypes (DENV1-4). While the infection with one distinct serotype confers life-long immunity, secondary infection with a different serotype increases the risk of a complicated course of disease significantly. Different models to explain this phenomenon have been postulated but a consensus is still lacking. Therefore, shifts in the serotype prevalence regularly lead to devastating outbreaks of dengue fever around the world [64]. Being transmitted to humans by sting of the *Aedes* mosquito, dengue fever is predominant in urban and suburban areas since these are the preferred breeding sites of the vector, but can also occur in rural areas. In Southeast Asia, *A. aegypti* and *A. albopictus* (shown in figure 12) are the main vectors [64].

Figure 12: *Aedes albopictus* mosquito (Centers for Disease Control and Prevention, Public Health Image Library, 2008).

After an incubation period of 3-14 days the infection clinically manifests in variable forms of severity, or in the case of primary infection may even be asymptomatic (50.0-90.0 % of the cases) [64]. Exact pathological mechanisms remain uncertain, but it has been postulated that the viral replication happens in the cells of the macrophage-mononuclear linage. The comparatively mild and self-limiting dengue fever is characterized by a sudden onset of high fever, severe headaches, retro-orbital pain, myalgia, gastro-intestinal symptoms and sometimes a skin rash. More severe and potentially fatal manifestations are the dengue hemorrhagic fever (DHF) and the dengue shock syndrome (DSS), which particularly affects children [65-66]. DHF is characterized by the same symptoms as dengue fever, with additional hemorrhagic pneumonia, hepatomegaly and internal bleeding, all due to an increased level of vascular permeability, leakage of plasma and disorders in hemostasis. Due to the overlap in symptoms, dengue fever and DHF are often difficult to distinguish from each other. DSS hits patients that have suffered from a severe loss of plasma that leads to a hypovolemic shock and circulatory failure. Usually it occurs after the first febrile episode has ended, typical signs include a sudden fall of temperature, a rapid pulse and hypotension. If immediate volume-replacement therapy is not applied, the patient is likely to die within 24 hours [64-65].

In general, oral or parenteral volume replacement and rehydration therapy is the most important component of dengue fever treatment. Furthermore, antipyretics are indicated in case of hyperpyrexia (> 39 °C), whereas salicylates should be avoided since they may enhance bleeding and acidosis [65].

Given the broad spectrum of clinical signs, laboratory testing is needed to confirm the diagnosis of a dengue virus infection. Depending on the stage of illness there are different approaches. In the early phase of disease, up to 5 days after onset of fever, direct viral isolation by sophisticated techniques, such as mosquito- and tissue culture- inoculation, or the detection of viral RNA by reverse transcriptase (RT)-PCR [67], are the most sensitive and reliable tools, followed by the detection of the viral antigen NS-1 [65]. In the subsequent phase of disease the detection of IgM-antibodies by ELISA or the rise in the IgG-antibody-titer in paired sera samples present the methods of choice [68]. Even though serological testing remains the most conventional method, there are several drawbacks. For example, paired convalescence sera samples are needed, which are often difficult to obtain from outpatients in rural settings. Furthermore, IgM-antibodies are often absent in the acute phase of the disease as well as in secondary infections, which have a higher potential for turning into DHF or DSS. Finally, IgM-antibodies can cross-react with other viruses from the same family, such as the Japanese encephalitis virus or the yellow fever virus. Consequently, a diagnostics algorithm for dengue fever should aim at the use of direct viral detection in the early phase and antibody detection in the late phase of the infection [68].

In the past decade, dengue fever has continued to be ranked in the top 10 causes of morbidity and mortality in Cambodia, (see appendices 7.3 and 7.4) with around 100,000 cases and more than 100 deaths reported annually [66]. While the fatality rate is decreasing, the disease still poses a serious public health threat, especially for children [9, 66]. The number of hospitalized dengue fever cases increases sharply during the rainy season, due to the better breeding conditions for the mosquitoes. In 2007 Cambodia has faced the last heavy outbreak of dengue fever with 404,165 estimated cases, 407 of which ended fatal [66, 69]. The “National Dengue Control Program” developed by the CNM in cooperation with several international organizations, has implemented a broad spectrum

of activities that will hopefully help to improve the disease control and prevent another outbreak in the future [16]. In Cambodia, the diagnosis of dengue fever is usually established clinically. Surveillance studies showed that the majority of the clinical diagnoses were positively confirmed by serology [69]. However, dengue fever cases are largely reported from urban areas and are likely to be underreported in rural areas. Furthermore, the national dengue fever case-definition only allows reporting of children less than 16 years of age. To estimate the burden of dengue fever in rural areas, IPC has conducted an active surveillance study in Cambodia's largest province, Kampong Cham, from 2006-2008. The findings confirm that dengue fever incidence was underestimated in rural areas, and the virus distribution is highly focal with incidence rates ranging from 1.5/1,000 person-seasons (rural) to 211.5/1,000 person-seasons (urban) [70]. A study on dengue as a cause of undifferentiated febrile illness in Vietnam showed that one third of the recruited study subjects in peripheral health posts had acute dengue fever [71]. Therefore, there is need to determine the importance of dengue fever among malaria-negative febrile patients in Cambodian health centers.

1.3.5 Influenza virus

Influenza, commonly referred to as the flu, is an infectious disease caused by RNA viruses of the family *Orthomyxoviridae*, which can affect birds and mammals. Influenza-infection is airborne and is usually transmitted between humans by coughs or sneezes, creating aerosols that contain the virus. It can also be transmitted by direct contact with bird- or other infected animal-droppings or nasal secretions, or even through contact with contaminated surfaces. There are 3 subtypes of the influenza virus, type A-C. The influenza A virus is the most virulent subtype and can cause the most severe disease in humans [72]. Based on the 2 major surface glycoproteins, the influenza A virus can be further subdivided into distinct serotypes based on the hemagglutinin (H)- and neuraminidase (N)-antigens which cause a specific immune-response in the host. Influenza pandemics, which spread around the world seasonally, are the result of the occasional antigenic shifting within the influenza A viruses [72-73]. Influenza B is less common than influenza A and almost exclusively infects humans. It is less genetically diverse than influenza A, with only one influenza B serotype, and usually causes a milder form of the disease. Even less common is

the influenza C virus, which infects humans, dogs and pigs, and sometimes causes both severe illness and local epidemics. The most common symptoms of the disease are chills, fever, sore throat, myalgia, severe headache, dry coughing, weakness, fatigue and general discomfort. In more serious cases, influenza viruses can cause pneumonia, which can be fatal, particularly for the young and the elderly. It has been reported that between 3 and 5 million cases of severe influenza disease occur each year, of which up to 1 million end fatal [73]. In routine medical practice the diagnosis is often established clinically. For the specific identification and sub-typing of the virus in cases of severe illness, pandemics or for research purposes, RT-PCR assays can be performed [73]. The treatment is mostly symptomatic, but anti-viral treatment with neuraminidase-inhibitors is available for severe cases or in the case of pandemics. Furthermore, seasonal vaccines are available for prevention measures [72].

In Cambodia, a hospital based surveillance system for influenza like illness (ILI) has been established in 5 sites with a weekly reporting and sampling scheme since 2006 [74]. In addition, surveillance of ARI cases has been established in 2 sentinel sites. In each of those sites naso-pharyngeal swabs are collected weekly to provide epidemiological data. Samples are tested at IPC in Phnom Penh, which is the national influenza reference centre in Cambodia [75]. Analysis of this data showed that influenza A viruses (mainly strains H1N1 and H3N2) circulate from June to December, with a clear seasonal peak in October during the rainy season. Furthermore, Cambodia is endemic for the avian influenza (H5N1) in poultry and has reported 8 fatal cases in humans to date [74-75].

1.3.6 Other possible causes of non-malaria febrile illness in Cambodia

There are numerous other possible causes of febrile illness in Cambodia, most of which are difficult to distinguish from malaria clinically. For example, enteric fever caused by the *Salmonella enterica* serotype *typhi* (*S. typhi*) and serotype *paratyphi* A, B and C (*S. paratyphi*) is endemic in Cambodia. Enteric fever (typhoid fever) affects humans worldwide, the transmission is usually oral-fecal, and therefore populations with unsafe water supply and limited sanitary facilities are especially at risk [76-77]. In the year 2000, it was estimated that typhoid fever caused 21.7 million illnesses and 217,000 deaths

worldwide, most of which occur in Southeast Asia [76]. Symptoms usually develop 1 to 3 weeks after exposure, and can be mild or severe. The classical symptoms include high fever, dry cough, malaise, headache, constipation or diarrhea, rose-colored spots on the chest, and enlarged spleen and liver [77]. The presence of clinical symptoms characteristic of typhoid fever or the detection of a specific antibody response is suggestive of typhoid fever but not definitive. If it is available, bone marrow aspirate culture is the gold standard for the diagnosis of typhoid fever. Since it requires a high level of expertise and equipment which are often not present in peripheral settings, blood culture is the mainstay of the diagnosis, even though it is less sensitive [77-80]. Serological methods like the Widal-Felix-test are generally not recommended due to their lack of sensitivity and specificity, but are still frequently used in remote settings where blood culture is not available. Some newer serological techniques include rapid diagnostic test strips which are very easy to handle and show promising results, but there are no official recommendations on their use yet [78-79, 81]. The current standard treatment of typhoid fever consists of fluoroquinolone-antibiotics like ciprofloxacin; former recommendations included chloramphenicol, ampicillin, amoxicillin and trimethoprim-sulfamethoxazole [77, 82]. In Cambodia, data about prevalence and incidence of typhoid and paratyphoid fever remains scarce. The latest study on typhoid fever in hospitalized children found 3.7 % (5/134) of positive blood cultures with *S. typhi* [83]. Another study, conducted from 2006-2009 among Cambodian patients presenting with acute fever of unknown origin, showed that *S. typhi* was detected in 0.9 % (41/4,985) of blood cultures, and showed reduced susceptibility to fluoroquinolones [82].

Another bacterial infection that can mimic malaria and is difficult to diagnose clinically is melioidosis. This illness is caused by the agent *Burkholderia pseudomallei,* a gram-negative aerobic saprophyte found in contaminated soil and water. It is thought that the majority of melioidosis cases result from percutaneous inoculation, more rarely from ingestion or inhalation of the agent [84]. Many infections are initially subclinical but may result in latency and can manifest even after several decades. Clinical symptoms include septicemia, cavitating pneumonia, bone and soft tissue infections, disseminated abscesses and lymphadenitis. The case fatality rates of melioidosis range from 15.0 to 50.0 %, partially due to the difficulties in diagnosis and treatment [85]. For the diagnosis, conventional

techniques including Gram stain, API 20NE-galleries and bacterial culture remain the mainstay, but are often not applicable in resource limited areas. Serological methods are only used in remote areas or for epidemiological purposes [86]. The treatment is difficult due to a natural resistance to a broad spectrum of antibiotics, including penicillins and aminoglycosides. Carbapenem-antibiotics, third-generation cephalosporins and sulfamethoxazole with trimethoprim are currently the first choice for empiric treatment [84, 87]. Southeast Asia and northern Australia are the main endemic foci of melioidosis [84-85]. In northeast Thailand, melioidosis was shown to be the third most common cause of death from infectious diseases after HIV/AIDS and tuberculosis, with a mortality rate of 46.2 % [85]. In Cambodia the available data on melioidosis is limited, a study conducted in 2008 showed that 16.0 % of examined Cambodian children carried *B. pseudomallei* antibodies [86]. The first prospective study on pulmonary melioidosis in Cambodia from 2007 to 2010 showed that mortality was very high (61.5 %) due to the lacking access to efficient antibiotics and under-recognition of the disease by clinicians [84]. Another recent study, conducted in a hospital in Phnom Penh, reported 58 patients from different provinces of Cambodia with melioidosis, the observed fatality rate was equally high with 52.0 % [87].

Last but not least, the Japanese encephalitis virus (JEV), genus *Flavivirus*, member of the *Flaviviridae* family, like dengue virus, can also cause malaria-like symptoms in humans and is difficult to diagnose clinically. Japanese encephalitis is a mosquito-borne disease with a high rate of mortality and disability. It is endemic to large parts of Asia and the Pacific, putting around 3 billion people at risk for the disease worldwide [88]. With large scale vaccination programs in India and China the worldwide incidence of the disease is decreasing, meanwhile the transmission is likely to increase in countries with rapid population growth and the lack of vaccination programs and surveillance, like Cambodia, Lao PDR or Bangladesh. JEV is mainly transmitted by the mosquito *Culex tritaeniorrhynchus*, which prefers to breed in irrigated rice paddies. Water-birds and pigs act as main reservoir and amplifying hosts [88]. Humans are a dead-end host for the virus and viremia typically remains very low. Most human infections are mild or can even be asymptomatic, but sometimes result in severe disease, especially in young children. The complicated course of disease is characterized by rapid onset of high fever, headache, neck

stiffness, disorientation, coma, seizures, spastic paralysis and death with a case fatality rate as high as 30.0 % and disability rate of 40.0 % of survivors [88]. Currently the treatment is entirely supportive. Laboratory-based diagnosis can be established using serological methods like hemagglutination inhibition test and IgM–capture enzyme-linked immunosorbent assay (MAC-ELISA), since direct virus detection from plasma or CSF by PCR remains very rare due to the extremely low viremia [89]. In Cambodia, human cases of Japanese encephalitis have been described since 1965, over the years pediatric hospital based studies revealed that 18.0-31.0 % of children with meningoencephalitis had JEV [90]. The latest Cambodian surveillance data from 2006 to 2008 showed that of 586 pediatric patients (mean age 6.2 years) presenting with meningoencephalitis, JEV was detected in 19.0 %. The percentage of Japanese encephalitis cases at individual sentinel sites ranged from 13.0-35.0 % of all meningoencephalitis cases, which occurred year-round [90]. Cambodia shares similar agrarian practices and ecologic characteristics with its neighboring countries Thailand and Vietnam, who have both demonstrated a considerable JEV burden and have introduced the vaccine into routine immunization programs in the 1990s. Since 2009, the JEV-vaccine is also used in 3 Cambodian provinces (Kampong Cham, Svay Rieng and Prey Veng) [91].

2 Material and methods

2.1 Study objectives and design

2.1.1 Study objectives

The aim of this cross-sectional, observational study was to identify the most common causes of acute undifferentiated febrile illness in basic health posts in rural areas of Cambodia with the primary objective to develop evidence to guide management of acute, malaria-RDT negative fever cases. Secondary objectives were to determine available diagnostic tools applicable in field conditions, to enhance further research and to build capacity for laboratory testing and research within the country. Finally, it was intended to show that an extensive AUFI-study is feasible in a peripheral, non-hospital setting and to provide a study protocol that may be used in other countries.

2.1.2 Study sites

The 3 study sites (C-1 to C-3) for this cross-sectional, observational study were located in basic health centers in rural areas in the west (C-1 and C-2 in Pailin City, Pailin province) and east (C-3 in Snoul, Kratie province) of Cambodia, as shown on the map in figure 13. Pailin city is at 371 km distance to Phnom Penh (Ministry of Health, Cambodia, 2009). The surrounding area is hilly and forested, with lots of little villages spread around. Many people here work in the forests or as gem- miners. Pailin City has been a base for research projects in the past, since multi-drug-resistant malaria parasite strains started to emerge from this area close to the Thai border [4].The sites C-1 and C-2 were chosen here because the malaria incidence in this area has dropped significantly during the last 5 years due to the extensive control measures that have been established in the region. Furthermore, these 2 health centers (Suon Komar and Oh Chra) were easily accessible and the staff had participated in research projects before.

Figure 13: 2 maps of Cambodia, displaying the 3 study sites C-1-3. Left: Schematic map; Right: Satellite map displaying exact locations of C-1 in red, C-2 in blue, C-3 in green (Tara Müller, 2009, created with Google earth®, coordinates provided by Ministry of Health).

Suon Komar Health Centre (C-1) lies within the compound of the referral hospital of Pailin city, and is therefore connected with its network, which makes it easy to reach for the people of the area; its operational district is covering 5,151 households with a total population of 22,336. Oh Chra Health Centre (C-2) lies in a more remote setting at the outskirts of town and its OD is covering 4,016 households with a total population of 14,283.

Figure 14: Left: Outskirts of Pailin City and the surroundings. Top right: Suon Komar Health Center (C-1). Bottom right: Oh Chra Health Center (C-2) (Tara Müller, 2010).

Figure 15: Left: Snoul town center; Right: Snoul Health Center (Tara Müller, 2010).

The site C-3 in Snoul was chosen to outline geographical differences compared to the Pailin area; it was the first time that a research project took place in this health center. Snoul Health Centre lies on the fringes of Snoul, a small town at the eastern side of the country, in Kratie province, 255 km from Phnom Penh. The health center is covering an operational district with 8,310 households with a total population of 43,867 (Ministry of Health, Cambodia, 2009). This remote area, close to the Vietnamese border, is characterized by vast rubber plantations and is mostly inhabited by plantation-workers and farmers. According to the national data, Kratie province is an area of low transmission for malaria [16].

2.1.3 Study duration

In Pailin the study started on the 1st of January 2008. After 3 months of pilot phase, the study period ended on the 31st of December 2010. In Snoul, the study commenced 4 months later, on the 1st of May 2008 and finished on the 31st of December 2010.

2.1.4 Subject population

Male and female persons who visited the 3 health centers during the study from January 2008 to December 2010 were eligible to participate in the study if they were between 7 and 49 years old on the day of recruitment The first study group, Group F (fever), consisted of outpatients that presented with an acute febrile illness, defined by a body temperature over 38.5 °C measured on tympanic membrane, which had lasted for not longer than 8 days. The patients had to be eligible for a malaria test according to the national guidelines, as explained in chapter 1.2.2. Lastly, the patients had to have an understanding of the study and agree to its provision by giving written informed consent (see appendix 9.6). In case of under-aged participants the parents or guardians had to agree. If the patients were in a critical clinical condition that warranted immediate hospitalization they were not enrolled in the study. An asymptomatic, comparative group, Group N (non-febrile) was recruited from family members or friends that accompanied the patients to the health center or people who consulted there for reasons not related to infectious diseases like minor injuries or pregnancy consulting. Inclusion criteria for the Group N were the absence of a febrile illness and the understanding and signature of the informed consent. Recruiting healthy Cambodian people for a study that involves blood drawing is very difficult, due to the general reluctance of Cambodians to give blood. Even though this is not an independent age- and gender-matched Group N, it was thought to be interesting to compare the febrile study population to asymptomatic people from the same background. In total, 1,475 individuals have participated in this study, 1,193 of which belonged to the febrile Group F, and 282 individuals belonged to the healthy Group N. Table 4 summarizes the inclusion and exclusion criteria for both study groups.

Table 4: Inclusion and exclusion criteria for Group F and Group N

	Inclusion criteria	**Exclusion criteria**
Group F	Age 7-49 years Eligible for malaria testing Fever (temperature > 38.5 °C) Fever duration < 8 days Informed consent	Critical clinical condition
Group N	No fever (temperature < 37.5 °C) Informed consent	

2.1.5 Sampling and data processing

Following the enrolment, the patient's history was taken and a physical clinical examination was performed to detect possible reasons for the fever, such as infected skin lesions, infections of the urinary tract or respiratory tract. Furthermore, a RDT for malaria was conducted. All of these findings, as well as the presumptive diagnosis and prescribed treatment of the health center staff, were documented on a designated form (appendix 9.7). Thereafter, the following samples were taken from each participant:

- 1 naso-pharyngeal swab, in viral transport medium (VTM)
- 15 ml of venous whole blood which were separated into 5 ml in an ethylene-diamine-tetraacetic-acid (EDTA)-tube, 5 ml in a dry tube and 5 ml in a blood culture bottle
- 1 capillary blood spot on filter paper
- 1 blood smear for microscopy, thick and thin film

The patient's data and specimen were anonymized immediately at health center level. Each sample was labeled with an ID containing the site (CX) and patient number (xxxx), e.g. C1-0001. The EDTA-tubes were stored at 4 °C, the blood culture bottles in an incubator at 37 °C and the VTM- and dry- tubes in a liquid nitrogen tank, all of which had been provided by the study sponsors and maintained by IPC. Every morning a taxi service brought the collected samples from the sites to the IPC laboratory in Phnom Penh within maximum 48 hours. The clinical data was forwarded to the CNM for translation. If the laboratory result could have had a direct bearing on the patient management and was available at the appropriate time, it was immediately sent back to the health center staff. Figure 16 gives an overview of the data-flow in the study.

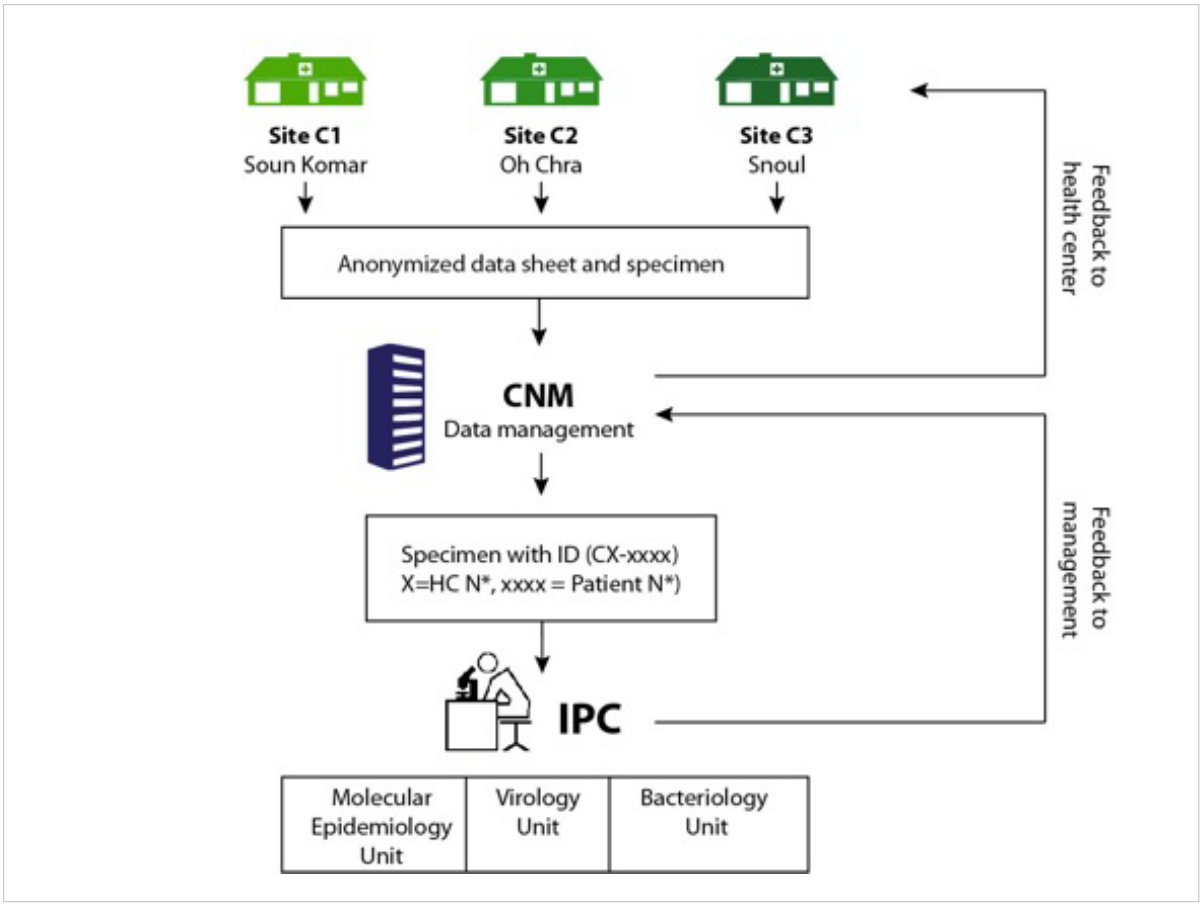


Figure 16: Flowchart of data and specimen processing within the study (Tara Müller, 2011).

2.1.6 Laboratory testing and ethical approval

Molecular diagnostic tests were run on all blood samples to detect the deoxyribonucleic acid (DNA) of *Plasmodium* spp., *Leptospira* spp., *Rickettsia* spp., *Orientia tsutsugamushi*, or the ribonucleic acid (RNA) of dengue and influenza virus. The testing took place in the Department of Molecular Epidemiology and the Department of Virology at IPC in Phnom Penh. Nested polymerase chain reaction (nPCR) was chosen as the main diagnostic tool in this study, since it is both highly sensitive and specific and no convalescence samples are needed in comparison to most routine serological methods. The positive amplified PCR products were further sent to Paris for nucleotide sequence analysis at Genopole®; the sequence was then compared to published sequences to assess the diagnosis with a specificity of 100 %. Even though in current literature realtime-PCR is considered as the new standard, it is often not available in study settings like this. That is why it was chosen

to use and evaluate nested PCRs as a primary diagnostic tool. The blood smears were analyzed with a high resolution microscope by experienced staff in the IPC laboratory for evaluation and quality assurance. In addition, the Department of Bacteriology evaluated blood culture bottles for all febrile individuals, to test for community acquired septicemia, typhoid fever and melioidosis. HIV, tuberculosis and viral hepatitis have not been evaluated.

All of the tests have been approved by the Cambodia Ethics Committee for Health Research on June 11th 2007.

2.2 On site diagnostics at health centers

The only diagnostic tests performed directly in the field were rapid diagnostic tests (RDTs) for malaria diagnosis. These tests were distributed by the WHO and are able to detect and distinguish *P. falciparum* (PF) and non-*falciparum* malaria parasites (non-PF). These tests (ICT MALARIA Cassette Test®, reference: ML02 25 TEST KIT) are a combination of HRP2 and pLDH, thus can differentiate between single infection with PF, single infection with non-PF malaria parasites, which are in Cambodia mostly due to *P. vivax*. Mixed infections with PF and non-PF malaria parasites can be detected, too. Figure 17 displays an image of the possible test results, a mixed infection would result in 3 pink lines on the test strip.

Blood smears for microscopic diagnosis have been prepared on site as well. Thick and thin blood films were fixed on the slide with methanol and were then stained with a modified Giemsa stain, Accustain® (Sigma-Aldrich®, Germany, reference: 058K4349), for 30 minutes. Slides were usually read in the field for on-site presumptive diagnosis and then sent to IPC for a second read by an experienced microscopist (see chapter 2.2.1).

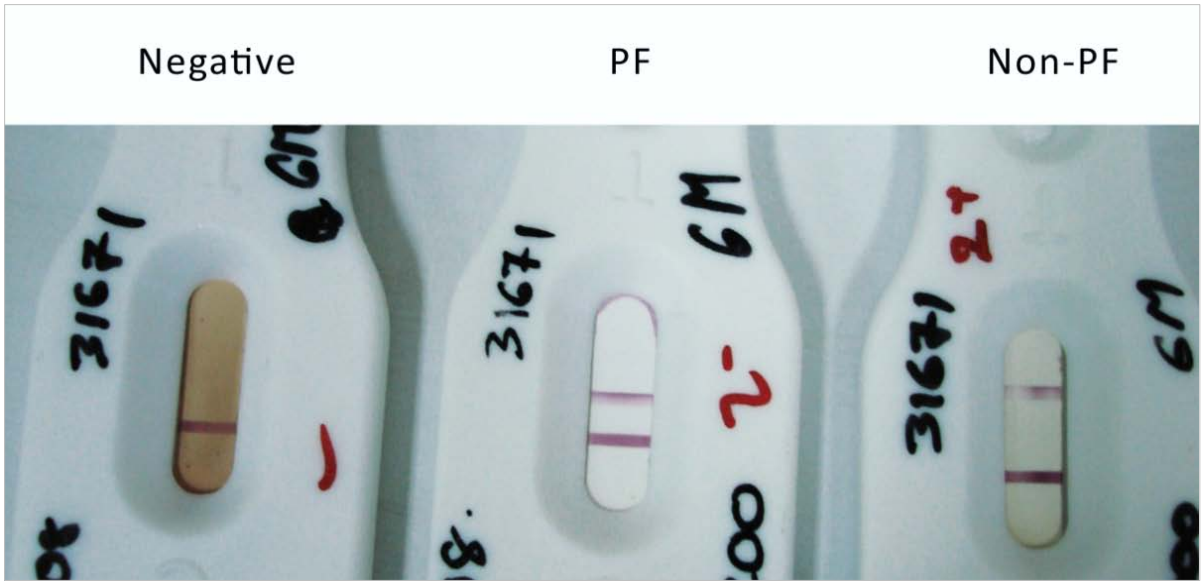


Figure 17: Malaria-RDT, showing different results. From left to right: Negative test, positive test for *P. falciparum* (PF), positive test for non-*falciparum* species (non-PF) (Tara Müller, 2009).

2.3 Processing and testing of samples

2.3.1 Microscopy

According to the WHO's "Basic Malaria Microscopy" manual, at least 100 fields of the thick film, which is equivalent of approximately 0.25 µl of blood, were examined using a 100 x oil-immersion objective to determine if a slide could be considered negative. An experienced microscopist can detect malaria parasites at densities of approximately 5-10 malaria parasites per microliter blood with this method [92]. If parasites were identified, they were counted in relation to the number of leucocytes to evaluate the parasite density (parasitemia). At least 200 leucocytes have to be counted; in case of low parasitemia (less than 99 parasites/200 leucocytes) 500 leucocytes had to be counted. Considering an average number of 8,000 leucocytes per microliter blood of any patient, the parasite count can be easily converted to parasites/µl blood using the following formula [92]:

$$\frac{\text{Number of counted parasites} \times 8{,}000}{\text{Number of leucocytes}} = \text{Number of parasites/µl blood}$$

The thin film was used to determine the parasite species and to identify mixed infections by 2 or more *Plasmodium* spp.. The specificity of this technique is highly depended on the level of training of the microscopist as well as the quality of the slide [92]. Furthermore, mixed infections are at risk to be underestimated because the identification of the minor species, like *P. malariae* or *P. ovale*, is very challenging [93]. Figures 18, 19 and 20 show microscope images of thin and thick films of the different malaria parasites, taken in the IPC laboratory in Phnom Penh.

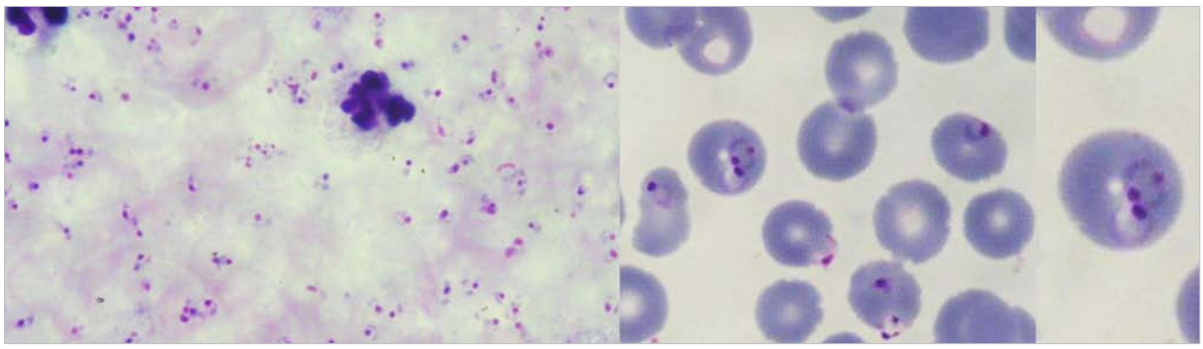


Figure 18: Microscopic images of *P. falciparum*. Left: Thick film, right: Thin film (100 x oil-immersion objective, Tara Müller, 2010).

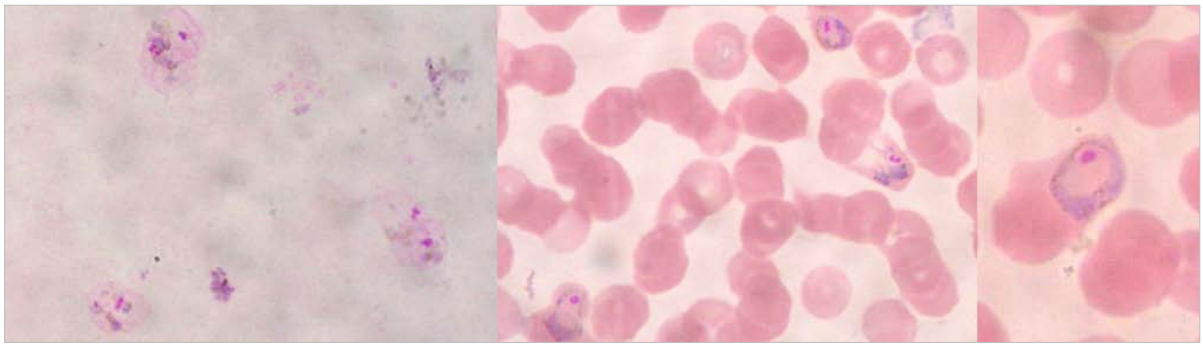


Figure 19: Microscopic images of *P. vivax*. Left: Thick film, right: Thin film (100 x oil-immersion objective, Tara Müller, 2010).

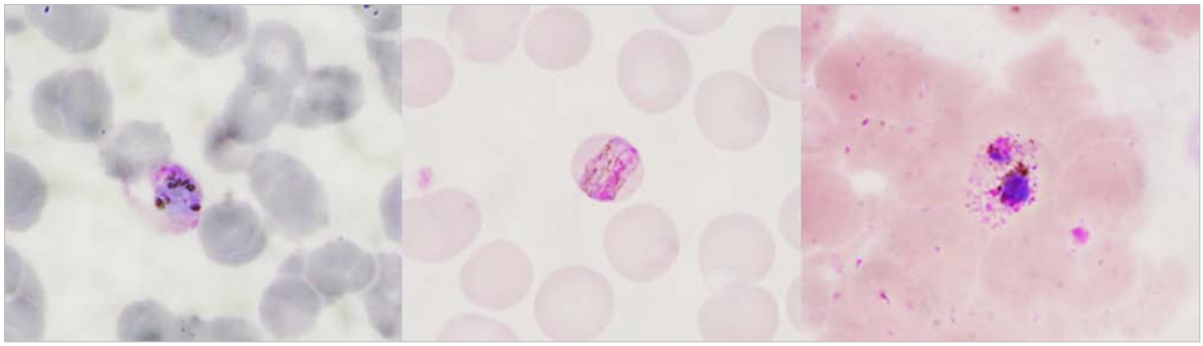


Figure 20: Microscopic images of a thin blood film with *P. knowlesi*, *P. malariae* and *P. ovale* from left to right (100 x oil-immersion objective, Tara Müller, 2010).

2.3.2 Blood culture

For each febrile patient 5 ml of whole blood were injected in an aerobic blood culture bottle (Pharmaceutical Factory No. 2, Vientiane, Lao PDR) based on tryptic hydrolysate of casein and soy peptone, blood to media ratio was 1:10. These were incubated at 37 °C in the field, and then sent back to IPC with the other samples in a special temperature surveillance container. The bottles were weighed before departure for the study site and then on return to measure the weight of blood and hence estimate the volume of blood added to the bottles. Upon arrival at IPC they were incubated and observed for another 5 days at 37 °C in the Department of Bacteriology. If bacterial growth was observed the cultures were furthermore tested for their antibiotic sensitivity, using standard bacteriological procedures.

2.3.3 C-reactive protein level detection

C-reactive protein (CRP) is an acute-phase protein whose plasma levels rise as a response to inflammatory processes in the body, especially bacterial infections. Other reasons for elevated levels can be a myocardial infarction, cancer, or trauma. CRP-levels were evaluated on all plasma samples using an immunoturbidimetric essay on latex particles. The human CRP agglutinates on the latex particles which are loaded with monoclonal anti-CRP-antibodies. The antigen-antibody-complexes can be measured by the loss of intensity

of a light beam (turbidimetry). All CRP-levels were evaluated using the Cobas Integra® 400 automate (Roche Diagnostics Limited, Switzerland), the cut-off value was 5 mg/l.

2.3.4 DNA and RNA extraction

After arrival at the Department of Molecular Epidemiology at IPC, the 5 ml of EDTA-blood samples were centrifuged for 10 minutes at 2,000 rpm to separate plasma from blood cells. All samples were stored at -80 °C. For the extraction of DNA, 200 µl of packed blood cells have been thawed, and processed according to the protocol of the QIamp® DNA Mini Kit (Qiagen®, Germany, reference: 51306). First 20 µl of Proteinase K and 200 µl of lysis buffer were added to the sample, mixed by pulse-vortexing for 15 seconds and then incubated in a water bath at 56 °C for 10 minutes. After centrifugation wash steps were performed according to the manufacturer's instructions, using the buffers AW1 and AW2. In the last step the DNA was eluted in 50 µl of Buffer AE and incubated for another 5 minutes to increase the DNA yield.

For the extraction of viral RNA the QIamp® Viral RNA Mini Kit (Qiagen®, Germany, reference: 52906) was used on 200 µl of plasma or 140 µl throat swab-sample, following the manufacturer's instructions.

2.3.5 DNA amplification

2.3.5.1 Detection threshold evaluation of nested PCR-assays

The detection thresholds of the nested-PCR assays were empirically evaluated by using dilution series of a positive control sample with a known DNA-concentration. The DNA concentration of the positive controls was measured with the Nanodrop®-spectrophotometer (Thermo Fisher Scientific, USA). However, this approach was used to approximate the sensitivity of the used assays in "real life" conditions. The obtained results will be mentioned in the following detailed description of the applied assays.

2.3.5.2 Reagents and conditions for nested PCR assays

For the detection of *Plasmodium* spp., *Leptospira* spp., *Rickettsia* spp. and *O. tsutsugamushi* specific nPCR assays have been performed in the Mastercycler® thermocycler (Eppendorf, Germany), using the following reagents:

- *Thermus aquaticus*-DNA-Polymerase I (Taq-Polymerase, 5 U/µl), $MgCl_2$ -solution (25 mM) and Reaction-Buffer package, FIREPOL® (BioDyne Solis, Estonia)
- Deoxynucleotid-triphosphates (dNTPs, 2mM) (BioDyne Solis, Estonia)
- Purified water (Fresenius Kabi, France, reference.: 343 470.4)

The conditions and volumes of the reagents for the primary and nested PCR reactions were applied as listed in table 5.

Table 5: Overview of primary and nested PCR reagents and conditions

Primary PCR reagents	***cytB***	***LEPTO***	***47kDA***	***gltA***	***ompB***
10x Buffer	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
dNTP (2 mM)	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
$MgCl_2$ (25 mM)	2.5 µl	2.0 µl	2.5 µl	2.5 µl	2.0 µl
Forward Primer (10 µM)	1.25 µl	1.25 µl	1.25 µl	1.25 µl	1.25 µl
Reverse Primer (10 µM)	1.25 µl	1.25 µl	1.25 µl	1.25 µl	1.25 µl
Taq-Polymerase (5 U/µl)	0.25 µl	0.25 µl	0.25 µl	0.25 µl	0.25 µl
H_2O	eq. 23 µl	eq. 23 µl	eq. 22 µl	eq. 23 µl	eq. 23 µl
DNA	2 µl	3 µl	3 µl	2 µl	2 µl
Final volume	25 µl	25 µl	25 µl	25 µl	25 µl
Primary PCR conditions	***cytB***	***LEPTO***	***47kDA***	***gltA***	***ompB***
Denaturation	94 °C	94 °C	94 °C	94 °C	94 °C
	3 min 30 s	3 min 30 s	3 min 30 s	3 min 30 s	3 min 30 s
Annealing	57 °C	63 °C	60 °C	55 °C	58 °C
	1 min 30 s	1 min 30 s	1 min 30 s	1 min 30 s	1 min 30 s
Extension	72 °C	72 °C	72 °C	72 °C	72 °C
	11 min 30 s	11 min 30 s	11 min 30 s	11 min 30 s	11 min 30 s
40 cycles	Hold at 4 °C	Hold at 4 °C	Hold at 4 °C	Hold at 4 °C	Hold at 4 °C
Nested PCR Reagents	***cytB***	***LEPTO***	***47kDA***	***gltA***	***ompB***
10x Buffer	5 µl	5 µl	5 µl	2.5 µl	5 µl
dNTP (2 mM)	5 µl	5 µl	5 µl	2.5 µl	5 µl
$MgCl_2$ (25 mM)	5 µl	5 µl	4 µl	2.5 µl	4 µl
Forward Primer (10 µM)	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Reverse Primer (10 µM)	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Taq-Polymerase (5 U/µl)	0.4 µl	0.4 µl	0.4 µl	0.4 µl	0.4 µl
H_2O	eq. 48 µl	eq. 47 µl	eq. 48 µl	eq. 48 µl	eq. 48 µl
Primary PCR product	2 µl	3 µl	2 µl	2 µl	2 µl
Final volume	50 µl	50 µl	50 µl	50 µl	50 µl
Nested PCR conditions	***cytB***	***LEPTO***	***47kDA***	***gltA***	***ompB***
Denaturation	94 °C	94 °C	94 °C	94 °C	94 °C
	3 min 30 s	3 min 30 s	3 min 30 s	3 min 30 s	3 min 30 s
Annealing	63 °C	61 °C	58 °C	55 °C	59 °C
	1 min 30 s	1min 30 s	1 min 30 s	1 min 30 s	1 min 30 s
Extension	72 °C	72 °C	72 °C	72 °C	72 °C
	11 min 30 s	11 min 30 s	11 min 30 s	11 min 30 s	11 min 30 s
30 cycles	Hold at 4 °C	Hold at 4 °C	Hold at 4 °C	Hold at 4 °C	Hold at 4 °C

2.3.5.2.1 CytB-nested PCR for the detection of Plasmodium spp. DNA

The mitochondrial gene *cytochrome B (cytB)* is coding for the inhibitory cytochrome bC-1 complex, which is a transmembrane electron transport-protein of the respiratory chain. The *cytB* gene of *Plasmodium* is highly conserved but still allows the differentiation of species by species-specific punctual mutations, called single nucleotide polymorphisms (SNPs) and is a frequently used molecular marker in malaria epidemiology and drug-resistance studies [94-95]. The primers shown in table 6 were used in this study and are specific for the DNA of all *Plasmodium* spp. which infect humans and primates [95]. The detection threshold of this technique was evaluated at approximately 0.1 genome copies/µl PCR-mix. As positive control DNA of *P. falciparum* reference strain 3D7 was extracted from 200 µl of packed blood cells, according to the protocol described under *2.2.2 DNA and RNA extraction*. Figure 21 shows a picture of an agarose gel with products of primary and nested *cytB*-PCR of a positive control (T+).

Table 6: Primary and nested PCR-primers for cytB-PCR

Primary PCR-primers	**Product size**
PF: 5'-AATGCCTAGACGTATTCCTGA-3' PR: 5'-CGAAGCATCCATCTACAGC-3'	1385 bp
Nested PCR-primers	
NPF: 5'-CTCGAGGAATTCGGATCC*AGAGAATTATGGAGTGGATGGTG-3' NPR: 5'-TCTAGAAAGCTTGGATCC*AAGCATAGAATGCACACATAAACC-3'	815 bp
*tag –sequence, needed for subsequent nucleotide sequencing	

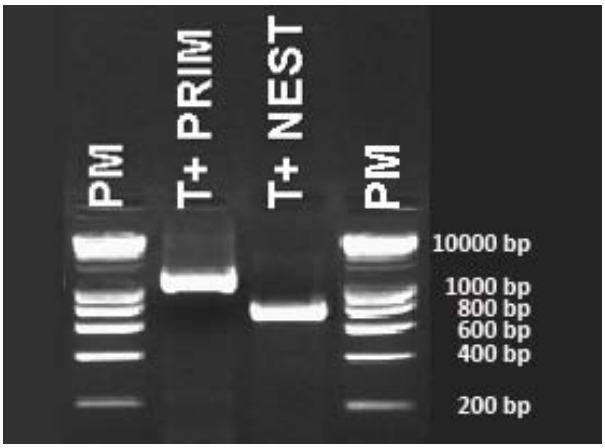


Figure 21: *CytB*-PCR products of primary PCR (T+PRIM) and nested PCR (T+NEST) on an agarose gel. PM: Smart ladder 200 bp (Tara Müller, 2009).

2.3.5.2.2 16SrRNA nested PCR for the detection of Leptospira spp. DNA

The nested PCR for the *16SrRNA* gene was performed as previously described by Merien *et al.*, 1992 and 2005. The pair of primers (LEPTO A/B) shown in table 7 amplifies a product of 331 bp [96] and the nested PCR primer pair (LEPTO C/D) a final product of 289 bp [38]. However, this PCR assay had been shown to detect all 7 pathogenic (*L. interrogans, L. kirschnerii, L. borgpetersenii, L. noguchii, L. alexandrii, L. weilii, L. santarosai*) as well as 3 saprophytic (*L. biflexa, L. meyeri, L. wolbachi*) and 2 *Leptospira* spp. of controversially discussed pathogenicity (*L. inadai, L. fainei*) that it was assessed for. The detection threshold of the assay was assessed to be 16 genome copies/µl [38]. DNA of *L. interrogans*, kindly provided by the Bacteriology Unit of IPC, was used as positive control (T+). Figure 22 shows a picture of an agarose gel with products of primary and nested PCR.

Table 7: Primary and nested PCR-primers for 16SrRNA-PCR

Primary PCR-primers	**Product size**
PF: 5'-GGCGGCGCGTCTTAAACATG-3' PR: 5'-TTCCCCCCATTGAGCAAGATT-3'	331 bp
Nested PCR-primers	
NPF: 5'-CTCGAGGAATTCGGATCC*CAAGTCAAGCGGAGTAGCAA-3' NPR: 5'-TCTAGAAAGCTTGGATCC*CTTAACTGCTGCCTCCCGTA-3' *tag –sequence, needed for subsequent nucleotide sequencing	290 bp

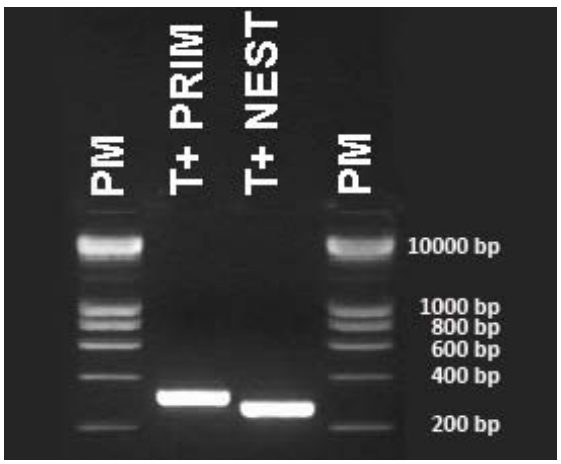


Figure 22: *16SrRNA*-PCR products of primary PCR (T+PRIM) and nested PCR (T+NEST) on an agarose gel. PM: Smart ladder 200 bp (Tara Müller, 2009).

2.3.5.2.3 GltA- and ompB- nested PCRs for detection of Rickettsia spp. DNA

Target genes for the detection and differentiation of SFG- and TG-*Rickettsia* were the citrate synthase gene *gltA*, which is present in TG- as well as SFG-*Rickettsia* [97], and the outer membrane protein B gene *ompB*, which is the dominant amplicon for SFG-*Rickettsia* [52]. The target gene *ompB* demonstrated an increased sensitivity over the *gltA* gene for detection of SFG-*Rickettsia* in both single-template and multiplex PCR assay [52]. Primers were chosen according to the previously described realtime multiplex PCR assay by Stenos *et al.* [97] and Paris *et al.* [62]. The original design of the *ompB* primers included coverage for 19 different SFG-*Rickettsia* (*R. rickettsii, R. conorii, R. honei, R. australis, R. felis, R. heilongjiangensis, R. helvetica, R. akari, R. amblyommii, R. hulinensis, R. IsraeliTT, R. japonica, R. massiliae, R. mongolotimonae, R. parkeri, R. rhipicephali, R. sibirica, R. slovaca*, and *R. africae*) [62]. The assay had been transformed into 2 nested PCRs using the primers shown in table 8 and 9. The primary PCR for *ompB* had a product of 444 bp size, the following nested PCR resulted in the final product which was 298 bp long. The products of the primary and nested *gltA*-PCR were 122 bp and 74 bp long respectively. DNA of *R. conorii*, kindly provided by Mahosot Wellcome Trust in Lao PDR, was used as positive control (T+). Figure 23 shows the PCR products of primary and nested *ompB*-PCR on an agarose gel.

Previously described sensitivities for both assays range from 1-26 genome copies/µl, depending on use of single-template or multiplex PCR (multiplex PCR was shown to decrease the sensitivity) [62]. However, by the technique used in this study, the *ompB* PCR showed a detection threshold of approximately 200 genome copies/µl.

Table 8: Primary and nested PCR-primers for ompB-PCR

Primary PCR-primers	**Product size**
PF : 5'-TAAAGCTAAAACCACCGGTGTC-3' PR : 5'-TTGTTTGCAACTGTTGTACCGG-3'	444 bp
Nested PCR-primers	
NPF: 5'-CTCGAGGAATTCGGATCC*CGACGTTAACGGTTTTTCATTCT-3' NPR: 5'-TCTAGAAAGCTTGGATCC*ACCGGTTTCTTTGTAGTTTTCGTC-3'	298 bp
*tag –sequence, needed for subsequent nucleotide sequencing	

Table 9: Primary and nested PCR-primers for gltA-PCR

Primary PCR-primers	**Product size**
PF : 5'-TCTATAAAGCTATGGGTATACCG-3' PR : 5'-TCTGCTGATTTTTTGTTCAGGG-3'	122 bp
Nested PCR-primers	
NF : 5'-CTCGAGGAATTCGGATCC*TCGCAAATGTTCACGGTACTTT-3' NR : 5'-TCTAGAAAGCTTGGATCC*TCGTGCATTTCTTTCCATTGTG-3'	74 bp
*tag –sequence, needed for subsequent nucleotide sequencing	

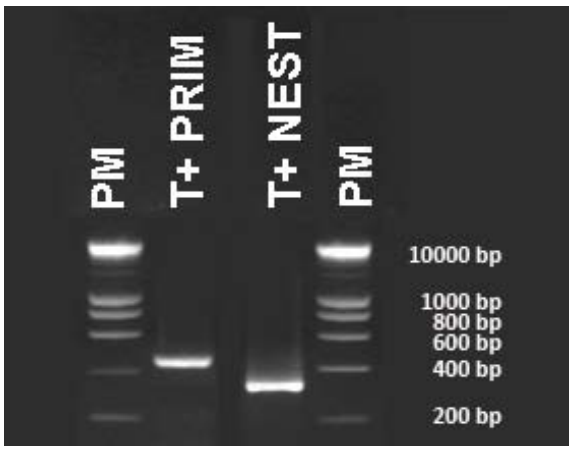


Figure 23: *OmpB*-PCR products of primary PCR (T+PRIM) and nested PCR (T+NEST) on an agarose gel. PM: Smart ladder 200 bp (Tara Müller, 2009).

2.3.5.2.4 47kDa nested PCR for detection of Orientia tsutsugamushi DNA

For the detection of *Orientia tsutsugamushi* DNA the gene *47kDa* outer surface protein or antigen, also known as “high temperature requirement A gene”, was chosen as a target. This marker was first described in a realtime-PCR assay by Jiang *et al.* 2004 [98], and was later also used in a multiplex realtime-PCR assay for the differentiation of *Orientia* and *Rickettsia* by Paris *et al.* 2008 [62]. The primers used for the primary PCR shown in table 10 were previously described by Paris *et al.* (47KDA PF/PR), whereas for the nested PCR, the primers previously described by Jiang *et al.* 2004 (47KDA F/R) were chosen, amplifying a final product of 118 bp. The detection threshold of this assay was approximately at 1,000 genome copies/µl. DNA of *O. tsutsugamushi* reference strain *Kato*, kindly provided by the Virology Unit of IPC, was used as a positive control (T+). Figure 24 shows the PCR products of primary and nested PCR on an agarose gel.

Table 10: Primary and nested PCR-primers for 47kDa-PCR

Primary PCR-primers	**Product size**
PF: 5'-AAGAGGAACAGTAACAAATGGC-3' PR: 5'-TACCACGACGAATTTTTTCTCC-3'	257 bp
Nested PCR-primers	
NPF: 5'-CTCGAGGAATTCGGATCC*AACTGATTTTATTCAAACTAATGCTGCT-3' NPR: 5'-TCTAGAAAGCTTGGATCC*TATGCCTGAGTAAGATACRTGAATRGAATT-3' *tag –sequence, needed for subsequent nucleotide sequencing	118 bp

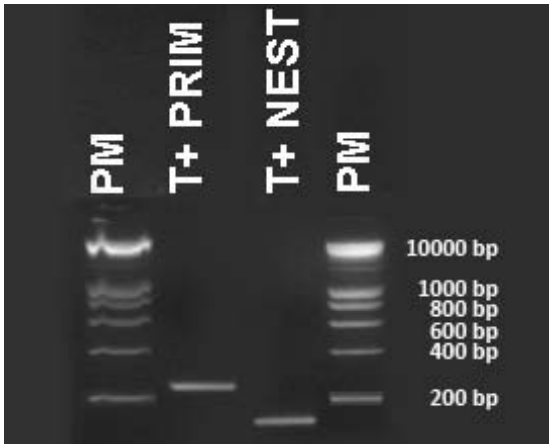


Figure 24: *47kDa*-PCR products of primary PCR (T+PRIM) and nested PCR (T+NEST) on an agarose gel. PM: Smart ladder 200 bp (Tara Müller, 2009).

2.3.5.3 Reagents and conditions for RT-PCR assays

2.3.5.3.1 Semi-nested RT-PCR assay for detection of dengue virus

The testing for dengue virus had been performed by the Department of Virology within IPC. The applied semi-nested reverse transcriptase PCR assay (RT-PCR), based on the *PrM/E* gene amplification [67], allows detection and differentiation of all 4 viral serotypes. In the first step the extracted viral RNA is converted into a copy-DNA (cDNA) by the enzyme reverse transcriptase (Qiagen®, one step RT-PCR kit, reference: 210212) and the primer D2. Then Taq-Polymerase and primer D1 were added for amplification of the cDNA under conditions as listed in table 11.

Table 11: PCR-primers, reagents and conditions for dengue virus RT-PCR

PCR-primers for dengue RT-PCR

D1(+): 5'-TCAATATgCTgAAACgCgCgAgAAACCg-3'
D2(-): 5'-TTgCACCAACAgTCAATgTCTTCAggTTC-3'

Reagents	Volume	Steps	Time	Temperature
H_20	13.1 µl	RT	60 min	45 °C
5x Buffer	5.0 µl	PCR activation	2 min	92 °C
dNTP (10 mM)	1.0 µl	PCR (35 cycles)	30 s	94 °C
Primer D1 (10 µM)	1.2 µl	Annealing	30 s	55 °C
Primer D2 (10 µM)	1.2 µl	Extension	1 min	72 °C
Qiagen, one step RT-PCR	1.0 µl	Final extension	10 min	72 °C
		Hold	∞	4 °C

In the following step a semi-nested PCR with 4 serotype-specific primers (TS1bis, TS2, TS3 and TS4) and primer D1 was performed, primers and conditions are listed in table 12. The different size of the final product determines the present serotype. For the RT-PCR-product a size of 511 bp demonstrates the general presence of dengue virus in the sample. The products of the subsequent semi-nested PCR can be interpreted as demonstrated in figure 25. The detection threshold of this technique was approximately 100 copies/ml plasma.

Table 12: PCR-primers, reagents and conditions for dengue virus serotype specific PCR

Dengue serotype specific PCR-primers	**Product size**
TS1bis(-): 5'-CgTCTCAgTgATCCggggRC-3'	482 bp
TS2(-): 5'-CgCCACAAgggCCATgAACAg-3'	119 bp
TS3(-): 5'-TAACATCATCATgAgACAgAgC-3'	290 bp
TS4(-): 5'-CTCTgTTgTCTTAAACAAgAgA-3'	392 bp

Reagents	Volume	Steps	Time	Temperature
H_20	28.6 µl	PCR activation	5 min	94 °C
10x Buffer	5.0 µl	PCR(25 cycles)	30 s	94 °C
dNTP (5 mM)	2.0 µl	Annealing	30 s	55 °C
MgCl2 (25 mM)	5.0 µl	Extension	1 min	72 °C
Primer D1 (10 µM)	1.0 µl	Final extension	10 min	72 °C
Primer TS1bis (10 µM)	1.0 µl		∞	4 °C
Primer TS2 (10 µM)	1.0 µl			
Primer TS3 (10 µM)	1.0 µl			
Primer TS4 (10 µM)	1.0 µl			
Taq pol (Promega, 5 u/µl)	1.0 µl			

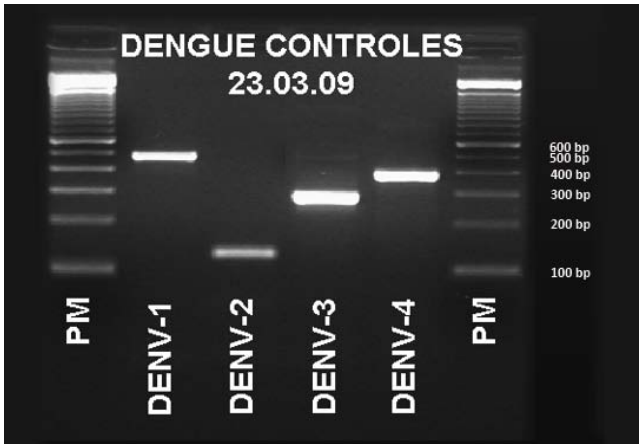


Figure 25: Dengue PCR products on agarose gel, showing the specific band of the 4 different dengue virus types (DENV1-4). PM: Smart ladder 100 bp (Tara Müller, 2009).

2.3.5.3.2 Multiplex RT-PCR for detection of influenza virus and influenza A subtyping

For the detection of influenza viruses type A and B, an in-house multiplex reverse transcriptase assay, amplifying the *M*-gene has been used. In the first step a multiplex RT-PCR has been performed, using the primers listed in table 13. After 40 cycles of PCR amplification, products were divided by their size into nucleic acids of influenza B virus (DNA band with the size of 365 bp) and nucleic acids of influenza A virus (DNA band with the size of 154 bp). Influenza B positive products were confirmed in a second semi-nested PCR using the primers B1 and MIB3 (see table 13) [99]. Influenza A virus positive products were further distinguished by a using real-time RT-PCR to detect the hemagglutinin and neuraminidase genes (H1, N1, H3, and N2). Primers and probes used for the realtime PCR are listed in table 13.

2.3.6 Gel electrophoresis

The PCR products were subsequently partitioned according to their size and visualized by gel electrophoresis. A 2.0 % agarose gel (Ultra Pure Agarose®, invitrogen™, Life Technologies, reference: 15510-027) with 0.005 % Ethidium-bromide (eurobio®, France, reference: GEPBET02) was used. A loading buffer containing 0.25 % bromophenol, 0.25 % xylene cyanol blue and 30 % glycerol was added (2 µl for 10 µl of PCR product).

Table 13: PCR-primers for the detection of influenza A and B virus

	Product size	Virus subtype
PCR-primers for RT-PCR to detect influenza A and B viral RNA		
GRAM/7Fw: 5'- CTTCTAACCGAGGTCGAAACGTA -3' GRAM/161Rv: 5'- GGTGACAGGATTGGTCTTGTCTTTA -3'	154 bp	Influenza A
B1: 5'- GAAAAATTACACTGTTGGTTCGGTG -3' B2B: 5'- AGCGTTCCTAGTTTTACTTGCATTGA -3'	365 bp	Influenza B
PCR-primers for semi-nested PCR to differentiate influenza B virus		
B1: 5'- GAAAAATTACACTGTTGGTTCGGTG -3' MIB3: 5'- CATGAAARCTCACACATCT -3'	260 bp	Influenza B
PCR-primers and probes for real-time PCR to subtype influenza A virus		
H1h-678Fw: 5'-CACCCCAGAAATAGCCAAAA-3' H1h-840Rv: 5'-TCCTGATCCAAAGCCTCTAC-3' H1h-715probe: CAGGAAGGAAGAATCAACTA[5']Fam[3']BHQ1	163 bp	Influenza A (H1)
H3h-177Fw: 5'-GAGCTGGTTCAGAGTTCCTC-3' H3h-388Rv: 5'-GTGACCTAAGGGAGGCATAATC-3' H3h-306probe: TTTTGTTGAACGCAGCAAAG[5']Fam[3']BHQ1	211 bp	Influenza A (H3)
N2h-1150Fw: 5'-GTCCAMACCTAAYTCCAA-3' N2h-1344Rv: 5'-GCCACAAAACACAACAATAC-3' N2h-1290probe: CTTCCCCTTATCAACTCCACA[5']Fam[3']BHQ1	194 bp	Influenza A (N2)
N1h-1134Fw: 5'-TGGATGGACAGATACCGACA-3' N1h-1275Rv: 5'-CTCAACCCAGAAGCAAGGTC-3' N1h-1206probe: CAGCGGAAGTTTCGTTCAACAT[5']Fam[3']BHQ1	142 bp	Influenza A (N1)

The loaded PCR-products were finally migrated in the gel floating in a Tris-Borate-EDTA–Buffer (Sigma Aldrich®, USA, reference: T4415-4L) at a voltage of 160 V for 45 minutes. 5 µl of DNA Smart Ladder 200 bp (Eurogentec®, Belgium, reference: MW-1700-02) were added in each gel as a molecular marker to determine the product's size. For dengue-PCR products a 100 bp DNA smart ladder (Invitrogen™, Life Technologies, reference: 15628-019) was used. Once the gel was fully migrated, pictures were taken with an UV-trans-illuminator (Biorad®, software: Quantity One® version 4.4.0), radiographing the gel with ultra-violet light, which makes the position of the PCR-products visible as a fluorescent band. The comparison of this band with the one of the according positive control and the correct expected size according to the marker gave the final positive or negative result.

2.3.7 Nucleotide sequence analysis

2.3.7.1 Nucleotide sequencing and alignments

Positive nested-PCR-products were sent to Genopole® Paris, France, for double strand nucleotide sequencing. Once the nucleotide sequences were sent back to the laboratory they were aligned with published reference sequences using NCBI-BLAST® (National Center for Biotechnology Information, USA), to determine the organism corresponding to the DNA sequence.

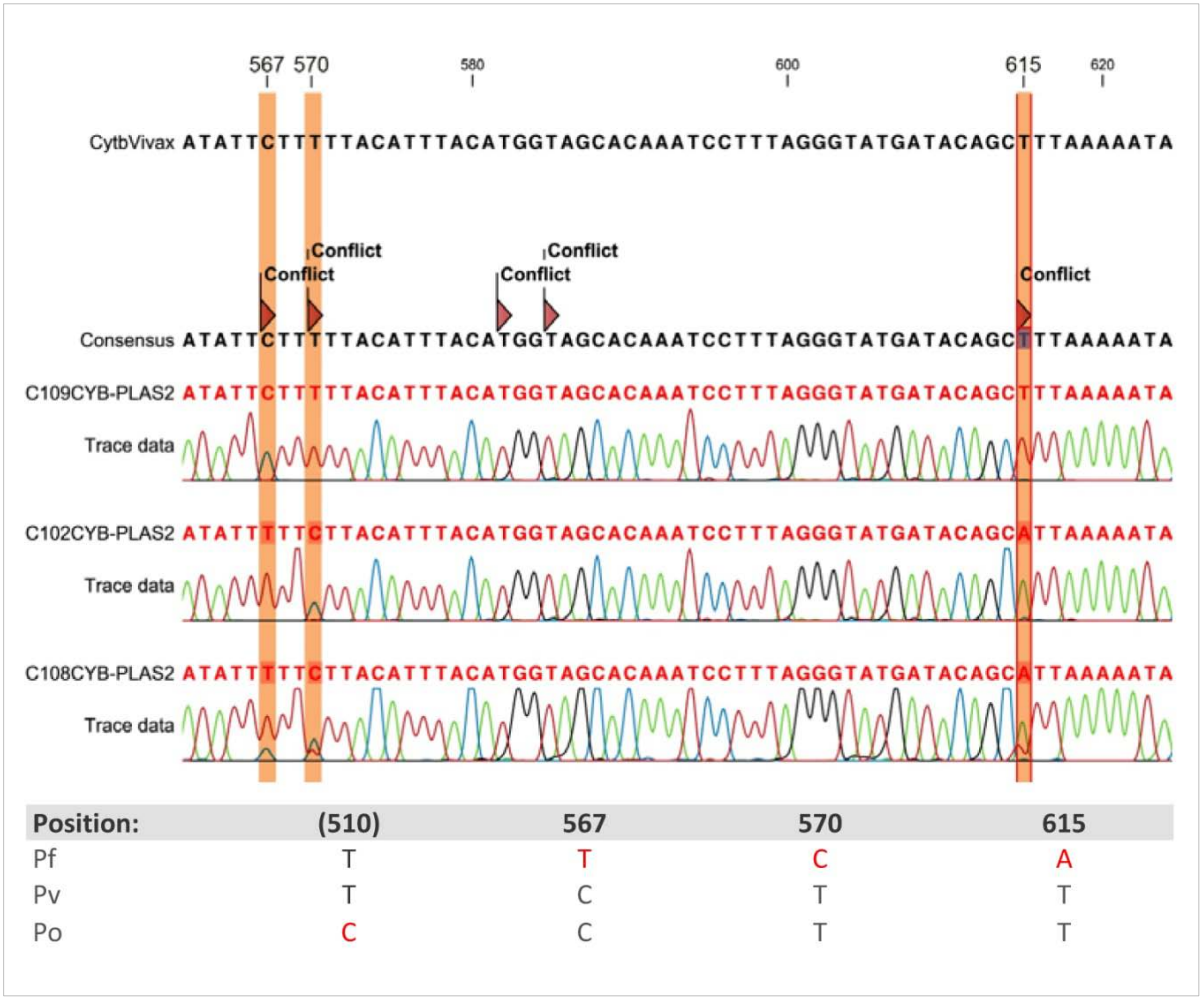
2.3.7.2 Analysis of single nucleotide polymorphisms to detect mixed *Plasmodium* infections

Within the fragment of the *cytB* gene amplified by the nPCR described earlier, several species-specific SNP combinations have been identified by Steenkeste *et al*., 2009 [95]. The analysis of these punctual mutations in the chromatogram with the software CLC Main Workbench® version 4.1.2 (CLC bio®, Denmark) allows the identification of the species and of mixed infections. Figure 26 shows the chromatograms of 3 nucleotide sequences assembled with a *cytB* reference sequence of *P. vivax*. C-109CYB-PLAS-2 is positive for *P. vivax*, C-102CYB-PLAS2 positive for *P. falciparum*, and C-108CYB-PLAS2 is an example for a mixed infection of the *P. vivax* and *P. falciparum*, which can be recognized by the double peaks at the predetermined positions (as an example position 567, 570 and 615 are highlighted in figure 26).

2.4 Data processing and statistical analysis

All of the laboratory data was recorded in a Microsoft Excel® database, including the results of microscopy, the PCR- and sequence-analysis results. All the clinical data, recorded on designated forms at the health centers, as explained earlier, was forwarded to CNM, where the anonymized forms were translated from the Cambodian scripture into English and recorded in a Microsoft Access® database. Statistical analysis was performed using the program Epi Info™ version 3.5.3 (Centers for Disease Control and Prevention). For the

comparison of results between the 2 groups F and N, Chi-square tests have been applied, p-values were calculated with the Mantel-Haenszel test [100]. If the sample size was smaller than n = 20, p-values were calculated using the Fisher's exact test [101]. For the comparison of means Student's t-test was applied [102]. Since the distribution of age and gender was different in Group F and Group N, the data was stratified by gender (male, female) and age (≥ 25 years, < 25 years). The significance level was α = 5.0 % (p < 0.05).



Position:	(510)	567	570	615
Pf	T	T	C	A
Pv	T	C	T	T
Po	C	C	T	T

Figure 26: Example of 3 nucleotide sequences, assembled with a *P. vivax* reference sequence. C-109CYB-PLAS-2 is positive for *P. vivax*, C-102CYB-PLAS2 positive for *P. falciparum*, and C-108CYB-PLAS2 is an example for a mixed infection with *P. vivax* and *P. falciparum*. The species specific peaks are highlighted in orange color (Tara Müller, 2009).

3 Results

3.1 Results overview

3.1.1 Overview of study population

In total 1,475 subjects were recruited in the 3 study sites during the study period from January 2008-December 2010. The sites C-1 and C-2 recruited 621 and 650 subjects respectively, whereas site C-3 only recruited 204 individuals. The subjects were divided in a febrile Group F and a non-febrile Group N. Figure 27 shows the distribution of recruitments between the sites as well as the percentage of enrolled subjects in the Groups F and N.

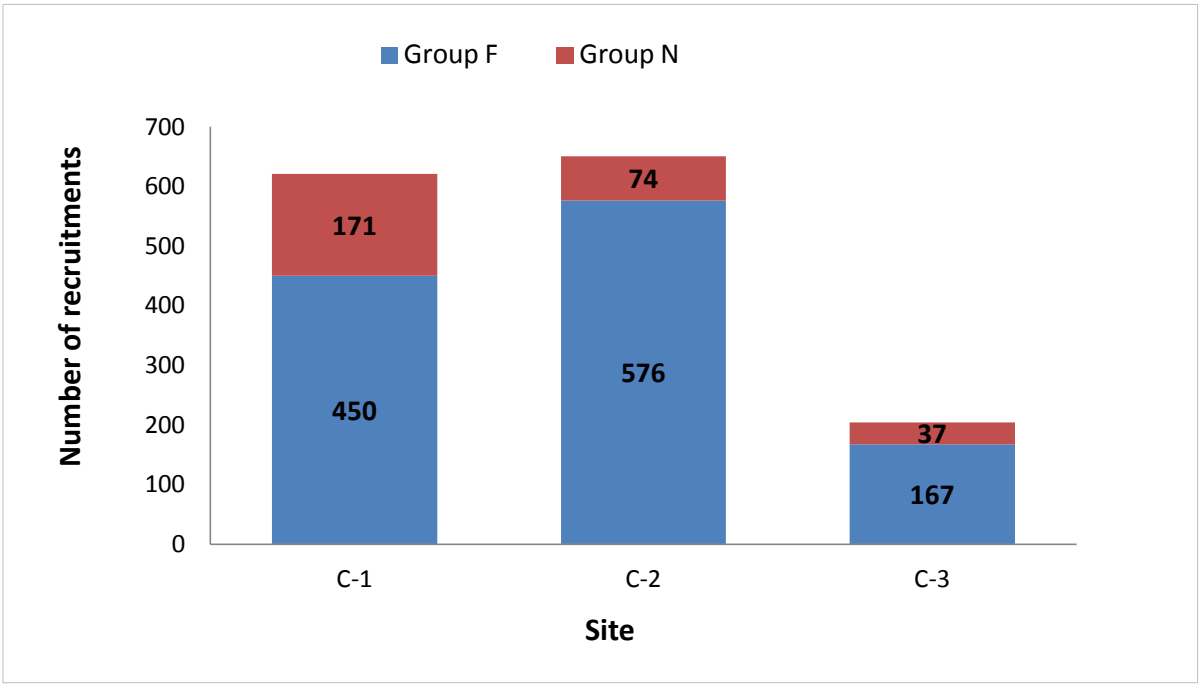


Figure 27: Number of recruitments per study site (C-1-3) in Group F (blue) and Group N (red).

In the study population 906 subjects were male and 569 were female (ratio: 1.59). In Group F the majority (67.1 %) of subjects were male, whereas in Group N the majority (62.8 %) of subjects were female, as shown in figure 28.

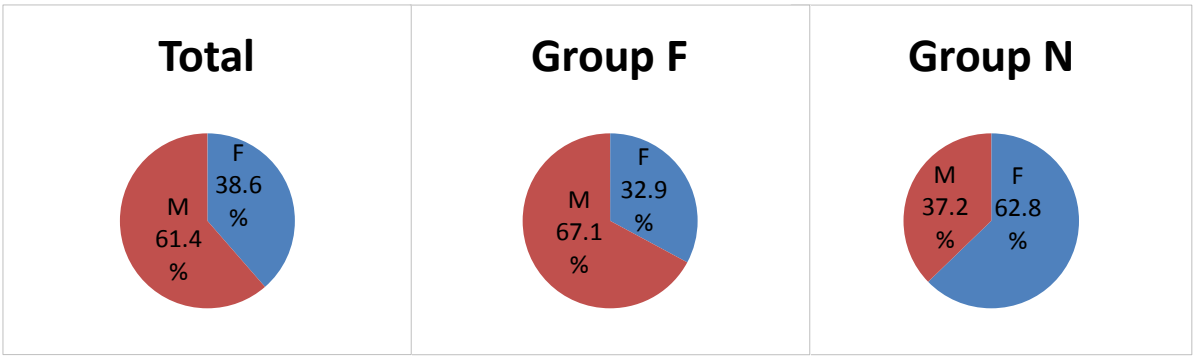
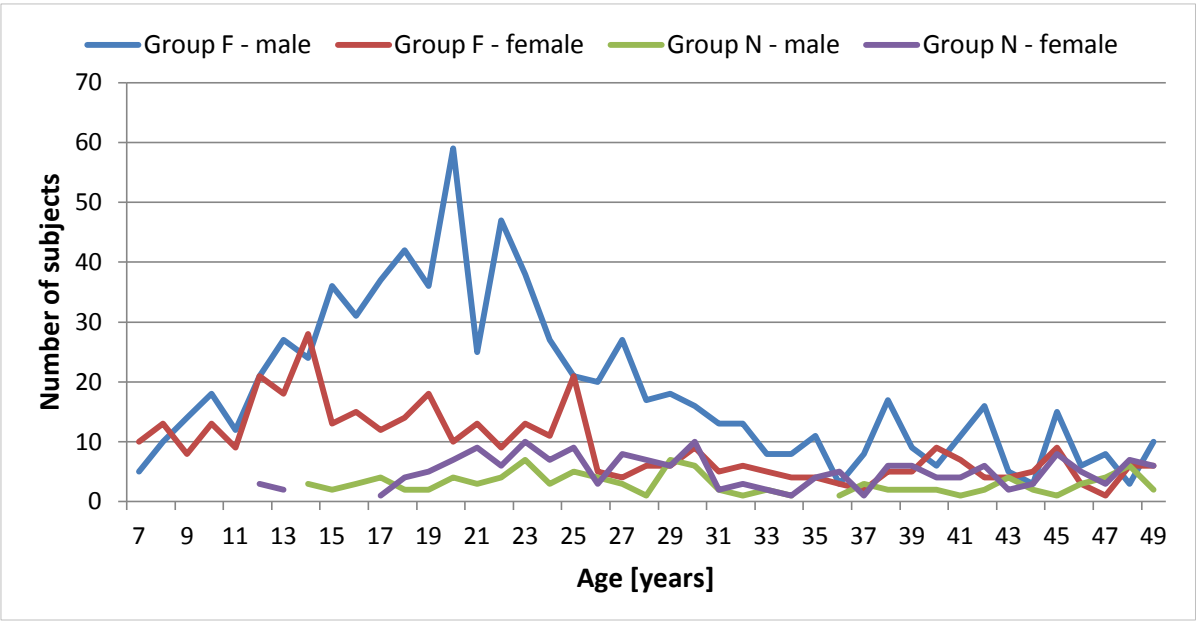


Figure 28: Gender distribution in the total study population, in Group F and Group N (M= male; F= female).

Age distribution in the study population, dived by group and gender is shown in figure 29. In Group F the median age for males was 22 years (mean 23.6, CI 95% [22.9; 24.3] years) and 20 years for females (mean 22.8, CI 95% [21.6; 23.9] years). In Group N the median age was higher than in Group F, the median age was 29 years in the male (mean 30.2, CI 95% [28.2; 32.3] years) as well as the female group (31.4, CI 95% [29.9; 32.9] years).



		N	Median [years]	Mean [years]	CI 95% [years]
Group F	**Male**	801	22	23.6	[22.9; 24.3]
	Female	392	20	22.8	[21.6; 23.9]
Group N	**Male**	105	29	30.2	[28.2; 32.3]
	Female	177	29	31.4	[29.9; 32.9]

Figure 29: Age distribution in study population, by group and gender.

As shown in figure 30, the number of recruitments in Group F was further affected by the season. Most febrile subjects were recruited during the rainy season from April to October, especially from July to September, the period of the heaviest rainfalls. In site C-3 the season had less impact on the number of recruitments, since it was rather low year round.

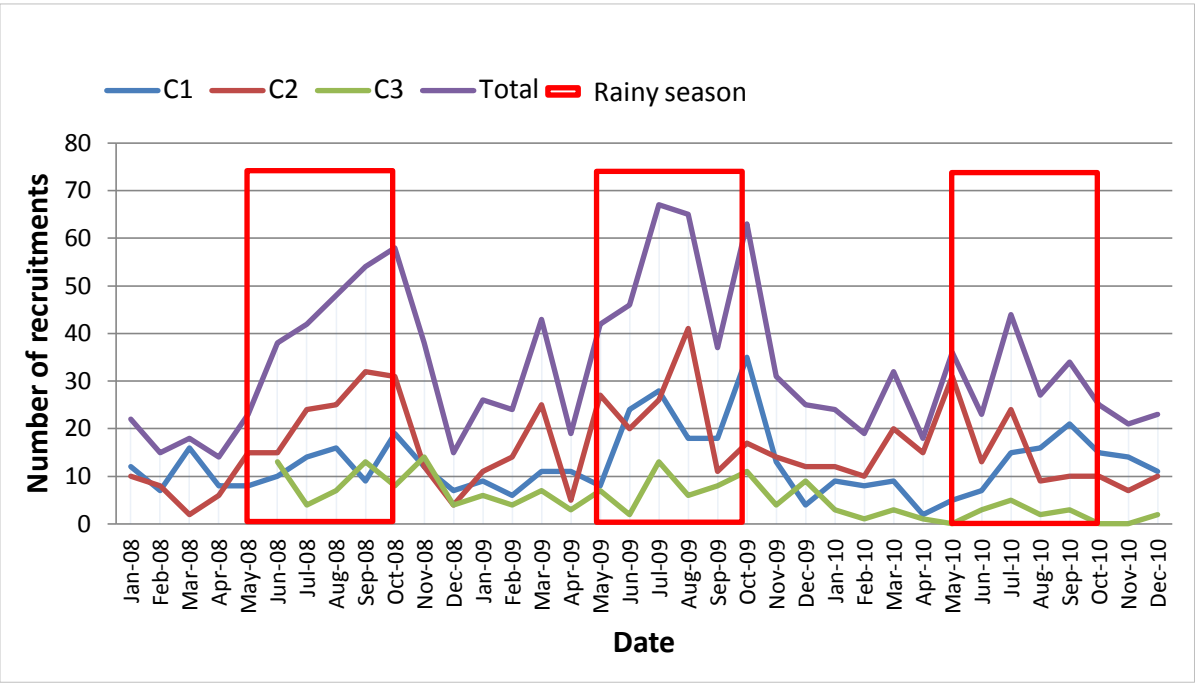


Figure 30: Seasonality of Group F recruitments in the study sites from January 2008 to December 2010, in total and by site (C-1-3). Rainy season (May-October) marked by red squares.

3.1.2 Overview of results

A laboratory based diagnosis was established in 73.2 % of the febrile patients in Group F (873/1,193). In 58.7 % of all samples 1 pathogen was detected, in 12.3 % 2 pathogens were simultaneously detected, in 1.2 % 3 and in 0.1 % even 4 pathogens were detected at the same time. In 33.1 % of the samples no pathogen was detected, as shown in table 14. In the asymptomatic Group N, 30.5 % had a positive test result for 1 of the investigated pathogens, 9.6 % had 2 and 0.4 % had 3 positive test results. Absence of a pathogen was significantly more frequent in Group N ($p < 0.01$), while mono-infections with 1 pathogen were significantly higher in Group F ($p < 0.01$). The presence of multiple positive test results

was not significantly more frequent in one of the groups (p = 0.22 for 2 pathogens; p = 0.29 for 3 pathogens).

Table 14: Number of simultaneously detected pathogens in samples of Groups F and N

	Total (n = 1,475)		**Group F (n = 1,193)**		**Group N (n = 282)**		
Number of pathogens	**n**	**%**	**n**	**%**	**n**	**%**	**p-value†**
1 pathogen	786	53.3	700	58.7	86	30.5	< 0.01
2 pathogens	182	12.3	155	13.0	27	9.6	0.22
3 pathogens	18	1.2	17	1.4	1	0.4	0.29
4 pathogens	1	0.1	1	0.1	0	0.0	n/a*
No pathogen found	488	33.1	320	26.8	168	59.6	< 0.01
Total	1,475	100.0	1,193	100.0	282	100.0	

**n/a: not applicable*

† P-values stratified by age (≥ 25 years/< 25years) and gender (m/f)

The most frequently detected pathogens in the whole study population were *P. vivax* (26.4 %), *P. falciparum* (20.7 %), pathogenic *Leptospira* spp. (9.5 %), dengue virus (5.4 %) and influenza A virus (5.9 %). Other detected pathogens were *O. tsutsugamushi* (3.7 %), influenza B virus (1.8 %), bacteria from blood culture (*Salmonella* spp., *E. coli, S. pneumoniae*, *E. cloacae*) (0.8 %) and SFG-*Rickettsia* spp. (0.2 %). Table 15 shows an overview of the results in Group F and Group N. The frequency of pathogenic *Leptospira* spp., *O. tsutsugamushi* or influenza B virus was not different in Group F and Group N (p-values > 0.05), whereas the detection of *Plasmodium* spp., dengue virus and influenza A virus was significantly more frequent in Group F (p-values ≤ 0.01).

Table 15: Overview of detected pathogens in the study population

	Total (n = 1,475)		Group F (n = 1,193)		Group N (n = 282)		
Detected pathogen	**n**	**%**	**n**	**%**	**n**	**%**	**p-value†**
***Plasmodium* spp.**	**754**	**51.1**	**676**	**56.7**	**78**	**27.7**	**< 0.01**
P. vivax	389	26.4	359	30.1	30	10.6	< 0.01
P. falciparum	306	20.7	277	23.2	29	10.3	< 0.01
P. vivax + falciparum	56	3.8	39	3.3	17	6.0	0.07
P. ovale	1	0.1	0	0.0	1	0.4	n/a
P. knowlesi	2	0.1	1	0.1	1	0.4	n/a
Pathogenic *Leptospira* spp.	**140**	**9.5**	**112**	**9.4**	**28**	**9.9**	**0.42**
L. interrogans	85	5.8	61	5.1	24	8.5	0.20
L. weilii	47	3.2	44	3.7	3	1.1	0.08
L. kmetyi	3	0.2	2	0.2	1	0.4	n/a
L. kirschnerii	1	0.1	1	0.1	0	0.0	n/a
L .santosaraii	1	0.1	1	0.1	0	0.0	n/a
L. genomospecies1	1	0.1	1	0.1	0	0.0	n/a
L. wolffii	1	0.1	1	0.1	0	0.0	n/a
L. noguchii	1	0.1	1	0.1	0	0.0	n/a
O. tsutsugamushi	**54**	**3.7**	**47**	**3.9**	**7**	**2.5**	**0.48**
***Rickettsia* spp.**	**3**	**0.2**	**2**	**0.2**	**1**	**0.4**	**n/a**
Dengue virus 1-4*	**80**	**5.4**	**75**	**6.3**	**5**	**1.8**	**0.01**
Influenza A virus	**87**	**5.9**	**83**	**7.0**	**4**	**1.4**	**0.01**
Influenza B virus	**26**	**1.8**	**23**	**1.9**	**3**	**1.1**	**0.77**
Cultured bacteria**	**9**	**0.8**	**9**	**0.8**	**-**	**-**	**n/a**

**For dengue virus testing only 1,468 samples were available*

***From 1,132 blood cultures: 1 S. typhi and 1 S. paratyphi, 1 E. coli,1 S. pneumoniae, 5 E. cloacae*

† P-values stratified by age (≥ 25 years/< 25years) and gender (m/f); n/a not applicable

Analysis of the CRP concentrations in plasma samples of Group F and N subjects showed that in Group F 81.3 % of patients had an elevated CRP level (≥ 5 mg/l), whereas in Group N only 25.2 % had an elevated level ($p < 0.01$), as shown in figure 31. Table 16 shows that the mean CRP concentration in samples of Group F was 48.6 mg/l (CI 95 % [45.2; 52.0]), whereas in Group N it was 9.9 mg/l (CI 95 % [6.5; 13.3]) ($p < 0.01$, Student's t-test).

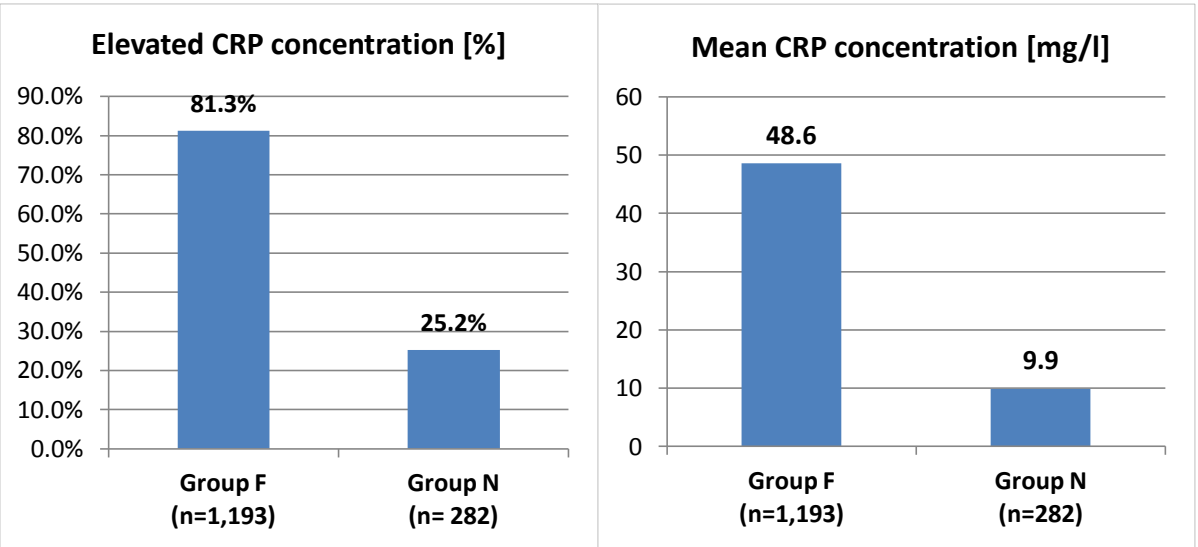


Figure 31: CRP concentrations in Groups F and N. On the left: Percentage of subjects with elevated CRP concentration (≥ 5.0 mg/l) [%]. On the right: Mean CRP concentrations [mg/l].

Table 16: CRP concentrations in plasma samples of Groups F and N

	Subjects	Elevated CRP	Mean CRP [CI 95 %]*	Range CRP	Median CRP
	n	n (%)	mg/l	mg/l	mg/l
Group F	1,193	970 (81.3)	48.6 [45.2;52.0]	0.2 - 389.6	25.7
Group N	282	71 (25.2)	9.9 [6.5;13.3]	0.1 -235.9	1.4

**Mean-CRP of Group F and Group N compared with Student's t-test p < 0.01.*

The CRP concentrations were further grouped into "not elevated" (< 5.0 mg/l), "elevated" (5.0-50.0 mg/l) and "highly elevated" (> 50.0 mg/l) and compared in 4 different categories of detected infections (malaria parasites, bacterial infections, viral infections and multiple infections), shown in table 17. The mean CRP concentration in viral infections was 12.5 mg/l (CI 95 % [10.1; 14.9]), which was significantly lower than in every other group ($p < 0.01$).

Table 17: CRP concentrations in different groups of detected pathogens

CRP concentration [mg/l]	Malaria parasites [n]	Bacteria [n]	Viruses [n]	Multiple pathogens [n]
< 5.0 (not elevated)	104	31	39	33
5.0-50.0 (elevated)	259	47	75	84
> 50.0 (highly elevated)	250	28	3	34
Total	613	106	117	151
Mean CRP concentration* [CI 95 %]	57.0 [52.4;61.7] mg/l	47.7 [34.9;60.4] mg/l	12.5 [10.1;14.9] mg/l	39.4 [29.7;49.0] mg/l

**Student's t-test results: Malaria vs. bacteria (p = 0.14), malaria vs. virus (p < 0.01), malaria vs. multiple (p = 0.01), virus vs. bacteria (p < 0.01), virus vs. multiple (p < 0.01), bacteria vs. multiple (p = 0.31).*

3.2 Details of detected pathogens

3.2.1 Malaria parasites

3.2.1.1 PCR results

The *cytB* nested-PCR, followed by nucleotide sequencing and SNP-analysis, was considered as the gold standard for malaria diagnosis in this study. As shown in table 18, 56.7 % of the Group F subjects had a positive test result for malaria by this PCR. This was significantly more common than in the asymptomatic Group N, in which 27.7 % had a positive test result ($p < 0.01$). The nucleotide sequencing results revealed that the predominant species in this study population was *P. vivax* (51.6 %) followed by *P. falciparum* (40.6 %). Both *Plasmodium* spp. were statistically more frequently detected in Group F than in Group N ($p < 0.01$). In 7.4 % of the positive samples there was evidence of a mixed-infection with both *Plasmodium falciparum* and *vivax*, which was not significantly more common in one or the other group ($p = 0.07$). For the first time ever in Cambodia, 2 human cases of the emerging simian malaria parasite *P. knowlesi* were detected, one of them in Group N, the other one in Group F. *P. ovale* was found only once (in Group N) and *P. malariae* was not detected at all in the investigated study population.

Table 18: CytB-nPCR and nucleotide sequencing results in Groups F and N

***CytB*-nPCR- results**

	Total		Group F		Group N		
***CytB*-PCR-result**	**n**	**%**	**n**	**%**	**n**	**%**	**p-value†**
Negative	721	48.9	517	43.3	204	72.3	<0.01
Positive	754	51.1	676	56.7	78	27.7	<0.01
Total	**1475**	**100.0**	**1193**	**100.0**	**282**	**100.0**	

Nucleotide sequencing results of positive PCR products

	Total		Group F		Group N		
Nucleotide sequencing result*	**n**	**%**	**n**	**%**	**n**	**%**	**p-value†**
PF	306	40.6	277	41.0	29	37.2	<0.01
PV	389	51.6	359	53.1	30	38.5	<0.01
PF/PV	56	7.4	39	5.8	17	21.7	0.07
PK	2	0.3	1	0.1	1	1.3	n/a
PO	1	0.1	0	0.0	1	1.3	n/a
Total	**754**	**100.0**	**676**	**100.0**	**78**	**100.0**	

**PF = P. falciparum, PV= P. vivax, PK= P. knowlesi, PO= P. ovale*

† P-values stratified by age (≥ 25 years/< 25years) and gender (m/f). n/a not applicable.

Table 19 shows the infection status of malaria parasites in Groups F and N. Mono-infection with only one kind of malaria parasite was significantly more frequently detected in Group F than in Group N ($p < 0.01$). It was observed that, when looking at mixed malaria infections (*P. falciparum* + *P. vivax*), without any other simultaneously detected pathogens, they were even more frequent in Group N than in Group F ($p = 0.02$). The detection of a second pathogen additional to the malaria parasites was more common in Group F (19.6 %) than in Group N (11.5 %) ($p < 0.01$).

Figure 32 shows which pathogens were simultaneously detected with malaria parasites. By frequency in descending order, these pathogens were *Leptospira* spp. (n = 58), influenza viruses (n = 31), dengue virus (n = 29), *O. tsutsugamushi* (n = 14) and bacteria from blood culture (n = 2)). In 8 cases 2 of the above mentioned pathogens were found simultaneously, in addition to the malaria parasites.

Table 19: Malaria parasite detection status in Groups F and N

	Total		Group F		Group N		
Detection status	**n**	**%**	**n**	**%**	**n**	**%**	**p-value†**
Mono -malaria	568	75.3	515	76.2	53	67.9	< 0.01
Mixed malaria	44	5.8	28	4.1	16	20.5	0.02
Malaria + other pathogen(s)	142	18.8	133	19.6	9	11.5	< 0.01
Total	754	100.0	676	100.0	78	100.0	-

† P-values stratified by age (≥ 25 years/< 25years) and gender (m/f).

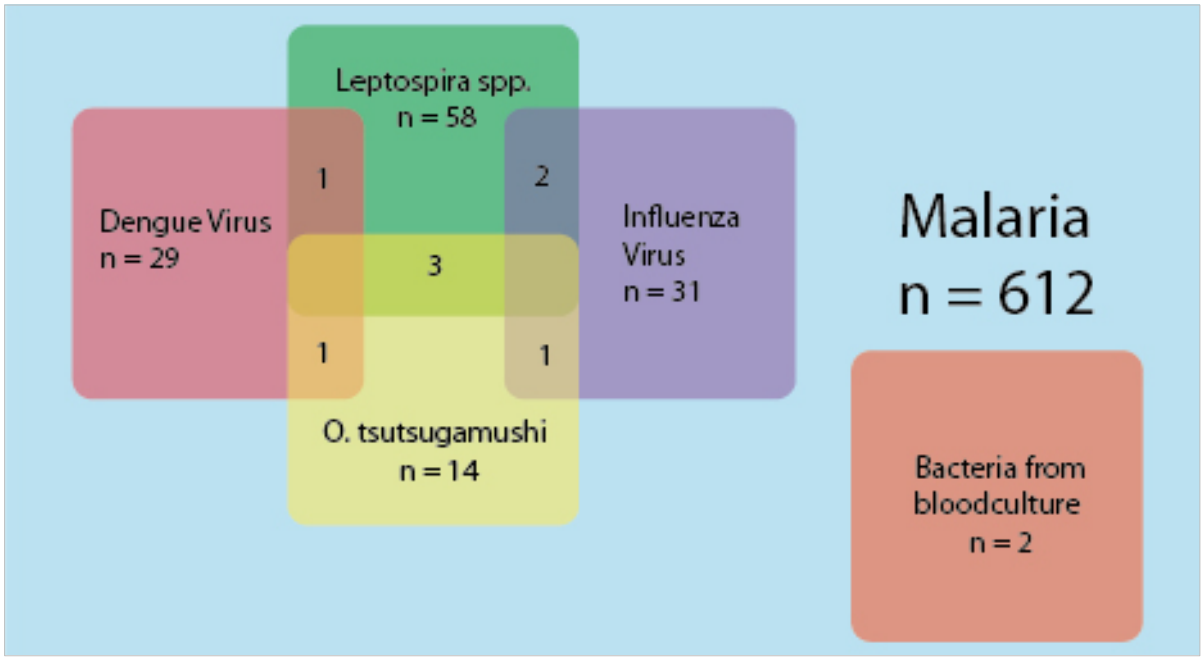


Figure 32: Simultaneously detected pathogens with malaria parasites in the study population. Of 754 subjects with positive malaria PCR, 612 subjects had malaria parasites only (single or mixed infections). In the remaining 142 subjects malaria parasites were simultaneously detected with pathogenic *Leptospira* spp., dengue virus, influenza virus or *O. tsutsugamushi*.
In 8 cases, 3 pathogens were detected simultaneously.

3.2.1.2 Microscopy and RDT results

By microscopy 33.9 % (501/1,475) of all slides were positive for malaria parasites. The detected species were 52.9 % *P. vivax*, 32.9 % *P. falciparum*, 12.7 % mixed *P. falciparum* with *P. vivax*, and 0.7 % mixed *P. falciparum* with *P. malariae*, 0.2 % *P. malariae* and 0.1 % *P. ovale*. The majority of positive slides (486/501) were detected in Group F, whereas only 15 positive slides were detected in Group N. By RDT, 26.0 % (383/1,475) of all tested

persons were positive for malaria parasites, 43.0 % of which were *P. falciparum*, 36.5 % were non-*falciparum* and 20.3 % were mixed *Plasmodium* infections. All of the positive RDT results were detected in Group F only. Figure 33 shows the performances of RDT and microscopy compared to the *cytB*-PCR in Group F (figure 33A) and Group N (figure 33B).

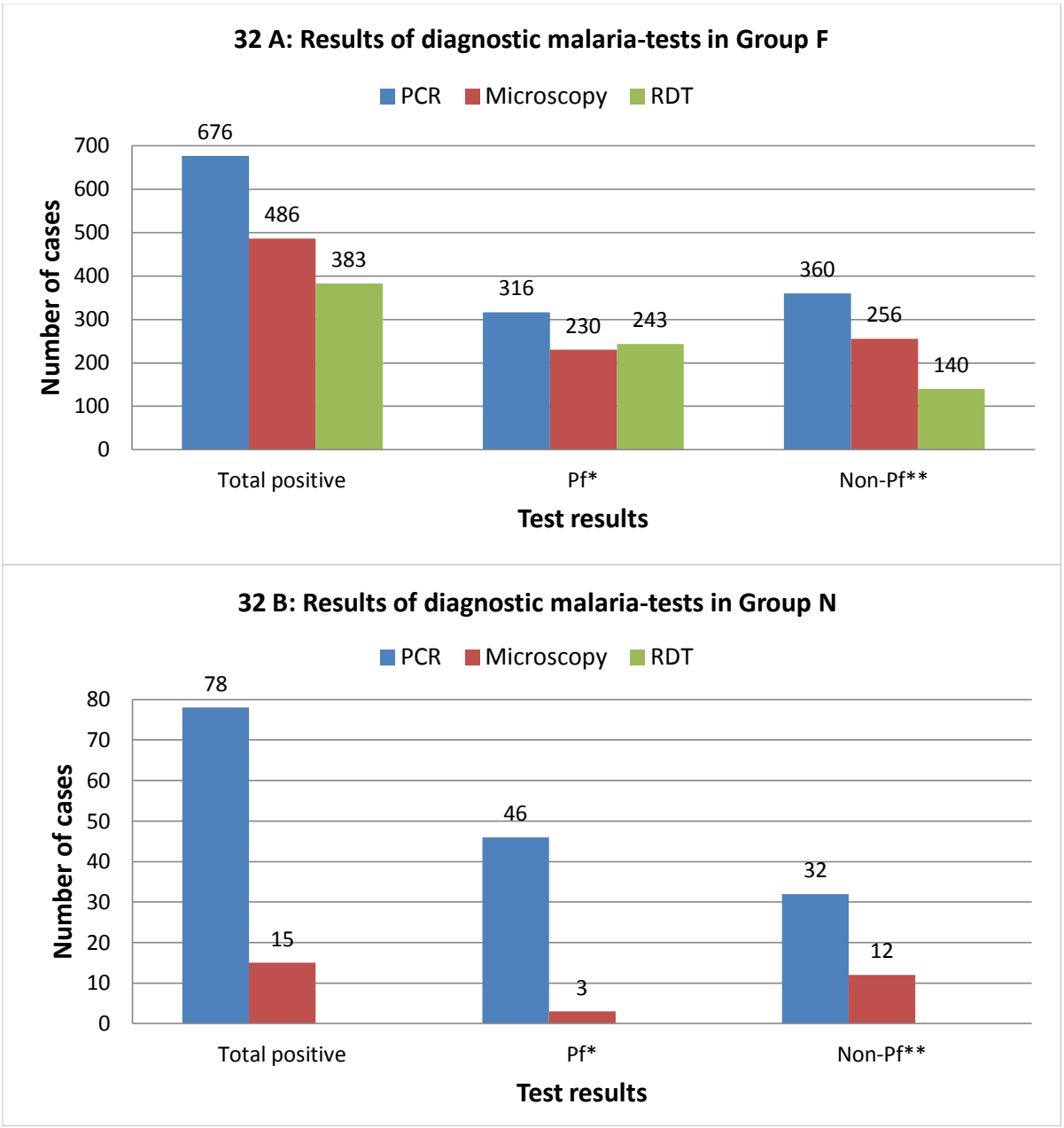


Figure 33: Comparison of the performance of different diagnostic tests for malaria in Group F (A) and Group N (B). (*Pf = *P. falciparum*, including mixed infections with *P. falciparum* and Non-*falciparum*; **Non-Pf = *P. vivax*, *P. ovale*, *P. knowlesi*).

Table 20 shows the detailed results of microscopy and RDT compared to the results of the *cytB*-PCR. For the detection of any malaria parasite in Group F the sensitivity for microscopy was 71.3 %, the specificity was 99.2 %. In the asymptomatic Group N where parasite density was low (see also table 21) sensitivity was only 19.2 %, whereas specificity was 100.0 %. For any *P. falciparum* detection (single or mixed), the sensitivity of microscopy was 59.5 % in Group F and only 2.2 % in Group N. The specificity for *P. falciparum* detection only, was 99.5 % in Group F and 100.0 % In Group N.

RDTs showed a sensitivity of 55.6 % and a specificity of 98.6 % for the detection of any malaria parasite in Group F. In Group N no RDT was positive. For the detection of any *P. falciparum* RDTs showed a sensitivity of 58.9 % and a specificity of 99.4 %.

Table 20: Comparison of results of 3 malaria diagnostic methods in Groups F and N

			Microscopy							RDT*			
	cytB* nPCR		**N**	**PF**	**PV**	**PM**	**PO**	**PF/V**	**PF/M**	**N**	**PF**	**N-PF**	**Mix**
Group F	**N**	**517**	513	4	-	-	-	-	-	510	4	2	1
	PF	**277**	66	134	30	2	-	41	4	89	113	12	63
	PV	**359**	105	17	215	-	1	21	-	183	42	124	10
	PF/V	**39**	22	7	8	-	-	2	-	27	6	2	4
	PK	**1**	1	-	-	-	-	-	-	1	-	-	-
	PO	**0**	-	-	-	-	-	-	-	0	-	-	-
Group N	**N**	**204**	204	-	-	-	-	-	-	-	-	-	-
	PF	**29**	24	1	4	-	-	-	-	-	-	-	-
	PV	**30**	25	1	4	-	-	-	-	-	-	-	-
	PF/V	**17**	14	-	3	-	-	-	-	-	-	-	-
	PK	**1**	-	1	-	-	-	-	-	-	-	-	-
	PO	**1**	-	-	1	-	-	-	-	-	-	-	-
Total		**1,475**	**974**	**165**	**265**	**2**	**1**	**64**	**4**	**1,092**	**165**	**140**	**78**

** N = Negative, PF = P. falciparum, PV = P. vivax, PM = P. malariae, PO = P. ovale, PK = P. knowlesi, N-PF = Non-P. falciparum, Mix= Non-P. falciparum and P. falciparum*

Microscopically evaluated parasite densities for *P. falciparum* mono-infections ranged from 10-364,000 parasites/µl blood, and from 10-116,533 parasites/µl blood for *P. vivax* mono-infections. Details of parasite densities regarding the status of infection are shown in table 21. It was observed that the mean level of parasite density was highest in mono-infections (26,031.8 parasites/µl blood CI 95% [20,175.8; 31,887.9] for *P. falciparum* and 9,474.6 parasites/µl blood CI 95% [7,680.1; 11,269.0] for *P. vivax*) and lowest in the asymptomatic subjects of Group N (2,414.6 parasites/µl blood CI 95% [0; 5,431.0] for *P. falciparum* and 758.2 parasites/µl blood CI 95% [0; 1,774.1] for *P. vivax*).

Table 21: Malaria parasite densities by microscopy regarding infection status [parasites/µl blood]

Status by PCR	*P. falciparum*					*P. vivax*			
	Mono-infection	+*P. vivax*	+Bacterial infection	+Viral infection	Asymptomatic (Group N)	Mono-infection	+Bacterial infection	+Viral infection	Asymptomatic (Group N)
Number of slides	160	16	16	5	5	208	24	10	5
Range of parasite count [p/µl]	10 – 364,000	48 – 160,000	9 – 80,000	15 – 10,200	9 – 8,967	10 – 116,533	60 – 39,882	22 – 13,333	125 – 3,075
Mean parasite count [p/µl]	26,031.8	18,168.0	8,138.8	2,113.0	2,414.6	9,474.6	6,779.5	3,925.1	758.2
CI 95%	20,175.8	0	0	0	0	7,680.1	3,039.7	1,230.8	0
	31,887.9	38,049.5	17,783.7	5,657.5	5,431.0	11,269.0	10,519.4	6,619.4	1,774.1

3.2.2 *Leptospira* species

In Group F, 11.4 % (136/1,193) of the samples showed a positive test result for the *Leptospira 16ssRNA*-nPCR. Interestingly, in Group N 11.0 % (31/282) had a positive test result, too (p = 0.72). The results of PCR and nucleotide sequencing are shown in table 22. Of the 167 positive PCR products, 140 (83.8 %) were identified as DNA from pathogenic *Leptospira* spp. (*L. interrogans*, *L. weilii*, *L. kirschnerii, L. noguchii, L. santarosai*, *L. genomospecies1*) and intermediate *Leptospira* spp. (*L. wolffii, L. kmetyi*) by nucleotide sequencing. The remaining 27 (16.2 %) PCR-products were identified as DNA of the non-pathogenic saprophyte *L. parva*. In total, *L. interrogans* was the most common species (50.9 %) followed by *L. weilii* (28.1 %), none of which was more frequent in Group F or N (p > 0.05).

Table 22: 16SrRNA-nPCR and nucleotide sequencing results in Groups F and N

***16SrRNA*-nPCR-results**

	Total		Group F		Group N		
Result	**n**	**%**	**n**	**%**	**n**	**%**	**p-value†**
Negative	1,308	88.7	1,057	88.6	251	89.0	0.72
Positive	167	11.3	136	11.4	31	11.0	0.72
Total	1,475	100.0	1,193	100.0	282	100.0	

Nucleotide sequencing results of positive PCR products

	Total		Group F		Group N		
Result	**n**	**%**	**n**	**%**	**n**	**%**	**p-value†**
L. parva (non-pathogenic)	27	16.2	24	17.6	3	9.7	0.42
L. interrogans	85	50.9	61	44.9	24	77.4	0.20
L. weilii	47	28.1	44	32.4	3	9.7	0.08
*L. kmetyi**	3	1.8	2	1.5	1	3.2	n/a
L. kirschnerii	1	0.6	1	0.7	0	0.0	n/a
L. noguchii	1	0.6	1	0.7	0	0.0	n/a
L. santarosai	1	0.6	1	0.7	0	0.0	n/a
*L. wolffii**	1	0.6	1	0.7	0	0.0	n/a
L. genomospecies1	1	0.6	1	0.7	0	0.0	n/a
(Total pathogenic *L.*spp.)	(140)	(83.8)	(112)	(82.4)	(28)	(90.3)	0.42
Total	**167**	**100.0**	**136**	**100.0**	**31**	**100.0**	

**Belong to the Leptospira spp. of intermediate pathogenicity*

† P-values stratified by age (≥ 25 years/< 25years) and gender (m/f)

Leptospira spp. were frequently detected simultaneously with various other pathogens, as is shown in table 23. The detection of *Leptospira* as the only pathogen was not significantly more frequent in Group F or Group N (p = 0.15). Simultaneous detection of a second pathogen was especially common with *P. vivax* and *P. falciparum.* In 10 cases *Leptospira* have been observed with 2 other simultaneous pathogens, all with either *P. vivax* or *P. falciparum* and a third pathogen (*O. tsutsugamushi*, dengue virus or influenza virus).

Table 23: Pathogenic Leptospira spp. detection status in Groups F and N

	Total		Group F		Group N		
Detection status	**n**	**%**	**n**	**%**	**n**	**%**	**p-value†**
Mono-detection	64	45.7	45	40.2	19	67.8	0.15
Leptospira + other pathogen(s)	76	54.3	67	59.8	9	32.2	0.15
Total	140	100.0	112	100.0	28	100.0	

† P-values stratified by age (≥ 25 years/< 25years) and gender (m/f)

3.2.3 *Rickettsia* species

The detection of rickettsial DNA in the given study setting was problematic. The results obtained with the 2 nested PCR essays (*ompB* and *gltA*) were not consistently reproducible. Subsequently an exchange of DNA samples with the study partners of the Wellcome Trust in Vientiane, Lao PDR was arranged for external quality control, which revealed problems with the quality of the stored DNA-samples from Cambodia. It is likely that the method of DNA extraction from frozen packed blood cells only provided very low concentrations of rickettsial DNA, at the detection limits of the assays. Furthermore, it is probable that the repeated thawing of the extracted DNA added to a degradation of its quality and thus made it difficult to reproduce the results. Moreover, the nucleotide sequencing of the very short *gltA*-PCR-product (74 bp) was infrequently successful. In an attempt to improve the results a realtime PCR strategy was employed (Paris *et al.*, 2008). Using this protocol, it was not possible to detect any of the previously positive samples, even though valid data was acquired with the positive controls (DNA of *R. conorii* and *R. typhii* from Lao PDR). In conclusion, the approach used in this study was not adequate for the difficult diagnostic of rickettsial infections. Nevertheless, in 3 cases the *gltA*-nPCR was confirmed in a second run.

3.2.4 *Orientia tsutsugamushi*

The nested PCR for the *47kDa*-gene of *O. tsutsugamushi* was positive in 3.7 % of all samples (54/1,475). There was no significant difference between the prevalence in Group F (3.9 %) and Group N (2.5 %), as shown in table 24 (p = 0.48). In 16 cases a second pathogen (5 *P. falciparum*, 7 *P. vivax*, 3 *Leptospira* spp., 1 *E. cloacae*) was detected simultaneously to *O. tsutsugamushi.* In 6 cases, 2 additional pathogens were detected (1 *P. vivax* with influenza virus, 1 *P. vivax* with *P. falciparum*, 1 *P. vivax* with *Leptospira* spp., 3 *P. falciparum* with *Leptospira* spp.) and in 1 case even 3 simultaneous pathogens were identified (*P. vivax*, *P. falciparum* and dengue virus type 4). Table 25 shows the status of detection of *O. tsutsugamushi,* alone or with other pathogens. Neither status was significantly more common in one of the 2 study groups (p = 0.92).

Table 24: 47kda-PCR and nucleotide sequencing results in Groups F and N

***47kDa*-nPCR + nucleotide sequencing result**	**Total**		**Group F**		**Group N**		
	n	**%**	**n**	**%**	**n**	**%**	**p-value†**
Negative	1421	96.3	1146	96.1	275	97.5	0.48
O. tsutsugamushi	54	3.7	47	3.9	7	2.5	0.48
Total	1475	100.0	1193	100.0	282	100.0	

† P-values stratified by age (≥ 25 years/< 25years) and gender (m/f)

Table 25: O. tsutsugamushi detection status in Groups F and N

	Total		**Group F**		**Group N**		
Detection status	**n**	**%**	**n**	**%**	**N**	**%**	**p-value†**
Mono-detection	31	57.4	25	53.2	6	85.7	0.92
O. tsutsugamushi + other pathogen(s)	23	42.6	22	46.8	1	14.3	0.92
Total	54	100.0	47	100.0	7	100.0	

† P-values stratified by age (≥ 25 years/< 25years) and gender (m/f)

3.2.5 Dengue virus

Of 1,473 tested samples 5.4 % had a positive test result in the multiplex RT-PCR essay for dengue virus. As shown in table 26, 6.3 % of Group F subjects had a positive test result,

which was significantly more frequent than in Group N, in which 1.8 % of test results were positive (p = 0.01).

Table 26: Multiplex dengue virus RT- and serotype-specific PCR results

Dengue RT-PCR result

	Total		Group F		Group N		
Results	**N**	**%**	**n**	**%**	**n**	**%**	**p-value†**
Negative	1,393	94.6	1,117	93.7	276	98.2	0.01
Positive	80	5.4	75	6.3	5	1.8	0.01
Total	1,473	100.0	1,192	100.0	281	100.0	

Serotype-specific PCR results

	Total		Group F		Group N		
Results	**N**	**%**	**n**	**%**	**n**	**%**	**p-value†**
DENV- 1	22	27.5	21	28.0	1	20.0	0.23
DENV- 1 and -2	1	1.2	1	1.3	0	0.0	n/a
DENV- 2	44	55.0	41	54.6	3	60.0	0.09
DENV- 3	5	6.3	4	5.3	1	20.0	0.77
DENV- 4	8	10.0	8	10.6	0	0.0	0.38
Total	80	100.0	75	100.0	5	100.0	

† P-values stratified by age (≥ 25 years/< 25years) and gender (m/f); n/a not applicable

Overall; the most prevalent serotype was DENV-2 (55.0 %) followed by DENV-1 (27.5 %), DENV-4 (10.0 %) and DENV-3 (6.3 %). In 1 sample DENV-1 and -2 were detected simultaneously. Dengue viruses were also detected simultaneously with other pathogens, particularly with malaria parasites (6 *P. falciparum*, 21 *P. vivax*, 4 both), but also with *Leptospira* spp. (n = 5) and influenza A virus (n = 1).

3.2.6 Influenza virus

Of 1,475 tested throat-swab samples 7.7 % were positive for influenza virus (5.9 % influenza A virus, 1.8% influenza B virus). Further sub-typing of influenza A viruses by real-time PCR was conducted on 58 out of 87 samples and revealed that type H3N2 was the most prominent sub-type (35.6 %), followed by H1N1 (28.7 %) and H3N3 (2.3 %). As shown

in table 27, the prevalence of influenza viruses was significantly higher in the Group F than in the Group N ($p < 0.01$). Influenza A virus was more frequently detected in Group F (7.0 %) than in Group N (1.4%) ($p = 0.01$). Influenza B virus was not significantly more commonly detected in one group (Group F 1.8 %, Group N 1.1 %) ($p = 0.77$).

Table 27: Influenza virus RT-PCR and influenza A sub-typing results in Groups F and N

Influenza virus RT-PCR results

	Total		Group F		Group N		
Results	**n**	**%**	**n**	**%**	**n**	**%**	**p-value†**
Negative	1,362	92.3	1,087	91.1	275	97.5	<0.01
Influenza B	26	1.8	23	1.9	3	1.1	0.77
Influenza A	87	5.9	83	7.0	4	1.4	0.01
Total	1,475	100.0	1,193	100.0	282	100.0	

Influenza A sub-typing real-time PCR results

	Total		Group F		Group N		
Results	**N**	**%**	**n**	**%**	**n**	**%**	**p-value†**
Subtype H1N1	25	28.7	24	28.9	1	25.0	0.18
Subtype H3N2	31	35.6	31	37.3	0	0.0	0.05
Subtype H3N3	2	2.3	2	2.4	0	0.0	n/a
No sub-typing	29	33.3	26	31.3	3	75.0	
Total	87	100.0	83	100.0	4	100.0	

† P-values stratified by age (≥ 25 years/< 25years) and gender (m/f), n/a not applicable

3.2.7 Bacteria from blood culture

The Bacteriology Unit at IPC evaluated 1,128 blood cultures from the 1,193 febrile patients in this study. In 65 cases no blood culture bottles were available in the field. The results are presented in table 28. Positive bacterial growth was observed in 9.5 % of the bottles, but in 8.7 % of these only floral bacteria, likely to be contamination, were identified (*Staph. non-aureus, Corynebacteria* spp., *Polymicrobical flora, Pseudomonas fluorescens* and *Pseudomonas putida)*. The growth of pathogenic bacteria was only observed in 9 cases (0.8 %) of these bottles and was considered as evidence of community acquired septicemia (CAS). Among these patients, the bacteria identified included *S. pneumoniae* (n = 1), *E. coli* (n = 1), *E. cloacae* (n = 5), *S. typhi* (n = 1) and *S. paratyphi* (n = 1). In 2 cases CAS (*E. cloacae*

and *S. paratyphi)* was associated with *P. vivax*. These results were immediately reported back to the health center, which was thus able to contact the patient and prescribe the corresponding treatment.

Table 28: Blood culture results after 1 week of incubation (Group F only)

Blood culture result	**n**	**%**
Negative	1,021	90.5
Positive	107	9.5
Total number of blood cultures	1,128	100.0
Growth of pathogenic bacteria		
Streptococcus pneumoniae	1	0.1
Enterobacter cloacae	5	0.4
Escherichia coli	1	0.1
Salmonella paratyphi	1	0.1
Salmonella typhi	1	0.1
Total	9	0.8
Growth of bacteria considered as contamination		
Staphylococcus non-aureus	53	4.7
Corynebacteria spp.	38	3.4
Polymicrobical flora	5	0.4
Pseudomonas fluorescens	1	0.1
Pseudomonas putida	1	0.1
Total	98	8.7

3.2.8 Established diagnoses of malaria-RDT negative fever cases

Upon consideration of the malaria RDT results in this study, illustrated in figure 34, one can see that only 32.1 % of the 1,193 febrile patients were malaria-RDT positive and 67.9 % were RDT-negative. In 37.0 % of these RDT-negative cases malaria parasites were detected using *cytB*-PCR as diagnostic method. Of these PCR-positive samples 43.6 % were identified as *P. vivax*, 32.0 % *Plasmodium* spp. plus a second pathogen (which means mostly low malaria-parasitemia), 18.3 % *P. falciparum* and 6.0 % mixed or other *Plasmodium* spp. (*P. knowlesi*, *P. ovale*). In 24.9 % of the RDT-negative cases, that were also negative for malaria by PCR, another pathogen was detected in the blood sample, namely influenza viruses (32.7 %), pathogenic *Leptospira* spp. (22.3 %), dengue virus (20.7 %),

O. tsutsugamushi (12.4 %), and in 11.9 % there was evidence of multiples of the above mentioned pathogens or CAS. In the remaining 38.1 % of the RDT-negative cases no pathogen could be detected, which indicates the need of further testing (see also discussion point 4.4.2).

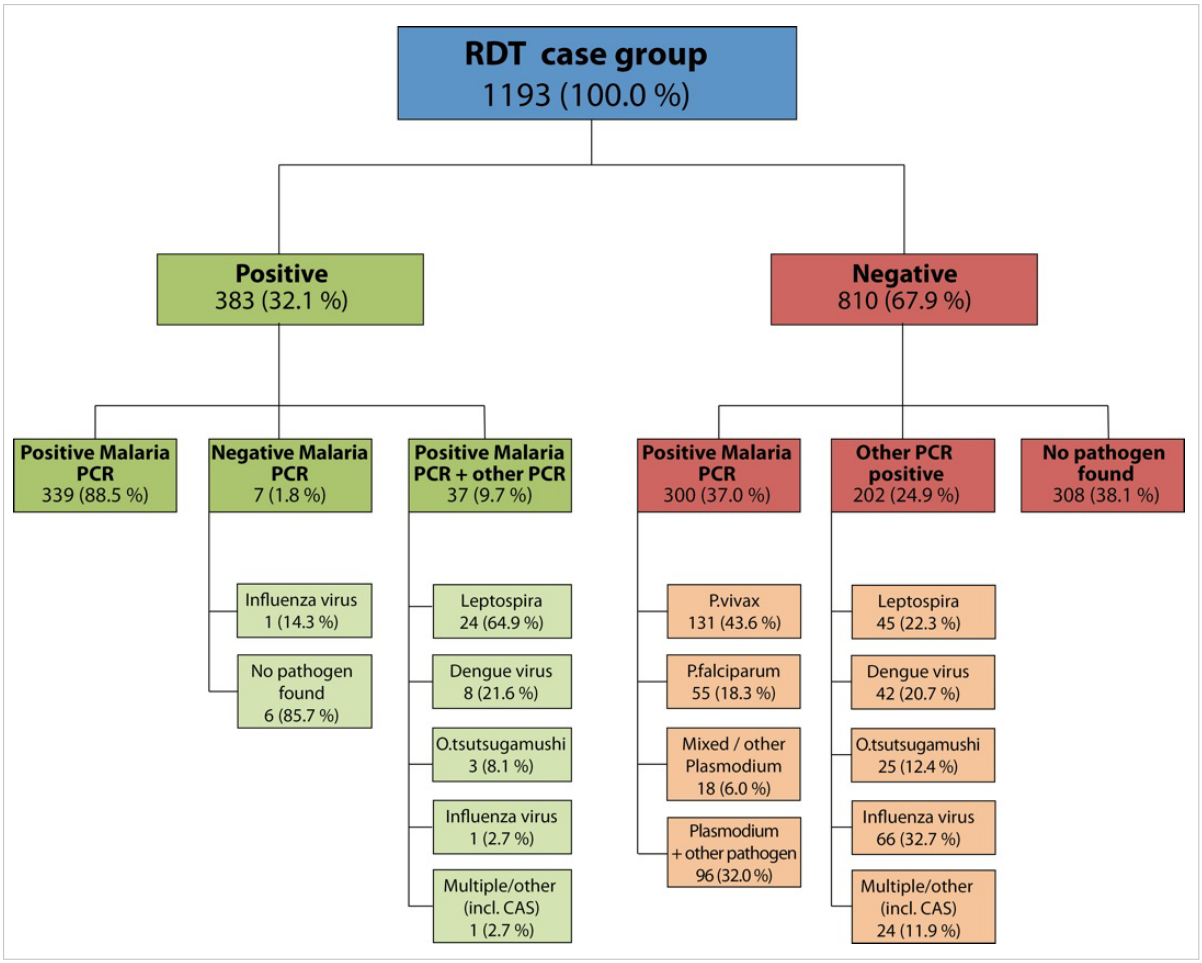


Figure 34: Study results dependant on malaria-RDT-status. In green: RDT positive cases (32.1 %); In red: RDT-negative cases (67.9 %) and the corresponding PCR results.

3.3 Seasonal and geographical distribution of detected pathogens

3.3.1 Seasonal trends

Some of the detected pathogens, like malaria parasites or dengue virus, showed distinct seasonal patterns in their prevalence, whereas others (like *Leptospira* spp.) seem to be

equally present year round. As shown in figure 35, the highest numbers of malaria parasites were detected during the rainy season from July to September, when the breeding conditions for the vector (*Anopheles dirus*) are optimal. Furthermore, an overall decrease of malaria case numbers can be observed, from 302 cases in 2008 and 301 cases in 2009, to 122 cases in 2010.

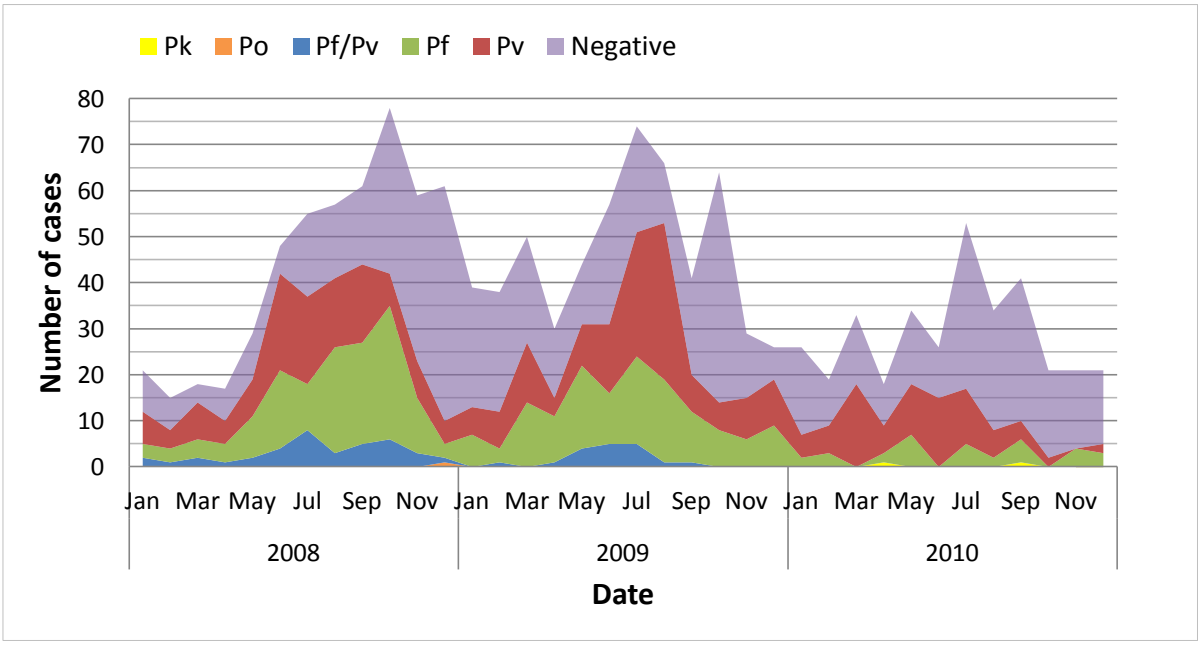


Figure 35: Seasonal prevalence of *Plasmodium* spp. prevalence in the study population from January 2008 to December 2010 (Pv = *P. vivax*, Pf = *P. falciparum*, Po = *P. ovale*, Pk = *P. knowlesi*).

Concordant with the national surveillance data, an annual epidemic wave of dengue virus can be clearly distinguished, with an increase of case numbers annually from May to October shown in figure 36. For influenza viruses the seasonal trend was also concordant with the national surveillance data, with an annual increase in prevalence from October to January as shown in figure 37.

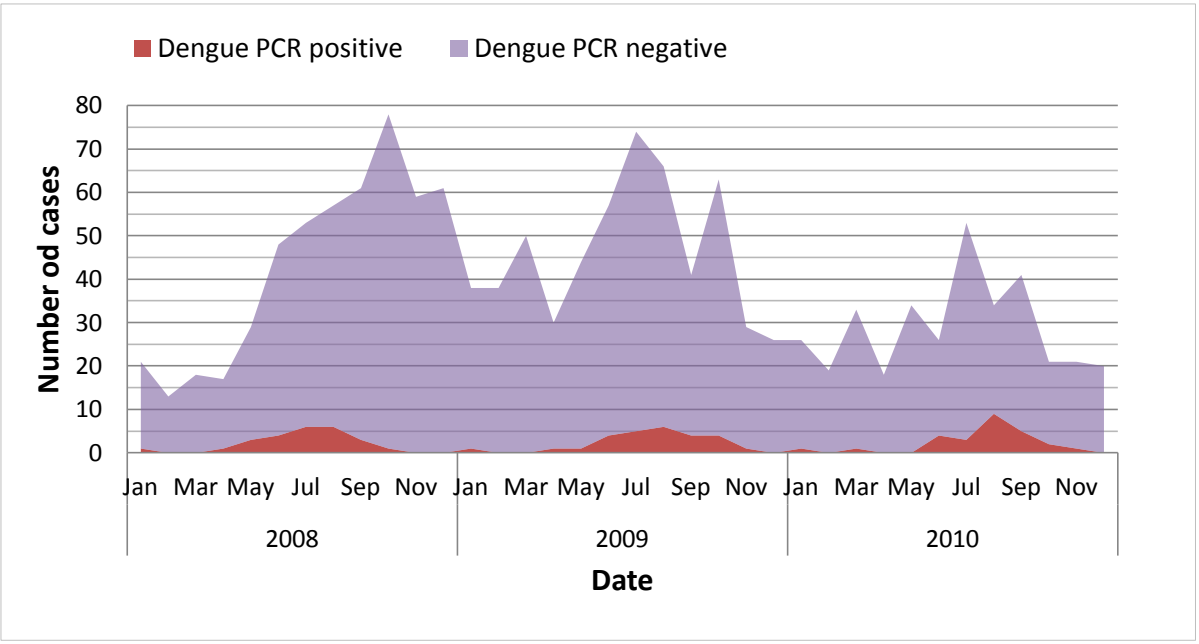


Figure 36: Seasonal prevalence of dengue virus in the study population from January 2008 to December 2010.

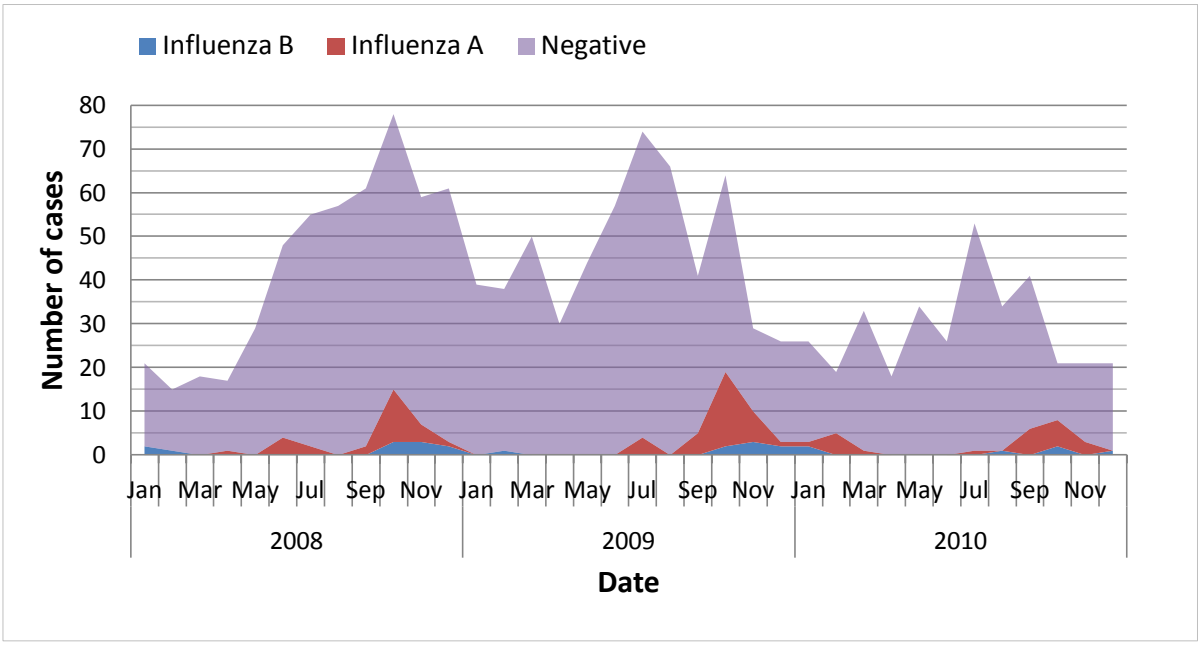


Figure 37: Seasonal prevalence of influenza virus A and B in the study population from January 2008 to December 2010.

For pathogenic *Leptospira* spp. there was no particular seasonal trend observable, indicating that they are present year round. The peaks shown in figure 38 could indicate

small regional outbreaks. Similarly, *O. tsutsugamushi* and *Rickettsia* spp. did not show any particularities in their seasonal prevalence.

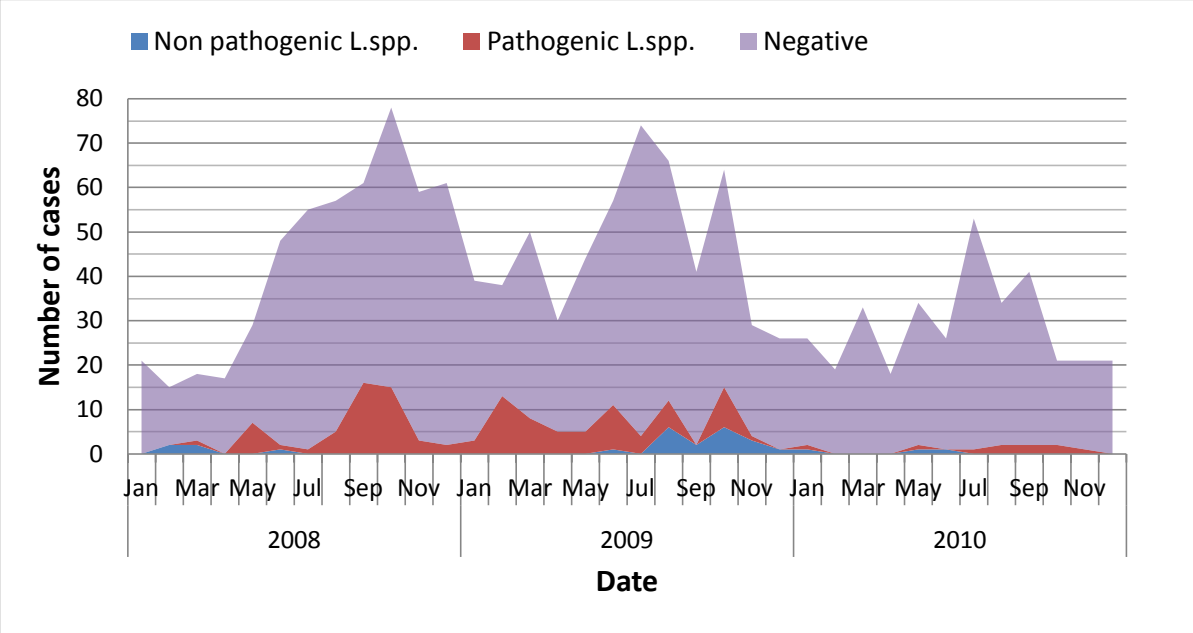


Figure 38: Seasonal prevalence of pathogenic and non-pathogenic *Leptospira* spp. (L. spp.) in the study population from January 2008 to December 2010

3.3.2 Geographical distribution

As explained above, 2 of the study sites were chosen in western Cambodia (site C-1 and C-2), whereas the third one was located in eastern Cambodia (site C-3). The sites C-1 and C-2 recruited 621 and 650 subjects respectively, whereas site C-3 in Snoul recruited only 204 individuals. The comparison of the distribution of malaria parasites between the 3 sites, shown in figure 39, revealed that site C-1 showed the lowest percentage of malaria positive cases, whereas the highest percentage was detected in site C-3 ($p< 0.01$). In sites C-1 and C-2 *P. vivax* was more prevalent compared to site C-3, where *P. falciparum* accounted for the majority of positive cases ($p < 0.01$). Interestingly, site C-3 is located in a province classified as low-transmission-area for malaria, whereas sites C-1 and C-2 are located in an area of high transmission. Both of the detected *P. knowlesi* positive samples came from site C-2, the only *P. ovale* positive sample from site C-3.

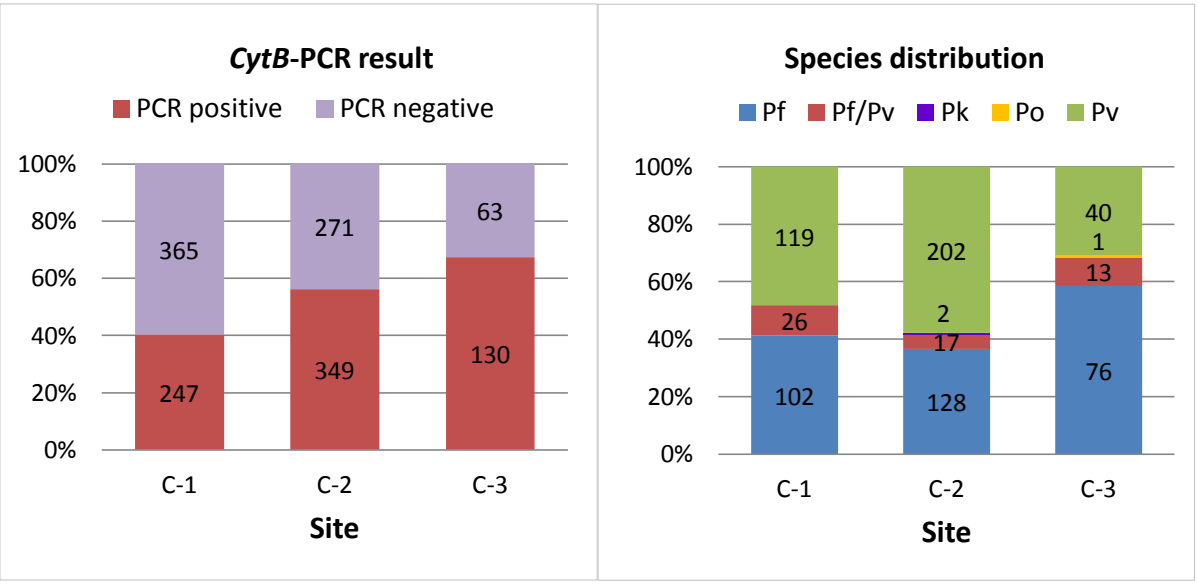


Figure 39: Geographical distribution of malaria parasite prevalence and species (Pv = *P. vivax*, Pf = *P. falciparum*, Po = *P. ovale*, Pk = *P. knowlesi*) in the 3 study sites (C-1-3).

As shown in figure 40, the prevalence of *Leptospira* spp. was around 10.0 % in all of the 3 study sites ($p = 0.42$). Some differences could be observed in the species distribution. In site C-3 *L. interrogans* was the uniquely detected species, whereas most of the less frequent species (*L. wolffii, L. santosaraii, L. kirschnerii, L. genomospecies1, L. kmetyi* and *L. noguchii)* were detected in site C-1 only ($p < 0.01$). The non-pathogenic saprophyte *L. parva* was detected in 27 samples, most of them from site C-1 ($p < 0.01$).

The distribution of dengue virus was similar in the 3 sites ($p = 0.57$) as shown in figure 41. However, it was observed that in site C-3 no dengue virus serotype 4 was detected and that in all 3 sites dengue virus serotype 2 was the most frequently detected serotype. There were no statistically significant differences in the serotype distribution between the 3 sites (DENV-1 $p = 0.78$, DENV-2 $p = 0.08$, DENV-3 $p = 0.40$, DENV-4 $p = 0.08$).

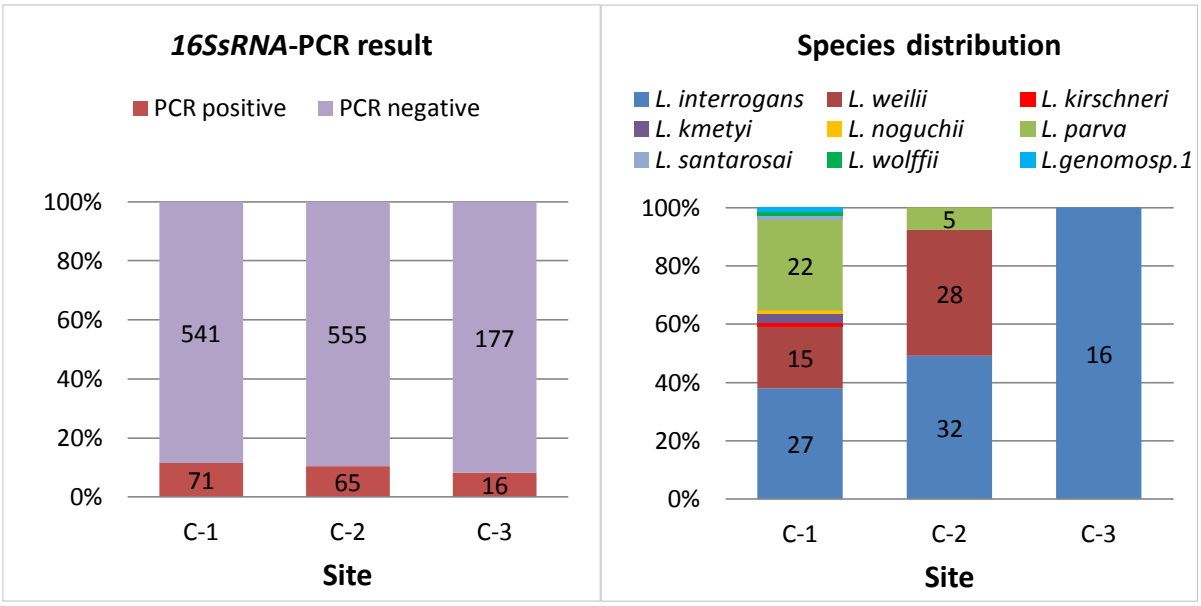


Figure 40: Geographical distribution of *Leptospira* prevalence and species distribution in the 3 study sites (C-1-3).

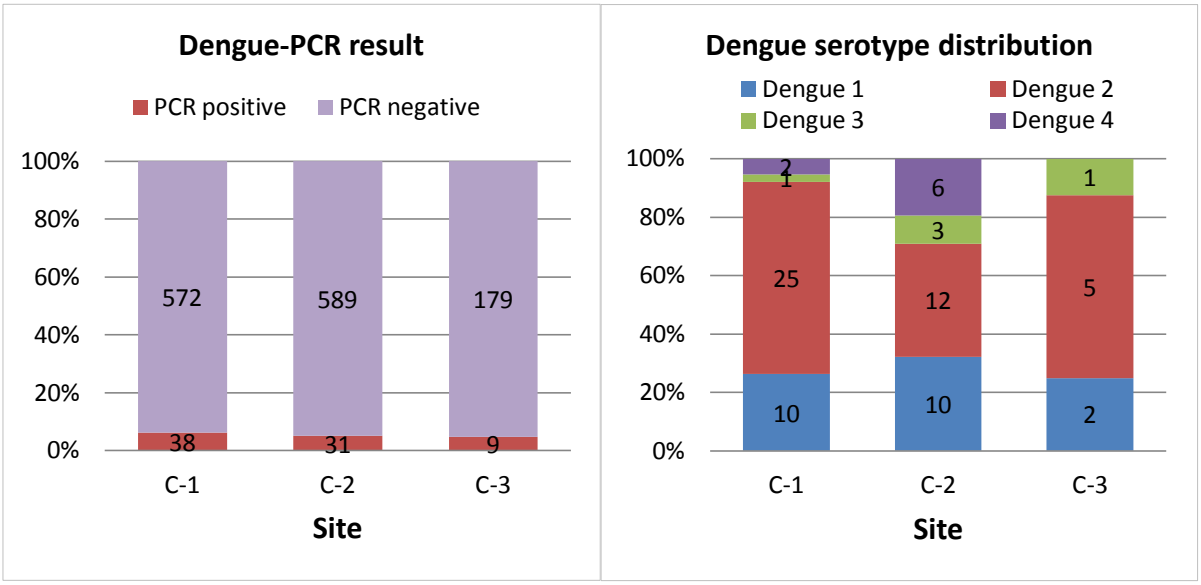


Figure 41: Geographical distribution of dengue virus prevalence and serotypes (dengue 1-4) in the 3 study sites (C-1-3).

Influenza viruses were most frequently detected in site C-1 ($p < 0.01$), as shown in figure 42. In C-1 over 80.0 % of the detected influenza viruses were influenza A viruses, whereas in C-3 almost 50.0 % were influenza B ($p = 0.04$). Since the sub-typing was not available for all detected influenza A viruses it is not possible to compare the distribution of subtypes between the 3 study sites.

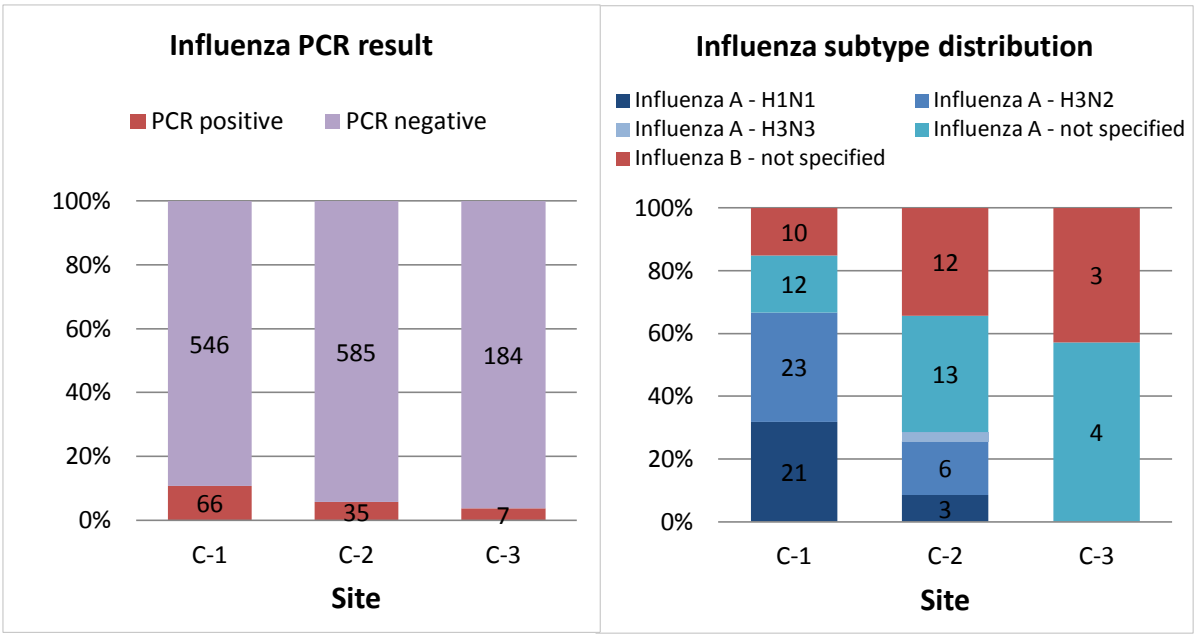


Figure 42: Geographical distribution of influenza virus prevalence and virus subtype distribution in the 3 study sites (C-1-3).

3.4 Association of laboratory results with clinical findings

3.4.1 Fever and additional symptoms

The mean duration of fever in the Group F was 2.6 days (range 1-10 days) and the mean fever temperature was 38.8 °C (range 37.8-42.2 °C), as shown in figure 43 (left). The most frequently reported symptoms in addition to fever were sore throat (41.7 %), cough (39.9 %), running nose (17.6 %), diarrhea (15.4 %) and vomiting (14.4 %), as shown in figure 43 (right). Table 29 shows the recorded symptoms corresponding to the pathogens detected in the laboratory. Respiratory symptoms like cough, sore throat and running nose were significantly more frequent when influenza virus A or B was detected. Other than that, no statistically significant difference of the distribution of symptoms was observed for the different pathogens detected by PCR.

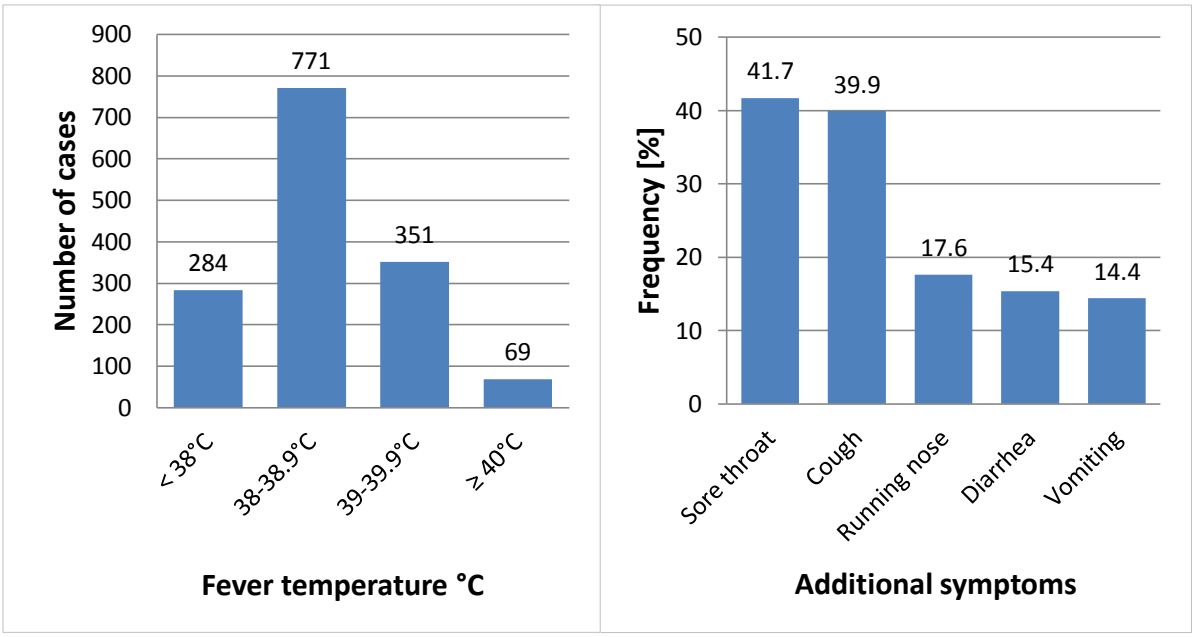


Figure 43: Left: Number of cases by measured body temperature [°C] in study population; Right: Frequency of additional symptoms to the fever [%].

Table 29: Recorded clinical symptoms matched with the different pathogens detected in the laboratory (Group F only)

Laboratory result	Clinical symptoms (multiple response was possible)							
	Cough	Sore throat	Running nose	Diarrhea	Vomiting	Pain Urinating	Rash	Ear-Ache
Malaria parasites (n = 676)	**37.7 %** [34.1; 41.4] (n = 255)	**38.5 %** [34.8; 42.1] (n = 260)	**17.5 %** [14.6; 20.3] (n = 118)	**18.3 %** [15.4; 21.3] (n = 124)	**15.1 %** [12.4; 17.8] (n = 102)	**3.0 %** [1.7; 4.2] (n = 20)	**1.2 %** [0.4; 2.0] (n = 8)	**3.8 %** [2.4; 5.3] (n = 26)
Pathogenic *Leptospira* (n = 111)	**40.5 %** [31.4; 49.7] (n = 45)	**51.4 %** [42.1; 60.6] (n = 57)	**20.7 %** [13.2; 28.3] (n = 23)	**18.9 %** [11.6; 26.2] (n = 21)	**20.7 %** [13.2; 28.3] (n = 23)	**2.7 %** [0.0; 5.7] (n = 3)	**0.9 %** [0.0; 2.7] (n = 1)	**3.6 %** [0.1; 7.1] (n = 4)
***O. tsutsu-gamushi* (n = 47)**	**57.4 %** [43.3; 71.6] (n = 27)	**55.3 %** [41.1; 69.5] (n = 26)	**19.1 %** [7.9; 30.4] (n = 9)	**36.2 %** [22.4; 49.9] (n = 17)	**19.1 %** [7.9; 30.4] (n = 9)	**4.3 %** [0.0; 10.0] (n = 2)	**2.1 %** [0.0; 6.3] (n = 1)	**12.8 %** [3.2; 22.3] (n = 6)
Dengue virus (n = 75)	**37.3 %** [26.4; 48.3] (n = 28)	**53.3 %** [42.0; 64.6] (n = 40)	**20.0 %** [10.9; 29.1] (n = 15)	**29.3 %** [19.0; 39.6] (n = 22)	**24.0 %** [14.3; 33.7] (n = 18)	**2.7 %** [0.0; 6.3] (n = 2)	**2.7 %** [0.0; 6.3] (n = 2)	**2.7 %** [0.0; 6.3] (n = 2)
Influenza A or B virus (n = 106)	**87.7 %** [81.5; 94.0] (n = 93)	**88.7 %** [82.6; 94.7] (n = 94)	**51.9 %** [42.4; 61.4] (n = 55)	**10.4 %** [4.6; 16.2] (n = 11)	**19.8 %** [12.2; 27.4] (n = 21)	**3.8 %** [0.1; 7.4] (n = 4)	**3.8 %** [0.1; 7.4] (n = 4)	**11.3 %** [5.3; 17.4] (n = 12)
Cultured bacteria (n = 9)	**33.3 %** [2.5; 64.1] (n = 3)	**55.6 %** [23.1; 88.0] (n = 5)	**11.1 %** [0.0; 31.6] (n = 1)	**22.2 %** [0.0; 49.4] (n = 2)	**55.6 %** [23.1; 88.0] (n = 5)	**0.0 %** (n = 0)	**0.0 %** (n = 0)	**11.1 %** [0.0; 31.6] (n = 1)

Age group and gender have been compared to the laboratory diagnosis in tables 30 and 31. All types of pathogens were mainly detected in the age groups 10-19 and 20-29 years. Viral infections (dengue and influenza virus) were more frequently detected in the age group 10-19 years than all other pathogens. Malaria parasites were more frequently detected in male study subjects (p = 0.02), whereas dengue virus was more frequently detected in females (p = 0.01). For the remaining pathogens no differences in gender distribution were observed.

Table 30: Age group distribution compared to pathogens detected in the laboratory (Groups F and N)

		Age group					
		< 10 years	**10-19 years**	**20-29 years**	**30-39 years**	**40-49 years**	**≥ 50 Years**
Laboratory results	**Malaria parasites (n = 754)**	**3.7 %** [2.4; 5.1] (n = 28)	**32.4 %** [29.0; 35.7] (n = 244)	**34.4 %** [31.0; 37.7] (n = 259)	**14.9 %** [12.3; 17.4] (n = 112)	**12.9 %** [10.5; 15.3] (n = 97)	**1.9 %** [0.9; 2.8] (n = 14)
	Pathogenic *Leptospira* spp. (n = 140)	**2.1 %** [0.0; 4.5] (n = 3)	**31.4 %** [23.7; 39.1] (n = 44)	**31.4%** [23.7; 39.1] (n = 44)	**18.6 %** [12.1; 25.0] (n = 26)	**12.9 %** [7.3; 18.4] (n = 18)	**3.6 %** [0.5; 6.6] (n = 5)
	***O. tsutsugamushi* (n = 54)**	**3.7 %** [0.0; 8.7] (n = 2)	**27.8 %** [15.8; 39.7] (n = 15)	**38.9 %** [25.9; 51.9] (n = 21)	**14.8 %** [5.3; 24.3] (n = 8)	**14.8** % [5.3; 24.3] (n = 8)	**0.0 %** (n = 0)
	Dengue virus (n = 80)	**8.8 %** [2.6; 14.9] (n = 7)	**50.0 %** [39.0; 61.0] (n = 40)	**28.8 %** [18.8; 38.7] (n = 23)	**6.3 %** [0.9; 11.6] (n = 5)	**3.8 %** [0.0; 7.9] (n = 3)	**2.5 %** [0.0; 5.9] (n = 2)
	Influenza A and B virus (n = 113)	**6.2 %** [1.8; 10.6] (n = 7)	**59.3 %** [50.2; 68.4] (n = 67)	**20.4 %** [12.9; 27.8] (n = 23)	**6.2 %** [1.8; 10.6] (n = 7)	**6.2 %** [1.8; 10.6] (n = 7)	**6.2 %** [1.8; 10.6] (n = 7)
	Cultured bacteria (n = 9)	**11.1 %** [0.0; 31.6] (n = 1)	**11.1 %** [0.0; 31.6] (n = 1)	**66.7 %** [35.9; 97.5] (n = 6)	**0.0 %** (n = 0)	**11.1 %** [0.0; 31.6] (n = 1)	**0.0 %** (n = 0)

Table 31: Gender distribution compared to pathogens detected in the laboratory (Groups F and N)

		Gender		
		Female (n = 388)	**Male (n = 765)**	**p-value**
Laboratory results	**Malaria parasites (n = 754)**	60.8 % (n = 236)	67.7 % (n = 518)	0.02
	Pathogenic *Leptospira* spp. (n = 140)	13.6 % (n = 53)	11.4 % (n = 87)	0.26
	***O. tsutsugamushi* (n = 54)**	4.1 % (n = 16)	4.9 % (n = 38)	0.52
	Dengue virus (n = 80)	9.5 % (n = 37)	5.6 % (n = 43)	0.01
	Influenza A and B virus (n = 113)	10.8 % (n = 42)	9.3 % (n = 71)	0.41
	Cultured bacteria (n = 9)	0.7 % (n = 3)	0.8 % (n = 6)	0.64

3.4.2 Clinical diagnosis and treatment

The most frequent presumptive clinical diagnoses established at health center level were upper ARI (51.3 %) and malaria (35.6 %). Furthermore, typhoid fever and diarrhea accounted for 6.6 % and 4.8 % of the clinical diagnoses respectively. The remaining 1.7 % of patients were diagnosed with lower ARI and other diagnoses (“breast swollen”, “neck swollen”, sexually transmitted disease, oral infection). The most commonly prescribed drugs were amoxicillin (n = 449), followed by anti-malarials (mefloquine + artemisinin, dihydroartemisinin + piperaquin, chloroquine, duocotexin) (n = 424). Other frequently prescribed antibiotics were penicillin, ampicillin, cotrimoxazol and metronidazol. Less frequently, therefore grouped as “other antibiotics” in table 37, were erythromycin, aminomycin, ofloxacin, ciprofloxacin, cloxacillin, and nalicixic acid. Paracetamol as additional treatment was prescribed in over 95.0 % of the cases, independent of the diagnosis. Only around 1.0 % of the patients didn’t receive any treatment or it was not recorded correctly on the form. The clinical diagnosis compared with the laboratory diagnosis is shown in table 32 and the prescribed treatment compared with the laboratory diagnosis is shown in table 33. These tables show that 61.9 % of the malaria infections detected by PCR (419/676) were captured by the clinical diagnosis at the health center. In

42 of the clinically diagnosed malaria cases an additional pathogen was detected by PCR. The majority (68.5 %) of the laboratory confirmed malaria cases were treated with anti-malarial drugs. Most of the cases with non-malaria pathogens were treated with amoxicillin, which can be used for mild forms of leptospirosis [35], but is not effective against *O. tsutsugamushi* and *Rickettsia* spp.. Of all detected cases of *Leptospira* spp., 57.7 % received an efficient antibiotic treatment whereas *Rickettsia* spp. or *O. tsutsugamushi* cases did not receive efficient drugs at all. Furthermore, all of the detected viral infections (dengue and influenza virus) were indiscriminately treated with antibiotics and paracetamol.

Table 32: Clinically established diagnosis compared to the laboratory established diagnosis (Group F only)

		Clinical diagnosis						
		Malaria	Upper ARI*	Lower ARI**	Typhoid fever	Diarrhea/Dysentery	Others***	Total of cases [n (%)]
Laboratory diagnosis	Malaria parasites	377	132	1	17	16	1	543 (45.5)
	Malaria parasites + other pathogen	42	73	1	11	5	0	133 (11.1)
	Leptospira spp.	1	32	1	7	4	0	45 (3.7)
	O. tsutsugamushi	0	21	0	1	3	0	25 (2.1)
	Rickettsia spp.	0	1	0	0	0	0	1 (0.1)
	Dengue virus	0	33	0	7	2	0	42 (3.5)
	Influenza A and B virus	1	62	1	1	0	1	66 (5.5)
	Cultured bacteria	0	4	1	0	0	0	5 (0.1)
	Multiple pathogens (excl. malaria parasites)	0	8	0	3	0	1	12 (1.0)
	No pathogen detected	**8**	**246**	**4**	**32**	**27**	**4**	**321 (26.9)**
	Total of cases (n)	**429**	**612**	**9**	**79**	**57**	**7**	**1193 (100.0)**

** Incl. pharyngitis, rhinitis, flu, viral infection, ear infection*

*** Incl. cough with blood, pneumonia*

**** Breast swollen, neck swollen, sexually transmitted diseases, oral infection*

Table 33: Laboratory diagnosis and prescribed treatment at health center level (Group F only)

		Prescribed treatment									
		Anti-malarials*	Amoxicillin	Penicillin/Ampicillin	Cotrimoxazol	Metronidazol/Mebendazol	Other Antibiotics**	Paracetamol (add.)	None (or not recorded)	Total of cases (n)	**Effective treatment (%)**
Laboratory diagnosis	Malaria parasites	372	104	15	26	21	5	523	8	543	**68.5**
	Malaria parasites + other pathogen	42	49	10	17	8	7	129	0	133	-
	Leptospira spp.	2	22	3	11	3	1	44	1	45	**57.7**
	O. tsutsugamushi	0	15	3	3	3	1	25	0	25	**0.4**
	Rickettsia spp.	0	0	1	0	0	0	1	0	1	**0.0**
	Dengue virus	0	24	3	9	4	2	42	0	42	-
	Influenza A and B virus	0	51	7	5	0	2	64	1	66	-
	Cultured bacteria	0	3	1	0	0	0	4	1	5	-
	Multiple pathogens (excl. malaria parasites)	0	6	1	3	1	1	12	0	12	-
	No pathogen detected	8	175	28	64	26	14	315	1	321	-
	Total of treatment	424	449	72	138	66	41	1160	11	1193	

** Mefloquine+artemisinin, dihydroartemisinin+piperaquin, chloroquine, duocotexin*

*** Erythromycin, aminomycin, ofloxacin, ciprofloxacin, cloxacillin, nalicixic acid*

4 Discussion

4.1 Applied methods

4.1.1 Study sites and sample size

In all of the 3 sites the recruitment of the required sample size took longer than expected. Initially it was planned to recruit 450 febrile subjects (Group F) and 150 non-febrile subjects (Group N) in each site in 2 years. After the extension of the study duration for 1 additional year, study site C-1 had recruited 452 febrile subjects and 170 non-febrile subjects, study site C-2 recruited 587 febrile and only 75 non-febrile subjects, and study site C-3 recruited only 164 febrile and 38 non-febrile subjects. Various factors contributed to this delay in recruitment. Firstly the number of visitors of the health centers was overestimated. All 3 health centers were located more or less close to a referral hospital which might have been consulted preferably. Furthermore, irregular opening hours in consequence of staff shortage at the health centers added to the low recruitment numbers. In addition, Cambodian people are generally reluctant to give blood samples, especially when they are not actually sick, therefore difficulties occurred to recruit an independent asymptomatic Group N. In study site C-3 (Snoul) the situation was especially problematic since this was the first time that recruitment for scientific purposes took place in this health center, in comparison to C-1 and C-2 where numerous research projects have been carried out already.

Another issue concerning the 3 study sites is a possible selection bias that cannot be completely ruled out. Since the health centers and their staff are supported and trained mainly by the CNM they became known to be specialized in malaria diagnostics and treatment. Thus it is conceivable that patients, who suspect that they may have malaria, primarily use the health centers as a first address for consultation, whereas patients who suspect they suffer from another or a more severe disease would primarily consult in a referral hospital or in the private sector. This would be an explanation for the high rate of malaria cases in this study population which will be discussed below in detail. Initially, the

study was planned in this setting because of the significant reduction of malaria in recent years in this area.

4.1.2 Diagnostic methods

4.1.2.1 Sample processing and quality control

One of the study's aims was to find a study protocol that is simple and suitable to be standardized, thus it could be used in other settings and countries. The idea was to have a set of PCRs that can all be run on the same sample, requiring 1 sole DNA-extraction from an EDTA-blood sample, even if this meant taking a loss in sensitivity for detection of *Rickettsia* spp. and *O*. *tsutsugamushi*.

A sample exchange with the laboratory in Lao PDR for external quality control showed that the Cambodian laboratory was able to identify all of the positive samples from Lao PDR, whereas the laboratory in Lao PDR was not able to detect more than 20.0 % of positive *Rickettsia*- and *O. tsutsugamushi*-samples from Cambodia. For the other pathogens similar problems were observed (*Leptospira* spp. 21.0 %, *P. vivax* 75.0 %, *and P. falciparum* 58.0 %). All of the Cambodian assays worked fine with the positive controls, as well as with the exchanged samples from Lao PDR, which shows that the problem lied in the quality of the blood- and DNA-samples and its degradation, rather than in the performance of the PCR assays. In retrospective, the DNA-extraction method could have contributed to the poor quality of the samples. The Qiagen® Kit is designed for extractions from fresh whole blood, whereas in this study, frozen packed blood cells were used, which are more viscous and might have restrained this method and result in non-pure DNA extracts. In addition, the storage conditions of blood- (-80 °C) and DNA-samples (-20 °C), as well as repeated thawing, possibly contributed to a further degradation of the sample quality [103]. This would explain why the results could not be reproduced on the samples which had been destabilized by this treatment.

4.1.2.2 PCR as diagnostic tool for non-malaria febrile illness

The nested-PCR assays used in this study showed great potential to serve as diagnostic tools for the differential diagnosis of non-malaria febrile illness. Nevertheless, it cannot be used as diagnostic tool alone, clinical and epidemiological context of the patient still have to be examined carefully. Furthermore, it was shown that it is difficult to run all these different PCR assays on only 1 sole sample of the patient, considering the different host-cells and courses of infection of the investigated pathogens. Details on the performance and advantages of each assay will be discussed in the following.

To begin with, the *16srRNA*-PCR showed to be a sensitive and reliable tool for the detection of leptospiral DNA. Furthermore, it allowed the genetic determination of species and their relatedness by nucleotide sequencing. Still, it has to be considered that it has a limited time frame in the course of the infection and blood might not always be the appropriate sample. As mentioned earlier, the PCR could also be used on pure plasma or urine samples instead of whole blood [38, 96]. Moreover, the diagnostic RT-PCR assays for dengue and influenza viruses worked equally well, and the results produced in this study correspond to the national surveillance data. Thus, these assays could be useful to establish a standardized protocol for the differential diagnosis of non-malaria febrile illness in Cambodia.

In contrast, the diagnosis of TG- and SFG-*Rickettsia* by nested PCR did not produce satisfactory results. Most positive PCR-results could not be confirmed in a second PCR-run. Also, the product of the *gltA*-assay was quite short (74 bp) for the nucleotide sequencing procedure, considering that the 20-50 bp of read after primer binding are generally poor quality, so that in many cases the sequencing result was not interpretable. A product size of more than 200 bp would be required for better results, which could be achieved by cloning the products into plasmids prior to sequencing. However, this technique was not available in this study setting. This is why, to rule out contamination or other problems with the nPCR-assay, a realtime PCR-assay by Paris *et al*. [62] was performed retroactively on the samples with positive nPCR. Although the assay worked well with controls (DNA extracted from cultured organisms), none of the nPCR-results could be confirmed. This can be explained by the poor quality of the extracted DNA-samples in combination with the

already very low concentration of DNA in the samples due to the choice of sample (packed cells instead of buffy coat). The detection of *O. tsutsugamushi* was less problematic, but the product of the *47kDa*-assay is also rather short for nucleotide sequencing (118 bp). To evaluate the genetic diversity of Cambodian *O. tsutsugamushi* strains the *56kDa* gene target has shown to be suitable [58], however for observational or screening purposes by nested PCR the *47kDA* target was sufficient.

4.1.2.3 Benefits of PCR over microscopy and RDT in malaria diagnosis

Compared to the PCR-results both microscopy and RDT were less sensitive and less specific in the detection and species determination of malaria parasites in this study. In the febrile Group F sensitivities for the detection of any malaria parasite were 71.3 % and 55.6 % for microscopy and RDT respectively. The sensitivity of RDTs did not increase when looking at *P. falciparum* detection only (58.9 %). In the asymptomatic Group N, where parasite density was low, the sensitivity of microscopy was only 19.2 %. Specificities of both tests were over 95.0 % in Group F as well as Group N. The lack of sensitivity of RDTs found in this study maybe be due to incorrect storing and handling of the tests, an effect that has been described before [14]. With RDT and microscopy as the only diagnostic tests a substantial proportion of parasite carriers would have been missed. The extend of this submicroscopic reservoir needs to be taken into account for effective surveillance, control and eradication programs [104-105]. Nevertheless, both techniques remain important tools in peripheral settings due to their feasibility and low cost. Especially RDTs will play an ever more important role in differentiation of malaria from other febrile illnesses in remote settings [14]. However, the quality of results from both of these methods rely on various factors, such as provision of high quality supplies, adequate work environment, storage facilities and external quality assurance. Both field microscopy and RDTs often fall short of these requirements at peripheral health services, and further improvement is desirable to a more accurate malaria management [14]. Furthermore, the results of this study underline the lack of performance of both microscopy and RDT in the case of low-level parasitemia in mixed or asymptomatic infections, which are extremely important in the context of malaria-eradication projects. The *cytB*-PCR is a highly sensitive and useful tool for large-scale epidemiological surveys and field studies [95].

4.1.2.4 Diagnostic value of CRP-level an clinical data for acute febrile illnesses

Concordant to the national data, it was observed in this study that male patients in the working age are the population group which is most affected by acute febrile illnesses [16], since they accounted for the biggest fraction of the febrile Group F. This is due to risk factors like working outside, in the forest or in mines, which are associated with higher exposure to mosquitoes and other vectors. Other than that, the clinical evaluation of the subjects recruited for this study has not revealed any predictor symptoms or risk factors to differentiate between the different types of febrile illnesses. This is not surprising, considering that all of the evaluated infectious diseases have a very similar panel of clinical symptoms. To get a more global clinical picture of each individual it would have taken a broader clinical questionnaire and a trained physician for a full clinical examination, which was not available in the setting of this study.

The evaluation of CRP-levels, as the only biological marker for infection in this study, did show an informative value for the presence of any infection (Group F vs. Group N), as well as for the cause of the infection (viral compared to bacterial/parasitic infection, see table 17). Thus, it should be considered to integrate the analysis of CRP-levels in an algorithm for the differential diagnosis of acute febrile illness. At least like this, viral infections could be more easily identified or ruled out as cause of the illness. Accessory information such as a full blood cell count could provide additional useful diagnostic clues [106].

4.2 Study results

4.2.1 Malaria

4.2.1.1 Malaria-PCR results in the national context

A very high prevalence of malaria was observed in this study. Of 1,193 febrile patients recruited for this study 56.7 % were carrying malaria parasites, and even in the non-febrile Group N 27.7 % turned out to be malaria-PCR positive. This was a surprise, considering that

the study was designed for a setting in which malaria does not play such an important role anymore. On the one hand the high rates can be attributed to the higher diagnostic sensitivity of PCR compared to microscopy and RDTs [104-105], taking into account that the *cytB*-PCR detects also gametocytes. On the other hand this could be also due to a possible selection bias caused by the specialization in malaria diagnostic and treatment of the study sites.

Interesting differences to the national data regarding the species distribution were observed. Whereas the national data suggests that 70.0 % of the malaria infections are caused by *P. falciparum* [16], in this study only 40.0 % of the malaria cases were identified as *P. falciparum*, whereas *P. vivax* was the predominant species (52.0 %). This can be explained by the higher sensitivity of PCR and nucleotide sequencing compared to RDT and microscopy for the detection of *P. vivax*, as was shown in table 20 and figure 33. The frequency of mixed *Plasmodium* infections (3.8 %) was comparable to the national data (5.0 %) [16].

In the Pailin area (sites C-1 and C-2), *P. vivax* was the predominant species even though this province is an area of high transmission for malaria and it was expected to find a bigger proportion of *P. falciparum* here. Surprisingly this species distribution did not apply to the Snoul area (site C-3) where *P. falciparum* was the predominant species, even though Kratie Province is considered as an area of low-transmission. Usually *P. vivax* is more likely to be found in areas of low-transmission [17]. This might be partially explainable by the Group N that has been bigger in Pailin and contributes significantly to the *P. vivax* case load. This could indicate that in populations in hyper-endemic areas like Pailin, asymptomatic *P. vivax* infections are common and might bare a possible protective effect against *P. falciparum* infections [105]. The seasonal trends of malaria prevalence observed in this study accord to the national data, confirming that the rainy season, from May to October, is the high season for malaria.

4.2.1.2 Importance of asymptomatic *Plasmodium* spp. infections

It has to be taken into account that a high rate of asymptomatic *P. vivax* infections was observed in the Group N, as well as the high rate of *P. vivax* as co-infection with another pathogen. Similar findings are reported in a survey of asymptomatic mobile Cambodians at the Thai border, which found a malaria prevalence rate of 2.4 % mostly *P. vivax* with low parasitemia [107]. It is possible that a high chronic infection rate in the population has been established, due to the protective effects of cross-species immunity. It has been shown previously that the severity of malaria symptoms is reduced in patients that have been pre-exposed to different species and that an infection with *P. vivax* is likely to protect from severe complications in the course of a *P. falciparum* infection [105, 108-109]. This effect might explain the lower mortality rates from *P. falciparum* in Southeast Asia compared with Sub-Saharan Africa [108]. These asymptomatic parasite carriers can act as a reservoir which is very difficult to control [104-105, 109]. To get a more specific overview of the malaria situation in regard of eradication measures, mass-screening programs by PCR are currently being set up in several Cambodian provinces.

4.2.1.3 Emergence of *P. knowlesi* infections in Cambodia

For the first time in Cambodia, 2 cases of infection with the simian *P. knowlesi* have been detected in this study. Both of the *P. knowlesi* infections were confirmed by additional PCR amplification and sequencing at the Genomic Platform, Pasteur Institute, Paris, France (Genopole®). Blood spots on filter paper were also sent blind to the Malaria Research Centre, Faculty of Medicine and Health Sciences, University Malaysia, Sarawak where they were identified as *P. knowlesi* single infections by real time and nested PCR. This finding confirms the spread distribution of human *knowlesi*-malaria cases throughout Southeast Asia and highlights the impact of strategies aiming to eradicate malaria from humans and the extensive deforestation in this region [110].

4.2.2 Identified causes of non-malaria febrile illness in Cambodia

4.2.2.1 Leptospirosis

Leptospirosis showed to play an important role as cause of undifferentiated febrile illness in this study population. 9.4 % of the Group F samples were positive for pathogenic *Leptospira* spp.. Interestingly, in the asymptomatic Group N 9.9 % also had a positive test result for pathogenic *Leptospira* spp.. This could be due to the fact that the Group N was recruited from family members or other accompanying persons, which were certainly exposed to the same risk-factors for the infection. However, compared to studies conducted in similar settings in Thailand and Lao PDR [25-26, 41], the results are not surprising, but underline even more the importance of this disease in the region. As already shown by the surveillance study of IPC and confirmed by this study, leptospirosis is a significant public health problem in Cambodia [44]. The awareness about leptospirosis needs to be raised urgently and more research on its epidemiology in Cambodia should be induced. Furthermore diagnostic tools for leptospirosis, such as PCR or MAT, should be made available in peripheral health services.

L. interrogans was by far the predominant species in this study, followed by *L. weilii,* both of which are classified as human pathogens [34]. Since no serological tests were applied, no information on the serovar could be obtained. This might be subject to further investigations on the plasma samples in stock. However, *L. genomosecies1, L. kirschnerii, L. santarosai* and *L. noguchii* are classified as pathogen *Leptospira*, whereas *L. wolffii and L. kmetyi* belong to the intermediate group whose pathogenicity and significance remains unclear [34]. Taking into account that the sensitivity of the PCR could be increased by using a different sample (plasma, urine) as explained earlier, it is likely that results of this study still underestimate the actual prevalence of leptospirosis in the study population.

4.2.2.2 Rickettsial infections and scrub typhus

Regarding the above mentioned difficulties with the rickettsial PCR assays it is doubtable if their results can contribute to a better understanding of the epidemiology of *Rickettsia* spp. in Cambodia. However, the detected species are plausible causative agents of acute

febrile illness and their finding should induce further investigations on the subject. Serological tests could be run on the plasma samples in stock and surveillance studies, using and evaluating nested or realtime-PCR on fresh buffy coat samples should be considered. Surprisingly, no cases of *R. typhii* infections were detected which was suspected to be one of the most prevalent species in Cambodia, due to the results of studies conducted in Lao PDR and Thailand, where murine typhus was identified as a major cause of acute febrile illness. Further clues to the diagnostic of rickettsial diseases could be added by a detailed clinical exam and risk factor analysis. A case control study conducted on febrile patients in Thailand showed that rickettsial disease was associated with the patients' report of rash or arthropod bite and a history of jungle trips. Furthermore, elevated liver enzymes and depressed platelet count were also useful differentiating markers of rickettsioses in the investigated population [28]. In addition, a study from Taiwan showed that rickettsial diseases (acute Q fever, scrub typhus and murine typhus) should be suspected in febrile patients who present with relative bradycardia, hepatomegaly, and elevated serum amino-transferases, but without leucocytosis [111]. These findings underline the importance to carefully look and ask for any history of arthropod bites or eschars in non-malarial febrile patients, as well as the importance of a full blood count and hepatic evaluation, to differentiate rickettsial diseases from other febrile illnesses.

O. tsutsugamushi, the causative agent of scrub typhus, was identified in 3.9 % of the Group F samples. In addition in the asymptomatic Group N 2.5 % of the samples were positive for *O. tsutsugamushi* too, which could be due to a similar exposure to risk-factors as already explained for leptospirosis. Being an intracellular organism which infects white blood cells, the sample choice (packed blood cells) was not ideal and it can be assumed that a different sample choice (buffy coat, peripheral blood mononuclear cells or skin-biopsies from eschar) could have resulted in a higher positivity rate. Nevertheless, these results emphasize the importance of scrub typhus in Cambodia and express the need of further research on the topic. To investigate the genetic relatedness of the different strains the *56kDA*-PCR assay [58] could be applied on the samples in stock, but regarding the sample quality problems with reproducibility have to be expected here, too.

4.2.2.3 Dengue fever and influenza infections

In 6.3 % of Group F, dengue virus was identified as the cause of non-malaria febrile illness. This figure is rather high, considering that patients were over the age of 7 years and were recruited in very rural, low-transmission areas for dengue fever, which is commonly more present in urban settings and young children [69]. In Group N, 1.8 % of samples were positive for dengue virus. These are considered as primary, asymptomatic infections. The observed annual epidemic wave is concordant with the national surveillance data [16].

Influenza viruses were identified in 8.9 % of the Group F samples and 2.5 % of the Group N. In total, 77.0 % of them were subtype A (most prevalent strains H3N2 and H1N1) and 33.0 % subtype B. This distribution as well as the seasonal findings is corresponding to the national surveillance data [75].

These results underline the importance of clinical training for health center staff to be able to recognize a viral infection and be aware of possible complications. Both, dengue and influenza infections were associated with a significantly lower CRP-level in the samples than malaria or bacterial infections (see table 17). Therefore, analysis of CRP-levels could be a possible helpful tool to distinguish viral infections as cause of non-malaria febrile illnesses in the future. Additional information could be obtained by a full blood count. A study from the Thai-Burmese border showed that patients with confirmed dengue infection had lower white blood cell counts (4.8 vs. 7.2, $p < 0.01$) and platelets (147 vs. 162, $p = 0.03$) than patients with non-dengue infections (typhoid fever, murine typhus, scrub typhus and leptospirosis) [112]. Furthermore, this work suggests that, in a clinical setting, a combination of at least 2 tests (IgM antibody ELISA, NS-1 antigen ELISA and/or realtime RT-PCR) are required to confidently diagnose acute dengue infection from a single blood specimen. The RT-PCR used in this study showed to be the most sensitive tool for the early diagnosis of dengue fever. Although cheap and field-deployable PCR systems are not yet available, progress has been made in the development and refinement of current techniques, which may result in nucleic acid detection becoming the standard for rapid dengue diagnosis even in resource-poor settings like rural Cambodia [112].

4.2.2.4 Bacteria from blood culture

The Department of Bacteriology of IPC evaluated blood culture bottles of the 1,128 febrile patients recruited in this study. Not only was this a logistic challenge, but also a cost-intensive measure. The results were disappointing, only 9 bottles (0.8 %) grew pathogen bacteria. Antibiotic resistance was tested on the positive cultures, the result of which was immediately transmitted to the health center staff, which would track down the patient and administer the correct treatment. It has to be considered that in this study setup several factors reduced the sensitivity of blood culture for the diagnosis of typhoid fever and other bacterial infections. First of all, only 1 bottle per patient, and only 5 ml of blood per bottle had been available, sensitivity would have increased with the number of bottles and the amount of blood administered to each bottle [24]. According to current guidelines, at least 2 sets of bottles with a minimum of 10 ml venous blood should be taken from adult patients with suspected bacteremia [113]. In addition, it was not evaluated if the patient did self-treat with antibiotics before consulting at the health center, which was probably the case and further reduces the sensitivity of blood culture [77, 80-81, 113]. The costs and lack of storage and incubation facilities in peripheral health services make blood culture a diagnostic tool reserved for special indications rather than routine diagnostics. Comparing these results to the ones of the simultaneous NMFI-study (see also 4.2.4) in Lao PDR, which was based in provincial hospitals it is remarkable that in Lao PDR 3.3 % of the blood culture bottles (the same bottles as used in the Cambodian study) grew pathogenic bacteria (of which 72.0 % were *S. typhi*). The difference is probably due to the different setting, considering that patients at provincial hospitals are more sick (more likely to have community acquired septicemia) as those coming to a peripheral health center. In addition in Lao PDR 2 bottles with 5 ml blood were taken from each patient, which increases the sensitivity significantly [24].

4.2.3 Simultaneous detection of pathogens

A high percentage of multiple simultaneously detected pathogens in 1 blood sample was observed in this study. In total 13.6 % of the samples showed evidence for the presence of more than 1 pathogen at the same time. Most frequently, these included malaria parasites

(*P. falciparum* and *P. vivax*) and a second pathogen (*Leptospira* spp., *Rickettsia* spp., *O. tsutsugamushi* or dengue virus). This seems to be an observation made more and more frequently due to the increase of interest in the above mentioned pathogens. A recent study in Thailand investigated possible acute co-infections in Thai malaria patients and found serological evidence for scrub typhus- (15.0 %), leptospirosis- (7.7 %) and murine typhus- antibodies (23.2 %) simultaneously to the *P. falciparum*-infection [114]. However, in this study only serological tests were applied and often there was no convalescence sample to differentiate between acute or previous exposure to the pathogen. The authors recommend to use culture or PCR to get a clearer picture of the prevalence of multiple infections in malaria patients [114]. Reports of malaria co-infections with other pathogens such as HIV or helminthes are numerous especially from sub-Saharan Africa. In Southeast Asia, however, reports on co-infections in malaria patients remain rare. Malaria in Southeast Asia is often associated with outside working conditions like logging, mining and agriculture, activities that put people at risk for leptospirosis, rickettsiosis and scrub typhus too, so it is likely to find simultaneous infections with more than 1 of these pathogens. Concurrent infections of malaria parasites and dengue virus are rare but have been reported from endemic areas before, where dengue viruses spread more and more to rural areas [115-117]. Additional dengue fever should be suspected if a malaria patient presents with prolonged fever (> 7 days), bleeding manifestations, retro bulbar headache, severe myalgia, thrombocytopenia or anemia [116-117], and an additional NS1-antigen test should be applied in these cases [115]. In many cases of malaria co-infections the involved species is *Plasmodium vivax,* in a rather low parasite density, as was shown in table 21 (parasite densities [parasites/µl blood] by microscopy regarding infection status). These cases could be interpreted as chronic asymptomatic infections with *P.* vivax plus a simultaneous acute infection with another pathogen. Unfortunately nothing is known about the interactions of these pathogens in co-infections so far, but it can be assumed that multiple infections complicate malaria and lead to treatment failure [114]. The awareness of the possibility of multiple infections should be raised and physicians should be suspicious of it in malaria cases with poor treatment response or atypical manifestations. In such cases an additional treatment with doxycycline, which is effective against all of the above-mentioned bacterial pathogens, could be a solution.

Within this study, 6 cases showed evidence of the simultaneous presence of pathogenic *Leptospira* spp. and *O. tsutsugamushi*, 3 of them even with additional malaria parasites. Reports from Thailand and Taiwan are describing similar findings. In Thailand, a study of 22 rice farmers with suspected leptospirosis, showed that 41.0 % had serological evidence of concurrent scrub typhus [118]. In Taiwan a retrospective analysis of co-infected patients was conducted, and concluded that, since the 2 diseases share main risk-factors (like rice farming), co-infection is not unusual and clinicians should be alert in cases of treatment failure or severe cases [119]. This awareness is very important because the first-line treatment for leptospirosis (penicillin) is not effective against scrub typhus (chloramphenicol) and vice versa, and untreated mortality rates for both diseases are high. Tetracycline antibiotics would present a choice of therapy that would cover both infections [118-119]. Other co-infections observed in this study were 5 cases of pathogenic *Leptospira* and dengue virus, as well as 1 case of *O. tsutsugamushi* and 1 case of influenza A virus with dengue virus.

4.2.4 Study on non-malaria febrile illness in Lao PDR

Simultaneously to this study, a partner study in Lao PDR was conducted by the Wellcome Trust-Mahosot Hospital-Oxford Tropical Medicine Research Collaboration (LOMWRU), Centre for Malariology, Parasitology & Entomology, Vientiane – funded by WHO/WPRO and FIND. The study settings in Lao PDR were different from the ones in Cambodia, so the results of the 2 studies are not comparable but rather complement each other. In Lao PDR the study was set up in 2 provincial hospitals, in the north and in the south of the country. 1,595 out- and in-patients were recruited for the study and convalescence samples were taken after 14 days to perform serological tests. The inclusion criteria were the same as in Cambodia, except that the lower age limit was 5 years in Lao PDR and 7 years in Cambodia. The samples taken were tested for the same diseases, except for influenza which was not tested in Lao PDR and Japanese encephalitis which was not tested in Cambodia (no convalescence samples). The results found in Lao PDR showed a very different pattern than the ones from Cambodia. In 66.1 % of the cases in Lao PDR no diagnosis could be established and malaria was only found in 1.2 % of the samples. Most frequent diagnoses by blood culture, PCR and serology were scrub typhus (8.7 %), dengue fever (8.3 %),

Japanese encephalitis (7.0 %), leptospirosis (3.9 %) and CAS (2.9 %) which was most commonly caused by *Salmonella enterica* serovar *typhi*. *Rickettsia* spp. (*R. typhii* and SFG-*Rickettsia*) were detected in 1.0 % of the samples by PCR and 7.0 % of the patients had elevated IgM-titers for *R. typhii*. In 1.3 % of the samples there was evidence for multiple pathogens [unpublished results]. These results underline the importance of scrub typhus, leptospirosis and dengue fever as common causes of non-malaria febrile illness in Southeast Asia. Furthermore it is interesting to see the high rate of JEV detected in Lao PDR, which would indicate promoting public vaccination against JEV. In Cambodia JEV could not be evaluated since PCR is not an appropriate tool for diagnostic and no convalescence samples were available for serological testing. The higher rate of community acquired septicemia is most probably due to the fact that the patients were recruited in a hospital which means that they were in a more severe condition than the out-patients recruited in the health centers in Cambodia.

4.3 Clinical implications of the study results

The diagnosis of non-malaria febrile illness in resource limited, rural areas remains a challenge [1]. The comparison of the clinical with the biological diagnoses in this study showed that whilst the clinical management of malaria is working well in Cambodia, the majority of malaria-RDT negative febrile patients did not receive an appropriate treatment. Furthermore, malaria RDTs showed a low sensitivity compared to microscopy and PCR and showed to be not sufficient to rule out malaria in this setting. Therefore, if RDT is negative but malaria is clinically suspected, a second test (microscopy or ideally PCR) should be run for confirmation. Furthermore, the results of this study indicate some important clues for the differential diagnosis of acute febrile illness in health centers. To begin with, typhoid fever and community acquired septicemia seem to be rather uncommon in this setting, even though it should be kept in mind that the applied diagnostic method is likely to miss bacterial blood stream infections, due to prior self-treatment with antibiotics and lack of sensitivity. On the other hand, malaria remains highly frequent and is often associated with other pathogens which can explain treatment failure. Furthermore, the burden of submicroscopic, low-parasitemia malaria was shown to be high, also amongst

asymptomatic people. Minor *Plasmodium* species and mixed species infections are likely to get more and more important while *P. falciparum*-malaria is rolling back. In addition, dengue and influenza viruses contributed significantly to the causes of febrile illness and cannot be treated with antibiotics. Therefore clinical training or additional tests to distinguish these viral infections from malaria and bacterial infections should be implied to reduce the unwarranted use of antibiotics and anti-malarials. Finally, the choice of empirically used antibiotics for new treatment algorithms should include effectiveness against *Leptospira*, *Rickettsia* and O. *tsutsugamushi* (e.g. doxycycline). At the same time, more research and surveillance is needed to determine the epidemiology of leptospirosis and rickettsial diseases in Cambodia.

4.3.1 Malaria

As mentioned earlier, the clinical data recorded for this study did not reveal any predictive clinical features to distinguish malaria from other febrile illnesses. This is partially due to the simple structure of the questionnaire, and the fact that the clinical examination in the health centers was not performed by a medical doctor. More detailed and precise questions and a full clinical exam would have allowed a more specific analysis of risk factors (*e.g.* working conditions, living situation, ITN coverage in household) and clinical features (*e.g*. hepatomegaly, splenomegaly, jaundice, and anemia). However in a setting like this, trained physicians are rarely available, and a questionnaire on this scale would have been difficult to complete for the health center staff. A complete blood count would provide valuable information on the severity of the infection, but is unfortunately not feasible in peripheral health centers at the moment. Furthermore it has been shown in a similar study that complete blood count did not provide discriminatory information for malaria, leptospirosis, rickettsiosis and dengue fever [32]. Nevertheless, it could be shown that the clinical diagnosis together with the RDTs performed in the field, succeeded to identify 61.9 % (419 out of 676) of the malaria cases correctly and provide the patient with the appropriate treatment.

4.3.2 Non-malaria febrile illness

Whereas the clinical presentation of the patients did not show any specific clues that could have helped to establish a differentiated diagnosis of febrile illness, CRP-analysis did prove to be beneficial to distinguish malaria and bacterial from viral infections. Hematological parameters and Procalcitonin as a marker for bacterial infections could be added to support the decision making for antibiotic treatment [120]. Most malaria-negative cases were clinically diagnosed as upper ARI and treated indiscriminately with antibiotics (mostly amoxicillin). Only 14.0 % of the malaria-negative cases received a treatment that was effective against the responsible pathogen identified in the laboratory. These findings underline the urgent need of specific diagnostic tools and clinical training of the health center staff for the effective management of malaria negative fever.

4.3.3 Developing a treatment algorithm for malaria RDT-negative fever

The current algorithm for malaria suspected cases was shown in figure 6. Patients with negative RDT for malaria are supposed to be referred to a hospital, especially if the case is severe and if patients are pregnant and children less than 5 years old. To develop an updated algorithm on management of malaria negative patients based on the results of this study, important considerations are:

- Regional disease burden and seasonality of infectious diseases
- Clinical condition of patients and severity of disease (case fatality rates with and without treatment)
- Availability and level of health care facilities and services

Further research is needed to identify disease burden and laboratory gaps in each level of health care. To develop clinical guidelines it is imperative to involve physicians, nurses and other health care professionals from various levels of health care as well as the policy makers [1]. Intervention packages have to be in line with the current clinical management efforts and the Ministry of Health's planning for human resources, facility upgrades and

laboratory strengthening. The following information would be needed to improve the diagnostic procedure for malaria-negative fever patients:

- Additional symptoms, clinical manifestation (full clinical exam by a physician)
- Local disease endemics and its seasonal variations
- Link between disease and patient occupation (risk factor analysis)
- Blood testing (complete blood count, platelets, liver enzymes)
- Information on self-medication prior to consulting the health facility

Malaria RDTs are currently the most important diagnostic tool available in peripheral health centers. As shown in figure 34, 67.9 % of the febrile patients in this study had a negative malaria RDT result. Many of them (37.0 %) were still carrying malaria parasites that could not be detected by the RDT. This shows that by negative RDT alone, malaria cannot be ruled out. For 24.9 % of the RDT-negative cases another etiology of fever was found, of which most were of viral origin (32.7 % influenza virus and 20.7 % dengue virus). These findings indicate that for the development of an updated algorithm in a region where malaria still is among the leading causes of febrile illness, it should be considered that patients with clinically suspected malaria but negative malaria-RDT should be tested for malaria using other more sensitive methods to detect non-*falciparum*- or *falciparum*-malaria cases with low parasitemia. If these additional tests do not reveal malaria as a result, the health professional has to determine between a viral and a bacterial cause of the fever, using clinical clues and blood analysis including a complete blood count and bio-markers like CRP or alternatively PCT. If the results point towards a bacterial cause of the fever, empiric antibiotic treatment with effectiveness against *Leptospira* spp. and *O. tsutsugamushi*, e.g. doxycycline should be prescribed. If the results point towards a viral cause of the fever (dengue or influenza virus) treatment should include paracetamol and fluids as well as surveillance or hospital-referral of the patient to prevent complications like DHS.

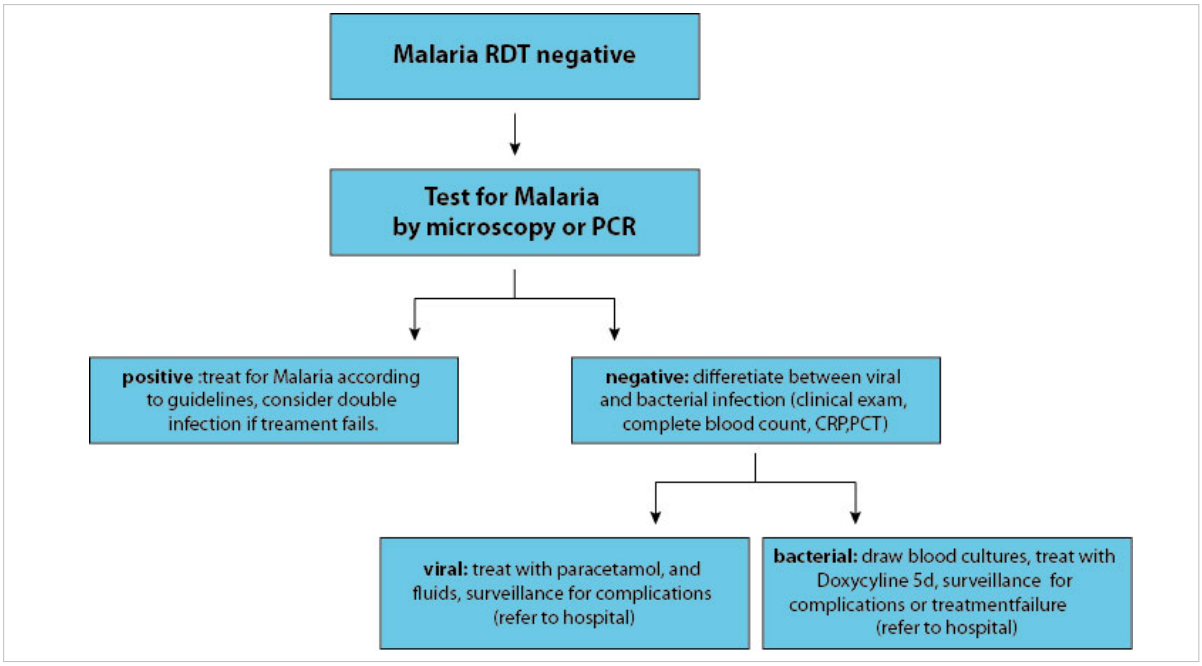


Figure 44: Example of a malaria-RDT negative case management algorithm.

4.4 Implications on further trials and diagnostic development

4.4.1 New diagnostic tools for acute febrile illness in the tropics

Developing novel pathogen detection methods is very expensive and should be accompanied by extensive epidemiological background research and cost-effectiveness studies. Requirements for a sub-national (e.g. regional hospital) clinical diagnostic test would be:

- High sensitivity of tests (90.0-95.0 %) for on-spot decision of clinicians
- Staff training on laboratory equipment operation
- Staff supervision and knowledge management
- Use of commercial tests, if not available homebrew assay with external quality assurance and -control

For scrub typhus and rickettsial diseases common diagnostics include either serology (lack of sensitivity) or culture (lack of feasibility), both of which are not adequate for field

conditions. PCR showed to be a good diagnostic tool for scrub typhus in this study but is hard to establish in peripheral settings. New techniques like LAMP PCR essays and a new serological RDT (by AccessBio®) are currently evaluated in Lao PDR and present promising options for the future [121]. Future NMFI trials should assess these methods and confirm positive results by culture. Furthermore the collaboration between different laboratories in Lao PDR, Cambodia and Thailand should be intensified to establish a local network that can easily exchange samples, compare results and techniques, assess new diagnostic tests and contribute to a larger epidemiological understanding of rickettsial diseases in the Indochinese region. Study investigators in further NMFI trials should follow-up the treatment and recovery of patients as this would strengthen the analysis of results.

Many different serological test and RDTs for the rapid diagnosis of leptospirosis have been evaluated in the last years, some of them showing promising results. For example, ELISA and DST (IgM dot-ELISA dipstick test) showed significantly higher sensitivity with early acute-phase sera than the reference or first-generation methods (MAT and IHA) while retaining high specificity and could be evaluated for the rapid detection of leptospirosis in the field [122]. The nPCR used in this study worked well but is time-consuming and sensitive to contamination, which is why it should be considered to additionally assess realtime PCR and LAMP PCR in future trials. Furthermore, these results should be compared to MAT (WHO/AO/OIE Collaboration centre), and culture in blood clot (Mahosot, Lao PDR). In general it should be tried to collect a larger number of samples by sentinel sites and during outbreaks, as well as conducting a prevalence study to better understand the epidemiology and meaning of symptomatic and asymptomatic carriage of *Leptospira* spp..

Positive results of blood cultures were rare in both, Cambodia and Lao PDR, and the prevalence of typhoid fever was by far lower than expected. This was surprising and maybe partially explained by the used technique, as explained earlier. To date the Widal-test was used in both countries sub-nationally but lacks specificity and accuracy [80]. Rapid diagnostic tests like Tubex™ and Typhidot® showed to be of low diagnostic value in peripheral settings, too [79]. The EnterocheckWB®-test [81] as well as recent serological dipstick tests [80] showed better results and could be assessed in further NMFI trials. A

new antigen test based on blood cultures is currently being evaluated in Lao PDR, depending on the results it should be considered to assess this test in Cambodia, too.

Both, the RT-PCR used in Cambodia, as well as the NS1 ELISA used in Lao PDR showed very good performance for diagnosis of dengue fever. New NS1-based RDTs are promising for dengue diagnostic in peripheral health care settings, especially when combined with IgM/IgG tests [123] and could be used as one of first-line tests for differential diagnosis of fever, especially during the dengue season. In Vietnam a recent study showed that the introduction of dengue RDTs to a peripheral health post through free market principles improved the quality of the diagnosis and decreases the prescription of antibiotics [124]. However, this effect is only sustainable and cost effective in combination with clinical training of health workers.

Last but not least, the influenza RT-PCR was shown to be a reliable test with a great performance but can currently be effectuated in central laboratories only. There is a variety of rapid diagnostic antigen-based tests that could be used in the field, especially during the influenza season. In Taiwan, where the same main subtypes of influenza A virus are circulating than in Cambodia (H1N1, H3N2), 4 rapid tests that also detect influenza B, have been currently evaluated and showed that the Formosa rapid diagnostic test fulfilled all criteria to be used in peripheral primary health care centers [125]. Furthermore the clinical training of heath center staff is essential to the process of distinguishing influenza from other febrile illness.

4.4.2 Etiologies of NMFI beyond the investigated pathogens

In the recruited Group F 26.8 % of the patients did not show any positive test result and thus the cause of their fever remains unknown. A variety of other pathogens come into consideration as cause for the fever, not all of which can be evaluated. For example, it has to be recalled that HIV infections, viral hepatitis and tuberculosis have not been evaluated due to ethical reasons but are likely to contribute to the burden of febrile illness. The parallel NMFI-study in Lao PDR found a high percentage of Japanese encephalitis in their study population, thus it would be interesting to test the Cambodian samples for this virus.

Unfortunately only serological testing would be conclusive and convalescence samples would be needed to evaluate this disease. A combined *Flavivirus* MAC-ELISA (JE–Dengue IgM Combo ELISA; Panbio® Diagnostics, Australia) was run on samples of patients with more than 5 days of fever, which applied only in 10 cases. Of these 10 samples, 2 had a positive result for JEV and 3 samples were positive for dengue virus. This underlines the importance to think about JEV as cause of febrile illness, especially in cases of prolonged fever or additional signs of meningitis.

Another viral disease that could be interesting to evaluate in the future is Chikungunya virus, regarding that there were several outbreaks reported from Thailand in 2008 and 2009 [126]. Further tests could include viral infections like Epstein-Barr-virus, Hepatitis A-, Hepatitis E-, Coxsackie virus and bacterial infections such as q-fever, brucellosis and melioidosis. Analysis by bacteriological methods like API 20E/NE gallery and culture of blood and additional samples like pus (from abscesses) or sputum would be helpful to determine the prevalence of melioidosis and other bacterial infections in a future study.

4.4.3 Lessons learned for further NMFI trials

Since this was the first time a NMFI trial was conducted in Cambodia it provides highly valuable information for the planning and design of further trials on the subject in Cambodia as well as in other countries. Further trials in Cambodia should include district hospitals to compare the disease burden to the one in health centers and eliminate the possible selection bias for malaria present in health centers. Since the recruitment was strongly depended on the rainy season it could be considered to limit a further trial to the wet season only and thereby increase the cost-effectiveness. Given that there is limited financial resource for NMFI-studies adequate funding should be sought from a single source to support and coordinate the whole activity. Piggy-backing on existing malaria activities in conducting the studies is recommended. Another important question to be asked is if it is useful to recruit control groups which are exposed to the same risk factors and might carry the same diseases, without being febrile on the day of recruitment. The prevalence of asymptomatic infections might be evaluated separately by a pure prevalence study.

On the clinical side, it is essential to use a standardized and revised data form with a stronger focus on the clinical exam (ideally performed by a medical doctor), as well as documentation of hematological parameters if available. Furthermore, questions about previous self-treatment and health care seeking behavior should be added. Additionally, epidemiological information and risk factor exposure should be included in the form and a follow-up of patients could give additional information about the outcome (mortality, morbidity). On the laboratory side, standardized and refined techniques and protocols for all pathogens should be applied in accordance with all study centers, so the data would be comparable between different sites e.g. in Lao PDR and Cambodia. Furthermore, new rapid diagnostic tests for NMFI as mentioned above should be assessed at the same time. Paired serum samples should be collected from all patients to be able to perform serological analyses. It should be considered to include additional pathogens in the screening (e.g. by multiplex-PCR for respiratory pathogens), and which added value this would have in terms of treatment (diseases for which treatment is not available anyway). For quality assurance of results, external quality control should be established from the beginning of the study.

Moreover, central data management and coordination should be developed to ensure standardized methods, data entry and analysis at all sites. Data collectors should have sufficient training to perform detailed clinical assessment. International collaboration is needed for research dissemination and utilization. The study results from Lao PDR and Cambodia should be published in parallel papers and disseminated by national partners in their countries. Lastly, organization of local symposia maybe considered to present the results and their implications to national programs, and to promote awareness of the topic.

5 Summary

5.1 Summary in English

Malaria has long been among the major health problems in Southeast Asia. In the past decade, the Cambodian government successfully implemented diverse strategies to fight malaria. The number of reported malaria cases, as well as case fatality rates, continue to decrease ever since. However, with the introduction and wide distribution of RDTs for malaria, it also turned out that in many clinically suspected malaria cases, no malaria parasites could be detected. There are currently no clear algorithms on management and treatment of patients with negative malaria-RDT in peripheral health posts. This poses problems in terms of public health, *e.g.* treating non-malarial patients with anti-malaria drugs or non-treatment of patients with other potentially fatal febrile illnesses. Therefore, improving clinical guidelines and diagnostic procedures is highly important. As a first step towards developing an algorithm for malaria-RDT negative fever management at peripheral health posts, a cross-sectional observational study was designed to investigate the causes of acute undifferentiated febrile illness in rural areas of Cambodia. From January 2008 until December 2010 1,475 study subjects have been recruited in 3 different sites, 2 of them at the western, and one of them at the eastern border of the country. Among the study subjects 1,193 were febrile out-patients (Group F, age 7-49 years, body-temperature ≥ 38.5 °C for not longer than 8 days) and 282 were non-febrile individuals who have been recruited as a comparative control group (Group N, most of them healthy accompanying persons). Of each subject, 15 ml whole blood, a blood smear and a nasopharyngeal throat swab were collected and sent to the central laboratory at the Cambodian Pasteur Institute in Phnom Penh for molecular, microscopic and bacteriological investigation. All samples were tested for malaria parasites (by RDT, microscopy and PCR), *Leptospira* spp., *Rickettsia* spp., *O. tsutsugamushi* (by PCR and sequencing), and dengue and influenza virus (by RT-PCR). In addition, blood culture bottles for the diagnosis of community acquired septicemia were tested within Group F. In 73.2 % of the 1,193 febrile outpatients, at least 1 pathogen could be identified in the taken samples, while in 26.8 % the etiology of the fever remained unknown. Most frequent pathogens detected amongst all recruited subjects, including asymptomatic Group N, were *P. vivax* (26.4 %), *P. falciparum* (20.7 %), pathogenic

Leptospira spp. (9.5 %), dengue virus (5.4 %), influenza virus A (5.9 %), *O. tsutsugamushi* (3.7 %), influenza virus B (1.8 %), *Rickettsia* spp. (0.2 %) and bacteria from blood culture (*Salmonella* spp., *E. coli*, *S. pneumoniae*, *E. cloacae*) (0.8 %). Furthermore, 2 cases of human infection with *P. knowlesi* were reported for the first time in Cambodia. The analysis of CRP-levels showed that CRP was significantly lower in subjects with viral infections than in bacterial infections and malaria, thus this marker could be used to rule out viral infections. The analysis of the clinical questionnaire did not provide useful information to help establish a clinical diagnosis. Difficulties occurred with the diagnostics of rickettsial disease and further research in collaboration with the study partners in Lao PDR should be enhanced on this subject. Given that 26.8 % of the study participants remained undiagnosed, it should be considered to conduct further tests on the stored samples. These could include tests for Japanese encephalitis virus, Chikungunya virus, Epstein-Barr-virus, Hepatitis A virus, Hepatitis E virus, Coxsackie virus and bacterial infections such as q-fever, brucellosis and melioidosis. However, the high incidence of a number of treatable causes of NMFI opened several questions on the need for revised clinical guidelines and better diagnostic tools. This study showed that of 1,193 febrile patients 67.9 % were malaria-RDT negative on admission, 37.0 % of which were actually carrying malaria parasites detected by more sensitive tests. This means that malaria cannot be ruled out by using RDT as the only diagnostic test in this kind of setting. In 24.9 % of the RDT-negative cases, other causes of febrile illness were detected in the samples, the majority of which were viruses (32.7 % influenza virus, 22.3 % *Leptospira* spp., 20.7 % dengue virus, 12.4 % *O. tsutsugamushi*, 11.9 % multiple pathogens and bacteria from blood culture). Lastly, it was shown that this kind of study protocol was feasible in a peripheral, non-hospital setting in rural areas of tropical countries like Cambodia.

These findings are helpful to establish a clinical algorithm and are making clear that tools to differentiate viral from bacterial disease as well as more sensitive diagnostic tests for malaria are needed urgently in rural areas of Cambodia.

5.2 Summary in German

Malaria war lange Zeit eines der vorherrschenden Gesundheitsprobleme in Südost Asien. In den letzten zehn Jahren wurden in Kambodscha erfolgreich verschiedene Strategien im Kampf gegen Malaria implementiert. Sowohl die Zahl der gemeldeten Malaria Fälle, als auch die Letalität sinken seitdem kontinuierlich. Durch die Einführung und landesweite Verteilung von Malaria-Schnelltests wurde jedoch gleichzeitig klar, dass in vielen der klinisch als Malaria diagnostizierten Fälle gar keine Malaria Parasiten nachgewiesen werden konnten. Aktuell gibt es keine klaren Richtlinien wie Patienten, deren Schnelltest für Malaria negativ ist, behandelt werden sollten. Dies birgt Probleme für das Gesundheitswesen, wie zum Beispiel die Fehlbehandlung von Malaria-negativen Patienten mit Antimalaria-Medikamenten oder die Nicht-Behandlung von Patienten mit potenziell tödlichen Fiebererkrankungen. Als ersten Schritt zur Entwicklung einer Leitlinie für das Management und die Behandlung von Malaria-negativen Fiebererkrankungen wurde eine beobachtende Querschnittstudie durchgeführt um die Ursachen für akute, undifferenzierte Fiebererkrankungen in ländlichen Gebieten Kambodschas zu untersuchen. Von Januar 2008 bis Dezember 2010 wurden insgesamt 1475 Personen in drei verschiedenen ambulanten Gesundheitszentren rekrutiert, zwei davon nahe der westlichen und eines an der östlichen Landesgrenze. 1193 der rekrutierten Personen waren ambulante Patienten mit Fieber (Gruppe F, Alter 7-50 Jahre, Körpertemperatur ≥ 38,5 °C, Fieber nicht länger als 8 Tage), die restlichen 282 Personen wurden als fieber-freie Vergleichsgruppe rekrutiert (Gruppe N, hauptsächlich gesunde Begleitpersonen). Von jedem Individuum wurden 15 ml Vollblut, ein Blutausstrich und ein Rachenabstrich abgenommen und zur molekularen, mikroskopischen und bakteriologischen Untersuchung in das zentrale Labor des Pasteur Institutes in der Hauptstadt Phnom Penh geschickt. Alle Proben wurden auf Malaria Parasiten (Schnelltest, Mikroskopie und PCR), Leptospiren, Rickettsien, *O. tsutsugamushi*, Dengue und Influenza Virus (PCR/RT-PCR), sowie ambulant erworbene bakterielle Sepsis (Blutkulturen) getestet. In 73,2 % der 1193 Fälle konnte mindestens ein Erreger in den Proben nachgewiesen werden, während in 26,8 % keine Ursache für das Fieber gefunden werden konnte. Die am häufigsten nachgewiesenen Erreger in allen Proben, inklusive der asymptomatischen Vergleichsgruppe, waren *P. vivax* (26,4 %), *P. falciparum* (20,7 %), pathogene *Leptospira* spp. (9,5 %), Dengue Virus (5,4 %), Influenza Virus A (5,9 %), *O. tsutsugamushi* (3,7 %),

Influenza Virus B (1,8 %), bakterielle Sepsis (*Salmonella* spp., *E. coli*, *S. pneumoniae*, *E. cloacae*) (0,8 %) und SFG-*Rickettsia* spp. (0,2 %). Außerdem wurde, zum ersten Mal in Kambodscha, in zwei Fällen *P. knowlesi* nachgewiesen. Die Analyse von CRP-Konzentrationen zeigte dass CRP bei viralen Infektionen signifikant niedriger war als bei bakteriellen Infektionen und Malaria, daher könnte dieser Parameter zum Ausschluss von viralen Infektionen genutzt werden. Die Auswertung des klinischen Fragebogens konnte keine hilfreichen Informationen zur Diagnostik beitragen. Die Diagnostik von Rickettsien gestaltete sich als schwierig, und weiterführende Forschung in Zusammenarbeit mit den Studienpartnern in Laos ist notwendig um ein besseres Verständnis dieser Erreger und ihrer Bedeutung in Kambodscha zu erlangen. Da nach wie vor bei 26,8 % der Patienten in Kambodscha kein Erreger nachgewiesen werden konnte, sollte in Betracht gezogen werden, weitere Tests durchzuführen. Denkbar wären zum Beispiel Tests für Japanische Enzephalitis Virus, Chikungunya Virus, Epstein-Barr-Virus, Hepatitis A Virus, Hepatitis E Virus, Coxsackie Virus oder bakterielle Infektionen wie Q-Fieber, Brucellose und Melioidose. Die hohe Zahl von behandelbaren Fieberursachen, die in dieser Studie nachgewiesen wurde konnten, unterstreicht die Notwendigkeit der Überarbeitung der aktuellen klinischen Richtlinien und der Verbesserung diagnostischer Mittel in ländlichen Gebieten Kambodschas. Es konnte gezeigt werden, dass die Mehrheit der ambulanten Patienten Malaria-Schnelltest negativ waren (67.9 %). In 37.0 % dieser RDT-negativen Patienten konnten mittels sensitiverer Methoden dennoch Malaria Parasiten nachgewiesen werden. Dies bedeutet, dass der Malariaschnelltest unter den vorherrschenden Bedingungen ungeeignet zum Ausschluss von Malaria war. In weiteren 24.9 % der Schnelltest-negativen Patienten konnten andere Erreger nachgewiesen werden, diese waren hauptsächlich Viren (32.7 % Influenza Virus, 22.3 % *Leptospira* spp, 20.7 % Dengue Virus, 12.4 % *O. tsutsugamushi*, 11.9 % mehrere oder andere Erreger). Zusätzlich konnte gezeigt werden, dass eine große Studie wie diese auch außerhalb des Krankenhausmilieus in tropischen Ländern wie Kambodscha überhaupt durchführbar ist.

Diese Ergebnisse tragen zur Findung klinischer Leitlinien zur Behandlung von akuten Fiebererkrankungen bei und verdeutlichen, dass sowohl Mittel zur Unterscheidung von viralen und bakteriellen Erkrankungen als auch sensitivere Tests zur Malaria Diagnostik in ländlichen Gebieten Kambodschas dringend benötigt werden.

6 References

1. Crump, J.A., S. Gove, and C.M. Parry, *Management of adolescents and adults with febrile illness in resource limited areas.* Bmj, 2011. **343**: p. d4847.
2. World Health Organization, I.H., *World Malaria Report 2010*. 2010: Geneva.
3. National Centre for Parasitology, E.a.M.C.C., *Annual Progress Report 2010*. 2010, Ministry of Health: Phnom Penh, Cambodia.
4. Wongsrichanalai, C., et al., *Epidemiology of drug-resistant malaria.* Lancet Infect Dis, 2002. **2**(4): p. 209-18.
5. National Institute of Public Health and National Institute of Statistics Phnom Penh, C. and M. ORC Macro Calverton, USA, *Cambodia Demographic and Health Survey 2010*. 2011, USAID, Measure DHS: Phnom Penh, Cambodia.
6. World Health Organization, W.P.R.O., *Country Health Information Profile Cambodia*. 2011, World Health Organization (Western Pacific Regional Office): Manila, Phillipines.
7. Knowles, J.C., *Health, Vulnerability and Poverty in Cambodia, Analysis of the 2004 Cambodia Socio-Economic Survey*. 2005, The World Bank: Washington DC, USA.
8. Chatterjee, P., *Cambodia's fight against malaria.* Lancet, 2005. **366**(9481): p. 191-2.
9. Ministry of Health, D.o.P.a.H.I., *National Health Statistics 2007*. 2008, Ministry of Health: Phnom Penh, Cambodia.
10. Huy, R., et al., *Cost of dengue and other febrile illnesses to households in rural Cambodia: a prospective community-based case-control study.* BMC Public Health, 2009. **9**: p. 155.
11. Sadanand, S., *Malaria: an evaluation of the current state of research on pathogenesis and antimalarial drugs.* Yale J Biol Med, 2010. **83**(4): p. 185-91.
12. Kantele, A. and T.S. Jokiranta, *Review of cases with the emerging fifth human malaria parasite, Plasmodium knowlesi.* Clin Infect Dis, 2011. **52**(11): p. 1356-62.
13. Tuteja, R., *Malaria - an overview.* FEBS J, 2007. **274**(18): p. 4670-9.
14. Bell, D., C. Wongsrichanalai, and J.W. Barnwell, *Ensuring quality and access for malaria diagnosis: how can it be achieved?* Nat Rev Microbiol, 2006. **4**(9): p. 682-95.
15. Delacollette, C., et al., *Malaria trends and challenges in the Greater Mekong Subregion.* Southeast Asian J Trop Med Public Health, 2009. **40**(4): p. 674-91.
16. National Centre for Parasitology, E.a.M.C.C., *Annual Progress Report 2009*. 2009, Ministry of Health: Phnom Penh, Cambodia.
17. Incardona, S., et al., *Large-scale malaria survey in Cambodia: novel insights on species distribution and risk factors.* Malar J, 2007. **6**: p. 37.
18. Yeung, S., et al., *Socially-marketed rapid diagnostic tests and ACT in the private sector: ten years of experience in Cambodia.* Malar J, 2011. **10**: p. 243.
19. Trung, H.D., et al., *Malaria transmission and major malaria vectors in different geographical areas of Southeast Asia.* Trop Med Int Health, 2004. **9**(2): p. 230-7.
20. World Health Organization, I.H., *Global Report on Antimalarial Drug Efficacy and Drug Resitance, 2000-2010*. 2010: Geneva.
21. Wongsrichanalai, C., et al., *Drug resistant malaria on the Thai-Myanmar and Thai-Cambodian borders.* Southeast Asian J Trop Med Public Health, 2001. **32**(1): p. 41-9.
22. National Centre for Parasitology, E.a.M.C., *National Treatment Guideline for Malaria*, M.o. Health, Editor. 2004.

23. Wilde, H. and C. Suankratay, *There is need for antigen-based rapid diagnostic tests to identify common acute tropical illnesses.* J Travel Med, 2007. **14**(4): p. 254-8.
24. Lee, A., et al., *Detection of bloodstream infections in adults: how many blood cultures are needed?* J Clin Microbiol, 2007. **45**(11): p. 3546-8.
25. Ellis, R.D., et al., *Causes of fever in adults on the Thai-Myanmar border.* Am J Trop Med Hyg, 2006. **74**(1): p. 108-13.
26. Leelarasamee, A., et al., *Etiologies of acute undifferentiated febrile illness in Thailand.* J Med Assoc Thai, 2004. **87**(5): p. 464-72.
27. Phuong, H.L., et al., *Acute undifferentiated fever in Binh Thuan province, Vietnam: imprecise clinical diagnosis and irrational pharmaco-therapy.* Trop Med Int Health, 2006. **11**(6): p. 869-79.
28. Pickard, A.L., et al., *A study of'febrile illnesses on the Thai-Myanmar border: predictive factors of rickettsioses.* Southeast Asian J Trop Med Public Health, 2004. **35**(3): p. 657-63.
29. Pradutkanchana, J., et al., *The etiology of acute pyrexia of unknown origin in children after a flood.* Southeast Asian J Trop Med Public Health, 2003. **34**(1): p. 175-8.
30. Sripanidkulchai, R. and P. Lumbiganon, *Etiology of obscure fever in children at a university hospital in northeast Thailand.* Southeast Asian J Trop Med Public Health, 2005. **36**(5): p. 1243-6.
31. Suttinont, C., et al., *Causes of acute, undifferentiated, febrile illness in rural Thailand: results of a prospective observational study.* Ann Trop Med Parasitol, 2006. **100**(4): p. 363-70.
32. McGready, R., et al., *Arthropod borne disease: the leading cause of fever in pregnancy on the Thai-Burmese border.* PLoS Negl Trop Dis, 2010. **4**(11): p. e888.
33. Victoriano, A.F., et al., *Leptospirosis in the Asia Pacific region.* BMC Infect Dis, 2009. **9**: p. 147.
34. Nalam, K., et al., *Genetic affinities within a large global collection of pathogenic Leptospira: implications for strain identification and molecular epidemiology.* PLoS One, 2010. **5**(8): p. e12637.
35. World Health Organization, I.L.S., *Human Leptospirosis:Guidance for Diagnosis,Surveillance and Control*. 2003, WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis, Royal Tropical Institute (KIT), Biomedical Research: Amsterdam.
36. Ariyapruchya, B., S. Sungkanuparph, and S. Dumrongkitchaiporn, *Clinical presentation and medical complication in 59 cases of laboratory-confirmed leptospirosis in Bangkok.* Southeast Asian J Trop Med Public Health, 2003. **34**(1): p. 159-64.
37. de Abreu Fonseca, C., et al., *Polymerase chain reaction in comparison with serological tests for early diagnosis of human leptospirosis.* Trop Med Int Health, 2006. **11**(11): p. 1699-707.
38. Merien, F., et al., *A rapid and quantitative method for the detection of Leptospira species in human leptospirosis.* FEMS Microbiol Lett, 2005. **249**(1): p. 139-47.
39. Suputtamongkol, Y., et al., *Strategies for diagnosis and treatment of suspected leptospirosis: a cost-benefit analysis.* PLoS Negl Trop Dis, 2010. **4**(2): p. e610.
40. Kawaguchi, L., et al., *Seroprevalence of leptospirosis and risk factor analysis in flood-prone rural areas in Lao PDR.* Am J Trop Med Hyg, 2008. **78**(6): p. 957-61.

41. Laras, K., et al., *The importance of leptospirosis in Southeast Asia.* Am J Trop Med Hyg, 2002. **67**(3): p. 278-86.
42. Seng, H., et al., *Leptospirosis in Takeo Province, Kingdom of Cambodia, 2003.* J Med Assoc Thai, 2007. **90**(3): p. 546-51.
43. Wuthiekanun, V., et al., *Clinical diagnosis and geographic distribution of leptospirosis, Thailand.* Emerg Infect Dis, 2007. **13**(1): p. 124-6.
44. Berlioz-Arthaud, A., et al., *[Hospital-based active surveillance of human leptospirosis in Cambodia].* Bull Soc Pathol Exot, 2010. **103**(2): p. 111-8.
45. Syhavong, B., et al., *The infective causes of hepatitis and jaundice amongst hospitalised patients in Vientiane, Laos.* Trans R Soc Trop Med Hyg, 2010. **104**(7): p. 475-83.
46. Sejvar, J., et al., *An outbreak of leptospirosis, Thailand--the importance of the laboratory.* Southeast Asian J Trop Med Public Health, 2005. **36**(2): p. 289-95.
47. Tangkanakul, W., et al., *Leptospirosis: an emerging health problem in Thailand.* Southeast Asian J Trop Med Public Health, 2005. **36**(2): p. 281-8.
48. Renvoise, A. and D. Raoult, *[An update on rickettsiosis.].* Med Mal Infect, 2008.
49. Parola, P. and D. Raoult, *Tropical rickettsioses.* Clin Dermatol, 2006. **24**(3): p. 191-200.
50. Watt, G. and P. Parola, *Scrub typhus and tropical rickettsioses.* Curr Opin Infect Dis, 2003. **16**(5): p. 429-36.
51. Phongmany, S., et al., *Rickettsial infections and fever, Vientiane, Laos.* Emerg Infect Dis, 2006. **12**(2): p. 256-62.
52. Parola, P., et al., *Emerging rickettsioses of the Thai-Myanmar border.* Emerg Infect Dis, 2003. **9**(5): p. 592-5.
53. Gimenez, D.F., *Staining Rickettsiae in yolk-sac cultures.* Stain Technol., 1964(39): p. 135-140.
54. Duffy, P.E., et al., *Murine typhus identified as a major cause of febrile illness in a camp for displaced Khmers in Thailand.* Am J Trop Med Hyg, 1990. **43**(5): p. 520-6.
55. Corwin, A.L., et al., *Short report: surveillance of rickettsial infections in Indonesian military personnel during peace keeping operations in Cambodia.* Am J Trop Med Hyg, 1997. **57**(5): p. 569-70.
56. Suputtamongkol, Y., et al., *Epidemiology and clinical aspects of rickettsioses in Thailand.* Ann N Y Acad Sci, 2009. **1166**: p. 172-9.
57. Kelly, D.J., et al., *Scrub typhus: the geographic distribution of phenotypic and genotypic variants of Orientia tsutsugamushi.* Clin Infect Dis, 2009. **48 Suppl 3**: p. S203-30.
58. Duong, V., et al., *Diversity of Orientia tsutsugamushi clinical isolates in Cambodia reveals active selection and recombination process.* Infect Genet Evol, 2010.
59. Moron, C.G., et al., *Identification of the target cells of Orientia tsutsugamushi in human cases of scrub typhus.* Mod Pathol, 2001. **14**(8): p. 752-9.
60. Walsh, D.S., et al., *Orientia tsutsugamushi in peripheral white blood cells of patients with acute scrub typhus.* Am J Trop Med Hyg, 2001. **65**(6): p. 899-901.
61. Sonthayanon, P., et al., *Rapid diagnosis of scrub typhus in rural Thailand using polymerase chain reaction.* Am J Trop Med Hyg, 2006. **75**(6): p. 1099-102.
62. Paris, D.H., et al., *Real-time multiplex PCR assay for detection and differentiation of rickettsiae and orientiae.* Trans R Soc Trop Med Hyg, 2008. **102**(2): p. 186-93.
63. Watt, G., et al., *Scrub typhus infections poorly responsive to antibiotics in northern Thailand.* Lancet, 1996. **348**(9020): p. 86-9.

64. Kyle, J.L. and E. Harris, *Global Spread and Persistence of Dengue.* Annu Rev Microbiol, 2008.
65. World Health Organization, I.H., *Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. New edition.* 2009, World Health Organization Geneva.
66. Beaute, J. and S. Vong, *Cost and disease burden of dengue in Cambodia.* BMC Public Health, 2010. **10**: p. 521.
67. Lanciotti, R.S., et al., *Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction.* J Clin Microbiol, 1992. **30**(3): p. 545-51.
68. Lolekha, R., et al., *Diagnosis of dengue infection using various diagnostic tests in the early stage of illness.* Southeast Asian J Trop Med Public Health, 2004. **35**(2): p. 391-5.
69. Huy, R., et al., *National dengue surveillance in Cambodia 1980-2008: epidemiological and virological trends and the impact of vector control.* Bull World Health Organ, 2010. **88**(9): p. 650-7.
70. Vong, S., et al., *Dengue incidence in urban and rural cambodia: results from population-based active Fever surveillance, 2006-2008.* PLoS Negl Trop Dis, 2010. **4**(11): p. e903.
71. Phuong, H.L., et al., *Dengue as a cause of acute undifferentiated fever in Vietnam.* BMC Infect Dis, 2006. **6**: p. 123.
72. Nicholson, K.G., J.M. Wood, and M. Zambon, *Influenza.* Lancet, 2003. **362**(9397): p. 1733-45.
73. Blair, P.J., et al., *Influenza epidemiology and characterization of influenza viruses in patients seeking treatment for acute fever in Cambodia.* Epidemiol Infect, 2010. **138**(2): p. 199-209.
74. Sreng, B., et al., *A description of influenza-like illness (ILI) sentinel surveillance in Cambodia, 2006-2008.* Southeast Asian J Trop Med Public Health, 2010. **41**(1): p. 97-104.
75. Mardy, S., et al., *Influenza activity in Cambodia during 2006-2008.* BMC Infect Dis, 2009. **9**: p. 168.
76. Crump, J.A. and E.D. Mintz, *Global trends in typhoid and paratyphoid Fever.* Clin Infect Dis, 2010. **50**(2): p. 241-6.
77. World Health Organization, I.H., *Background document: The diagnosis, treatment and prevention of typhoid fever*. 2003, The Department of Vaccines and Biologicals: Geneva.
78. Keddy, K.H., et al., *Sensitivity and specificity of typhoid fever rapid antibody tests for laboratory diagnosis at two sub-Saharan African sites.* Bull World Health Organ, 2011. **89**(9): p. 640-7.
79. Naheed, A., et al., *Clinical value of Tubex and Typhidot rapid diagnostic tests for typhoid fever in an urban community clinic in Bangladesh.* Diagn Microbiol Infect Dis, 2008. **61**(4): p. 381-6.
80. Gasem, M.H., et al., *Evaluation of a simple and rapid dipstick assay for the diagnosis of typhoid fever in Indonesia.* J Med Microbiol, 2002. **51**(2): p. 173-7.
81. Anusha, R., R. Ganesh, and J. Lalitha, *Comparison of a rapid commercial test, Enterocheck WB((R)), with automated blood culture for diagnosis of typhoid fever.* Ann Trop Paediatr, 2011. **31**(3): p. 231-4.

82. Kasper, M.R., et al., *Emergence of multidrug-resistant Salmonella enterica serovar Typhi with reduced susceptibility to fluoroquinolones in Cambodia.* Diagn Microbiol Infect Dis, 2010. **66**(2): p. 207-9.
83. Wijedoru, L.P., et al., *Typhoid Fever among Hospitalized Febrile Children in Siem Reap, Cambodia.* J Trop Pediatr, 2011.
84. Rammaert, B., et al., *Pulmonary melioidosis in Cambodia: a prospective study.* BMC Infect Dis, 2011. **11**: p. 126.
85. Limmathurotsakul, D., et al., *Increasing incidence of human melioidosis in Northeast Thailand.* Am J Trop Med Hyg, 2010. **82**(6): p. 1113-7.
86. Wuthiekanun, V., et al., *Burkholderia pseudomallei antibodies in children, Cambodia.* Emerg Infect Dis, 2008. **14**(2): p. 301-3.
87. Vlieghe, E., et al., *Melioidosis, phnom penh, Cambodia.* Emerg Infect Dis, 2011. **17**(7): p. 1289-92.
88. Erlanger, T.E., et al., *Past, present, and future of Japanese encephalitis.* Emerg Infect Dis, 2009. **15**(1): p. 1-7.
89. Srey, V.H., et al., *Etiology of encephalitis syndrome among hospitalized children and adults in Takeo, Cambodia, 1999-2000.* Am J Trop Med Hyg, 2002. **66**(2): p. 200-7.
90. Touch, S., et al., *Epidemiology and burden of disease from Japanese encephalitis in Cambodia: results from two years of sentinel surveillance.* Tropical Medicine & International Health, 2009. **14**(11): p. 1365-1373.
91. Touch, S., et al., *A cost-effectiveness analysis of Japanese encephalitis vaccine in Cambodia.* Vaccine, 2010. **28**(29): p. 4593-4599.
92. World Health Organization, I.H., *Basic Malaria Microscopy, Part I. Learner's Guide*. Basic Malaria Microscopy. 2010: WHO. 75.
93. Mayxay, M., et al., *Mixed-species malaria infections in humans.* Trends Parasitol, 2004. **20**(5): p. 233-40.
94. Ekala, M.T., et al., *Sequence analysis of Plasmodium falciparum cytochrome b in multiple geographic sites.* Malar J, 2007. **6**: p. 164.
95. Steenkeste, N., et al., *Towards high-throughput molecular detection of Plasmodium: new approaches and molecular markers.* Malar J, 2009. **8**: p. 86.
96. Merien, F., et al., *Polymerase chain reaction for detection of Leptospira spp. in clinical samples.* J Clin Microbiol, 1992. **30**(9): p. 2219-24.
97. Stenos, J., S.R. Graves, and N.B. Unsworth, *A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group Rickettsiae.* Am J Trop Med Hyg, 2005. **73**(6): p. 1083-5.
98. Jiang, J., et al., *Development of a quantitative real-time polymerase chain reaction assay specific for Orientia tsutsugamushi.* Am J Trop Med Hyg, 2004. **70**(4): p. 351-6.
99. Buecher, C., et al., *Use of a multiplex PCR/RT-PCR approach to assess the viral causes of influenza-like illnesses in Cambodia during three consecutive dry seasons.* J Med Virol, 2010. **82**(10): p. 1762-72.
100. Woolson, R.F. and J.A. Bean, *Mantel-Haenszel statistics and direct standardization.* Stat Med, 1982. **1**(1): p. 37-9.
101. Dupont, W.D., *Sensitivity of Fisher's exact test to minor perturbations in 2 x 2 contingency tables.* Stat Med, 1986. **5**(6): p. 629-35.
102. Zou, K.H., et al., *Hypothesis testing I: proportions.* Radiology, 2003. **226**(3): p. 609-13.

103. Farnert, A., et al., *Sampling and storage of blood and the detection of malaria parasites by polymerase chain reaction.* Trans R Soc Trop Med Hyg, 1999. **93**(1): p. 50-3.
104. Okell, L.C., et al., *Submicroscopic infection in Plasmodium falciparum-endemic populations: a systematic review and meta-analysis.* J Infect Dis, 2009. **200**(10): p. 1509-17.
105. Steenkeste, N., et al., *Sub-microscopic malaria cases and mixed malaria infection in a remote area of high malaria endemicity in Rattanakiri province, Cambodia: implication for malaria elimination.* Malar J, 2010. **9**: p. 108.
106. Lathia, T.B. and R. Joshi, *Can hematological parameters discriminate malaria from nonmalarious acute febrile illness in the tropics?* Indian J Med Sci, 2004. **58**(6): p. 239-44.
107. Kitvatanachai, S., et al., *A survey on malaria in mobile Cambodians in Aranyaprathet, Sa Kaeo Province, Thailand.* Southeast Asian J Trop Med Public Health, 2003. **34**(1): p. 48-53.
108. Whitehorn, J., et al., *A mixed malaria infection: is Plasmodium vivax good for you?* Trans R Soc Trop Med Hyg, 2010. **104**(3): p. 240-1.
109. Marangi, M., et al., *Prevalence of Plasmodium spp. in malaria asymptomatic African migrants assessed by nucleic acid sequence based amplification.* Malar J, 2009. **8**: p. 12.
110. Khim N, S.S., Kim S, Mueller T, Fleischmann E, Singh B, et al. , *Plasmodium knowlesi infection in humans, Cambodia, 2007–2010.* Emerg Infect Dis, 2011. **17 October 2011**(10).
111. Lai, C.H., et al., *Epidemiology of acute q Fever, scrub typhus, and murine typhus, and identification of their clinical characteristics compared to patients with acute febrile illness in southern taiwan.* J Formos Med Assoc, 2009. **108**(5): p. 367-76.
112. Watthanaworawit, W., et al., *A prospective evaluation of diagnostic methodologies for the acute diagnosis of dengue virus infection on the Thailand-Myanmar border.* Trans R Soc Trop Med Hyg, 2011. **105**(1): p. 32-7.
113. Ntusi, N., et al., *Guideline for the optimal use of blood cultures.* S Afr Med J, 2010. **100**(12): p. 839-43.
114. Singhsilarak, T., et al., *Possible acute coinfections in Thai malaria patients.* Southeast Asian J Trop Med Public Health, 2006. **37**(1): p. 1-4.
115. Carme, B., et al., *Concurrent dengue and malaria in Cayenne Hospital, French Guiana.* Emerg Infect Dis, 2009. **15**(4): p. 668-71.
116. Kaushik, R.M., et al., *Concurrent dengue and malaria due to Plasmodium falciparum and P. vivax.* Trans R Soc Trop Med Hyg, 2007. **101**(10): p. 1048-50.
117. Abbasi, A., et al., *Clinical features, diagnostic techniques and management of dual dengue and malaria infection.* J Coll Physicians Surg Pak, 2009. **19**(1): p. 25-9.
118. Watt, G., K. Jongsakul, and C. Suttinont, *Possible scrub typhus coinfections in Thai agricultural workers hospitalized with leptospirosis.* Am J Trop Med Hyg, 2003. **68**(1): p. 89-91.
119. Lee, C.H. and J.W. Liu, *Coinfection with leptospirosis and scrub typhus in Taiwanese patients.* Am J Trop Med Hyg, 2007. **77**(3): p. 525-7.
120. Schuetz, P., W. Albrich, and B. Mueller, *Procalcitonin for diagnosis of infection and guide to antibiotic decisions: past, present and future.* BMC Med, 2011. **9**: p. 107.

121. Blacksell, S.D., et al., *Accuracy of AccessBio Immunoglobulin M and Total Antibody Rapid Immunochromatographic Assays for the Diagnosis of Acute Scrub Typhus Infection.* Clin Vaccine Immunol, 2010. **17**(2): p. 263-6.
122. Bajani, M.D., et al., *Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis.* J Clin Microbiol, 2003. **41**(2): p. 803-9.
123. Fry, S.R., et al., *The diagnostic sensitivity of dengue rapid test assays is significantly enhanced by using a combined antigen and antibody testing approach.* PLoS Negl Trop Dis, 2011. **5**(6): p. e1199.
124. Phuong, H.L., et al., *Randomised primary health center based interventions to improve the diagnosis and treatment of undifferentiated fever and dengue in Vietnam.* BMC Health Serv Res, 2010. **10**: p. 275.
125. Tsao, K.C., et al., *Performance of rapid-test kits for the detection of the pandemic influenza A/H1N1 virus.* J Virol Methods, 2011. **173**(2): p. 387-9.
126. Pongsiri, P., et al., *Entire genome characterization of Chikungunya virus from the 2008-2009 outbreaks in Thailand.* Trop Biomed, 2010. **27**(2): p. 167-76.

7 List of abbreviations

ACT – Artemisinin combination therapy

AIDS – Acquired immunodeficiency syndrome

ARI – Acute respiratory infection

AUFI – Acute undifferentiated febrile illness

bp – Base pairs

CAS – Community acquired septicemia

cDNA – Copy deoxyribonucleic acid

CNM – Cambodian National Centre for Parasitology, Entomology and Malaria Control

CRP – C-reactive protein

CSF – Cerebrospinal fluid

DENV1-4 – Dengue virus serotype 1-4

DHF – Dengue hemorrhagic fever

DIC – Disseminated intravascular coagulation

DNA – Deoxyribonucleic acid

dNTP – Deoxy-nucleotid-triphosphate

DSS – Dengue shock syndrome

EDTA – Ethylene-diamine-tetra-acetic-acid

ELISA – Enzyme-linked immunosorbent assay

GDP – Gross domestic product

GFATM – Global Fund to fight AIDS, Tuberculosis and Malaria

HDI – Human Development Index

HIS – Health information system

HIV – Human immunodeficiency virus

HRP2 – Histidine-rich protein 2

IFA – Immunofluorescence assay

IFAT – Indirect fluorescent antibody test

Ig – Immunoglobulin

ILI – Influenza like illness

IPC – Institut Pasteur du Cambodge (Cambodian Pasteur Institute)

ITN – Insecticide-treated bed nets

JEV – Japanese encephalitis virus

LAMP – Loop-mediated isothermal amplification

Lao PDR – Lao People Democratic Republic

MAC-ELISA – IgM-capture enzyme-linked immunosorbent assay

MAT – Microscopic agglutination test

MDR – Multi-drug resistance

MoH – Ministry of Health

n/a – Not applicable

NGO – Non Governmental Organization

NMCP – National Malaria Control Program

nPCR – Nested polymerase chain reaction

OD – Operational district

PCR – Polymerase chain reaction

PF – *Plasmodium falciparum*

pLDH– *Plasmodium* lactate dehydrogenase

PM – *Plasmodium malariae*

PO – *Plasmodium ovale*

PV – *Plasmodium vivax*

RBC – Red blood cell

RDT – Rapid diagnostic test

RNA – Ribonucleic acid

RT-PCR – Reverse transcriptase polymerase chain reaction

SFG – Spotted fever group (*Rickettsia*)

SMCC – Society of Malaria Control in Cambodia

SNP – Single nucleotide polymorphism

Spp. – Species

Taq-Polymerase – *Thermus aquaticus* DNA-Polymerase

TG – Typhus group (*Rickettsia*)

USAID – United States Agency for International Development

UV – Ultraviolet

VMW – Village malaria worker

VTM – Viral transport medium

List of abbreviations

WHO – World Health Organization

WHO/WPRO – World Health Organization, Western Pacific Regional Office

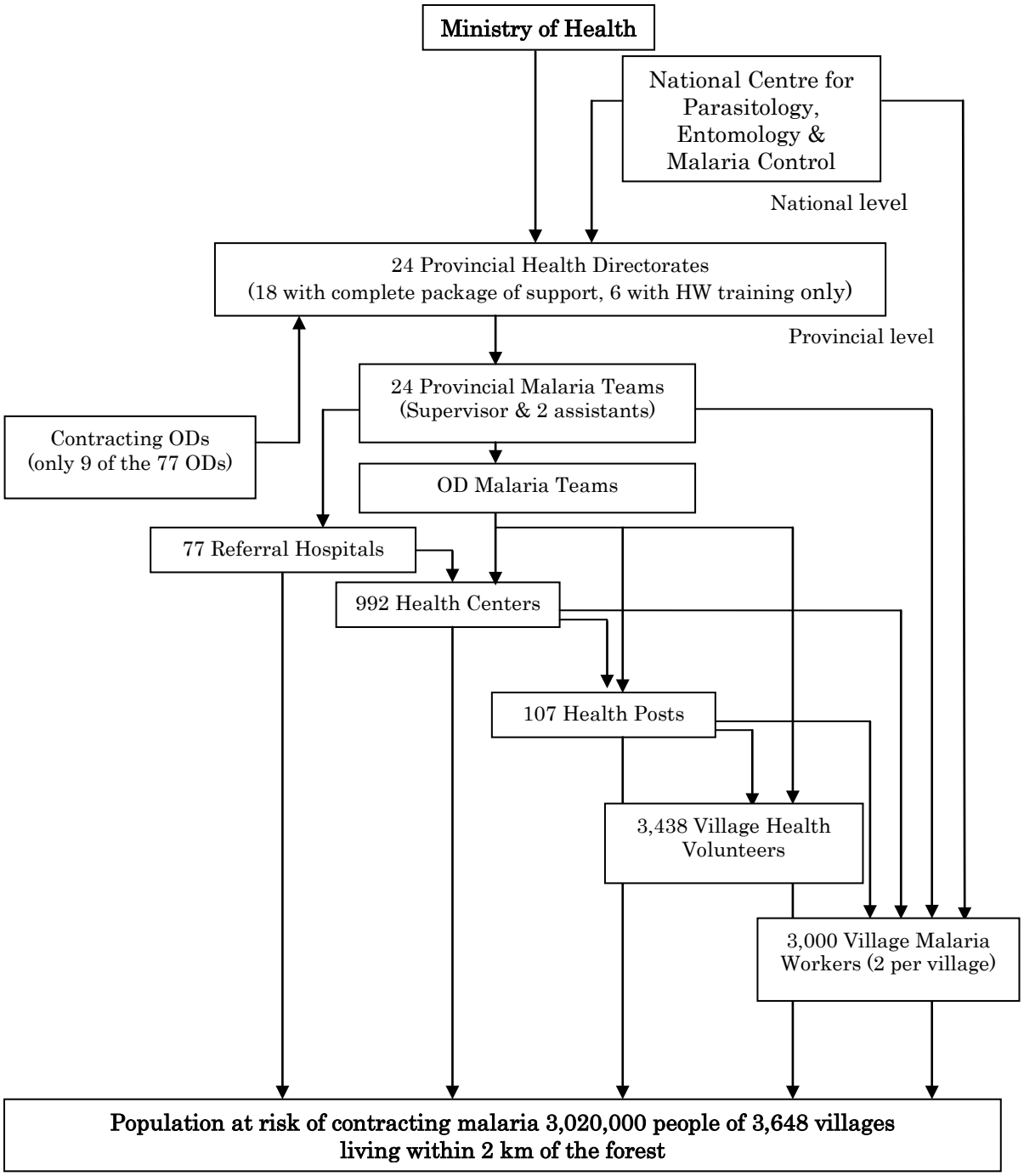
8 List of figures

9 Appendices

9.1 Socio-demographic indicators of Cambodia

Table 38: Socio-demographic indicators of Cambodia (source: Ministry of Health, Cambodia, 2007)	
Population	
Total	14,363,519
Urban	15.0 %
Rural	85.0 %
Males	48.3 %
Females	51.7 %
Gender ratio (number of males for 100 females)	93.5
Distribution of population by age group	
0-4 years	11.1 %
0-14 years	38.6 %
5-14 years	27.5 %
15-49 years	26.0 %
Annual Population Growth Rate	1.81 %
Male life expectancy at birth	58
Female life expectancy at birth	64
Number of households	2,530,000
Average household size	5.1
Population density per km²	74
Health	
Infant Mortality Rate	65 per 1,000 live births
Under 5 Mortality Ratio	83 per 1,000 live births
Maternal Mortality Ratio	472 per 100,000 live births
Crude Birth Rate	25.6 per 1,000 population
Contraceptive Prevalence Rate (any method)	27.0 %
Households with access to safe drinking water	44.0%
Urban	72.0 %
Rural	40.0 %
Households with toilet facility within premise	22.0 %
Urban	55.0 %
Rural	16.0 %
Education	
Adult literacy (age >15)	73.6 %
Male	84.7 %
Female	64.1 %
Urban (both sexes)	83.8 %
Rural (both sexes)	71.7 %
Economics	
Government expenditures on health care per capita per year	4.64 US$

9.2 Flowchart of Cambodia's health system structure



Source: CNM Annual Progress report, 2010, Cambodia.

9.3 Leading causes of mortality and morbidity in Cambodia

Table 39: Leading causes of mortality and morbidity in inpatient care, Cambodia 2005 and 2010 (source: WHO, National Health Statistics 2005 and 2010).

Year	Leading causes of morbidity	Number of cases	Rate per 100,000 population
2005	1. Acute respiratory Infections	2,236,262	17,084.44
	2. Diarrhea	356,273	2,721.51
	3. Tuberculosis	21,406	163.52
	4. Traffic accidents	14,035	107.21
	5. Dengue Hemorrhagic Fever	9,965	76.12
	6. Malaria	6,412	48.98
	7. Dysentery	5,129	39.18
	8. Meningitis	1,692	12.92
	9. Mine accidents	438	3.35
	10. Breast cancer	393	3.0
2010	1. Acute respiratory Infections	78,288	555.23
	2. Diarrhea	49,347	349.98
	3. Tuberculosis	28,384	201.30
	4. Typhoid fever	15,252	108.17
	5. Dengue fever	12,500	89.10
	6. Gynecological pathology	11,190	79.36
	7. Traffic accident	10,591	75.11
	8. High blood pressure	10,036	71.18
	9. Cataract	6,092	43.21
	10. AIDS	5,038	35.73
	Leading causes of mortality	**Number of cases**	**Rate per 100,000 population**
2005	1. Acute respiratory Infections	818	6.25
	2. Tuberculosis	313	2.39
	3. Malaria	296	2.26
	4. Road accidents	281	2.15
	5. Dengue Hemorrhagic Fever	190	1.45
	6. Meningitis	163	1.25
	7. Diarrhea	38	0.29
	8. Mine accidents	31	0.24
	9. Other tetanus	28	0.21
	10. Liver cancer	20	0.15
2010	1. Acute respiratory Infections	1,135	8.05
	2. Traffic accidents	495	3.51
	3. High blood pressure	468	3.32
	4. AIDS	280	1.99
	5. Tuberculosis	261	1.85
	6. Cardiovascular disease	256	1.82
	7. Meningitis	196	1.39
	8: Dengue	38	0.30
	9. Other tetanus	32	0.23
	10. Liver cancer	17	0.12

9.4 Main health problems among inpatients in Cambodian hospitals

Table 40: Main health problems and fatality rates among inpatients in Cambodian referral hospitals by age group (source: Ministry of Health, Cambodia, 2007).

	Age group									
	0-4 years		5-14 years		15-49 years		≥50 years		Total	
Disease	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths
Diarrhea	4,962	7	812	2	1,936	22	482	3	8,192	34
Dys-entery	926	2	378	0	723	0	222	0	2,249	2
Cholera	15	0	0	0	12	0	6	0	33	0
ARI	16,228	375	5,693	31	6,895	182	5,231	150	34,047	738
Simple malaria	409	1	755	1	2,608	2	290	0	4,062	4
Severe malaria	299	20	546	35	1,592	142	212	25	2,649	222
Simple dengue	3,996	1	6,203	0	676	0	19	0	10,894	1
Severe dengue	1,475	86	3,365	143	405	22	6	0	5,251	251
Typhoid fever	699	3	2,818	5	4,349	9	895	1	8,761	18
Total	29,009	495	20,570	217	19,196	379	7,363	179	76,138	1,270

Table 41: Main health problems and fatality rates among inpatients in Cambodian national hospitals by age group (source: Ministry of Health, Cambodia, 2007).

	Age group									
	0-4 years		5-14 years		15-49 years		≥50 years		Total	
Disease	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths
Diarrhea	6,503	16	101	0	143	2	70	3	6,817	21
Dys-entery	1,400	1	65	0	36	0	36	0	1,537	1
Cholera	3	0	1	0	5	2	0	0	19	2
ARI	18,778	280	1,380	14	926	23	465	30	21,549	347
Simple malaria	15	0	34	0	43	0	12	0	104	0
Severe malaria	198	1	193	1	56	12	13	1	460	15
Simple dengue	1,315	0	2,425	1	102	0	1	0	3,843	1
Severe dengue	5,872	103	9,896	102	7	2	1	0	15,776	207
Typhoid fever	86	0	265	0	132	1	56	0	539	1
Total	34,170	401	14,360	118	1,450	42	654	34	50,644	595

9.5 List of all currently recognized *Rickettsia* species

Table 42: List of all currently recognized Rickettsia species (Source: Renvoise et.al, 2008 [48]).

Species	Pathogenicity or disease
Typhus group	
Rickettsia typhi	Murine typhus
Rickettsia prowazekii	Epidemic typhus
Rickettsia Canadensis	Unknown pathogenicity
Not classified	
Rickettsia belli	Unknown pathogenicity
Spotted-fever-group	
Rickettsia akari	Rickettsialpox
Rickettsia australis	Queensland tick typhus
Rickettsia asiatica	Unknown pathogenicity
Rickettsia felis	Flea-borne spotted fever
Rickettsia Helvetica	Aneruptive fever
Rickettsia tamurae	Unknown pathogenicity
Rickettsia massiliae	Spotted fever
Rickettsia aeschlimannii	Spotted fever
Rickettsia montanensis	Unknown pathogenicity
Rickettsia rhipicephali	Unknown pathogenicity
Rickettsia sibirica subsp. sibirica	Siberian tick typhus
Rickettsia rickettsii	Rocky Mountain spotted fever
Rickettsia sibirica subsp. mongolitimonae	LAR
Rickettsia slovaca	TIBOLA-DEBONEL*
Rickettsia africae	African tick bite fever
Rickettsia conorii subsp. Conorii	Mediterranean spotted fever
Rickettsia conorii subsp. Israelensis	Israeli spotted fever
Rickettsia conorii subsp. Caspia	Astrakhan fever
Rickettsia conorii subsp. Indica	Indian tick bite typhus
Rickettsia heilongjiangensis	Far Eastern tick-borne rickettsiosis
Rickettsia japonica	Japanese or oriental spotted fever
Rickettsia peacockii	Unknown pathogenicity
Rickettsia raoulti	DEBONEL-TIBOLA*
Rickettsia parkeri	Spotted fever
Rickettsia honei	Flinders Island spotted fever
Species described but not yet recognized	
Rickettsia amblyommii	Unknown pathogenicity
Rickettsia andeana	Unknown pathogenicity
Rickettsia hulinii	Unknown pathogenicity
Rickettsia cooleyi	Unknown pathogenicity
Rickettsia martinet	Unknown pathogenicity
Rickettsia tarasevichiae	Unknown pathogenicity
Rickettsia monacensis	Spotted fever
Rickettsia marmionii	Australian spotted fever

* TIBOLA (tick-borne lymphadenitis) or DEBONEL (dermacentor-borne necrosis erythema lymphadenopathy)

9.6 Consent form sheet

CONSENT FORM SHEET

PROTOCOL TITLE:
A cross-sectional observational study to identify the causes of acute non-malaria febrile Illness in out-patients in rural Cambodia.

VOLUNTARY CONSENT TO PARTICIPATE:
The study is sponsor by WPRO in collaboration with the National Centre for Parasitology, Entomology and Malaria Control in Phnom Penh and the University of Munich and funded by USAID. The project is under the direction of Dr. Siv Sovannaroth from the National Centre for Parasitology, Entomology and Malaria Control (Monivong Blvd., Phnom Penh, Cambodia, P.O. box 1062, Tel.: 855 23 211 926/216 855 16 364 537, Fax. 855 23 996 202)

I hereby confirm that I fully understand what they has been explained in the information sheet by project representatives in a way that is understandable and satisfactory to me. I have been informed of the advantages and disadvantages of this research study that I shall be given.
I do not require further information to make my decision as to whether or not I want to donate blood.

___/___/___	_____	_____
Date (dd/mm/yyyy)	Name of participant	Signature or fingerprint
___/___/___	_____	_____
Date (dd/mm/yyyy)	Name of witness	Signature or fingerprint
___/___/___	_____	_____
Date (dd/mm/yyyy)	Name of field investigator	Signature

Thank you very much for your cooperation.

9.7 Example of clinical data sheet

1 Basic info							
Country site	*C-1*	Visit-Nr.	*1*	ID-Nr.	Visit-date		
Age	*23*	Gender	*F*	*C-1-0133*	*01.05.2009*		
2 Medical History							
Days ill	*3*	Days fever	*3*	Days cough	*1*	Days diarrhea	*0*
Days sore throat	*1*	Days stiff neck	*0*	Days pain to urinate	*0*	Days running nose	*0*
Days vomiting	*0*	Days rash	*0*	Days earache	*0*		
3 Symptoms at presentation (1=positive, 0=negative)							
Fever °C	*39*	Cough	*1*	Diarrhea	*0*		
Sore throat	*1*	Running nose	*0*	Meningism	*0*		
Earache	*0*	Rash	*0*	Malaria RDT	*1*		
Pain to urinate	*0*	Vomiting	*0*	Malaria species	*P. falciparum*		
4 Presumptive diagnosis							
Malaria							
5 Treatment							
A+M, Paracetamol							
6 Collected specimen sent to laboratory							
EDTA blood	*1*	Blood culture	*1*	Throat swab	*1*	Malaria slide	*1*
Dry tube	*1*	Serum	*1*	Clot	*1*	Filter paper	*1*

9.8 Review of study results on acute febrile illness

Table 43: Review of study results on acute febrile illness (Tara Müller, 2010)

Study title	Causes of acute febrile illness in rural Thailand	Etiologies of acute febrile illness in Thailand	Arthropod borne disease: the leading cause of fever in pregnancy on the Thai-Burmese border	Causes of fever in adults on the Thai-Myanmar border	Etiologies of obscure fever in children at a university hospital in northeast Thailand
Author	Suttinont, *et al*.	Leelarasamee, *et al.*	McGready, *et al*.	Ellis, *et al.*	Sripanidkulchai, *et al.*
Country	Thailand	Thailand	Thailand	Thailand	Thailand
Study period	2001-2002	1991-1993	2004-2006	1999-2002	2002-2003
Study population	845 patients in 5 different hospitals	1,137 Out-patients from 10 community-based hospitals	203 pregnant women in antenatal clinics	613 in- and out-patients at AFRIMS in a rural area	25 children with obscure fever in a university hospital
Malaria	Excluded	excluded	24.2 % (slide)	25.3 % (slide)	-
Leptospirosis	36.9 % (Culture/ MAT/IFAT)	1.1 % (MAT)	2.4 % (MAT/Culture)	17.0 % (ELISA/MAT)	8.0 % (IFA/MAT)
Murine typhus	2.8 % (MIA/ Westernblot)	5.3 % (Weil-Felix-Test/Serology)	7.1 % (RealtimePCR/ Invitro-isolation/ Serology)	1.5 % (IFA)	0.0 % (IFA)
SFG-*Rickettsia*	1.3 % (MIA/ Westernblot)	-	-	3.3 % (IFA)	-
Scrub typhus	19.9 % (MIA/ Westernblot)	7.5 % (Weil-Felix-Test/Serology)	5.7 % (Realtime PCR/ In-vitro-isolation/ Serology)	1.1 % (IFA)	0.0 % (IFA)
Dengue fever	5.2 % (ELISA)	5.7 % (ELISA)	9.5 % (ELISA)	1.5 % (ELISA)	40.0 % (ELISA)
Japanese encephalitis	0.4 % (ELISA)	0.6 % (ELISA)	-	0.2 % (ELISA)	0.0 % (ELISA)
Influenza	4.6 % (ELISA)	6.0 % (ELISA)	-	-	-
Q-fever	-	-	-	14 of 133 screened cases* (ELISA)	-
Septicemia	0.8 % (Culture)	3.2 % (Widal test/ IFAT)	-	-	-
Chikungunya Fever	-	1.1 % (ELISA)	-	-	-
Melioidosis	0.4 % (Culture)			7 of 44 screened cases* (ELISA)	
Gastroenteritis or typhoid-fever	-	-	1.4 % (Culture)	3.4 % (Realtime-PCR)	-
Others	-	-	31.3 %	26.8 %	4.0 %
No diagnosis established	**31.7 %**	**61.3 %**	**19.0 %**	**52.0 %**	**48.0 %**

Table 43: Review of study results on acute febrile illness (Tara Müller, 2010)

Study Title	The etiology of acute pyrexia of unknown origin in children after a flood	Febrile illness in Malaysia - an analysis of 1,629 hospitalized patients	Murine Typhus and Leptospirosis as causes of acute undifferentiated fever, Indonesia	Etiology of acute undifferentiated febrile illness in the Amazon Basin of Ecuador	Syndromic surveillance: etiologic study of acute febrile illness in dengue suspicious cases with negative serology
Author	Pradutkanchana, *et al.*	Brown, *et al.*	Gasem, *et al.*	Manock, *et al.*	Silva, *et al.*
Country	Thailand	Malaysia	Indonesia	Ecuador	Brazil
Study Period	2000	1975-1979	2005-2006	2001-2004	2008
Study Population	180 pediatric patients with acute fever in a referral hospital	1,629 hospitalized patients from rural areas	67 In- and 70 Out-Patients in hospital	533 Patients at 2 Hospitals	144 patients at a central public health laboratory
Malaria	-	6.2 % (slide)	-	12.5 % (slide)	-
Leptospirosis	27.2 % (IFA/MAT)	7.0 % (Hemolytic HL)	10.0 % (PCR/MAT/ELISA)	13.2 % (ELISA)	13.9 % (Bio-Manguinho-test)
Murine typhus	0.0 % (IFA)	0.5 % (IFA)	7.0 % (IFA)	-	-
SFG-*Rickettsia*	0.0 % (IFA)	0.3 % (IFA)	0.0 % (IFA)	5.9 % (ELISA)	-
Scrub typhus	1.1 % (IFA)	19.3 % (IFA/Mouse-inoculation)	0.0 % (IFA)		-
Dengue fever	29.4 % (HI)	7.0 % (Mosquito inoculation/ Culture)	-	5.3 % (Realtime-PCR/IFA)	11.8 % (ELISA)
Japanese encephalitis	-	-	-	-	-
Influenza	-	-	-	-	-
Q-fever	-	-	-	4.90 % (ELISA)	-
Septicemia	-	0.4 % (Culture)	-	-	-
Chikungunya fever	-	-	-	-	-
Melioidosis	-	-	-	-	-
Gastroenteritis or typhoid-fever	-	7.4 % (Widal-Test)	-	-	-
Others	-	-	-	3.3 % (viral infections, brucellosis RT-PCR)	3.9 % (Rubella)
No diagnosis established	**39.5 %**	**37.1 %**	**-**	**59.9 %**	**72.9 %**

***** These results were not felt to be convincing evidence of acute infection and were not included in the etiologic diagnoses.

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