INFECTIOUS ETIOLOGIES OF FEBRILE ILLNESSES IN CAMEROON

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ABSTRACT

Background: Diagnosis of febrile illnesses in malaria-endemic countries focuses primarily on confirming or ruling out malaria, since malaria is considered a major health threat. But because of the high rate of malaria over-diagnosis, other febrile illnesses are often misdiagnosed or overlooked. Thus, there remains a large gap in our understanding of pathogen profiling in most malaria-endemic countries. Because knowing the cause of fever is the first step in deciding appropriate treatment and improving disease outcomes, the overall objective of this dissertation research was to identify infectious causes of febrile illnesses in Cameroon.

Methods: We recruited 550 febrile patients seeking care at selected hospitals in Cameroon. Blood samples were collected from patients and used to conduct malaria tests by thick film microscopy, rapid diagnostic testing and PCR. Plasma samples were tested for antibodies against dengue, West Nile, chikungunya and respiratory viruses, *Leptospira interrogans* and *Salmonella typhi*, using RDT, microsphere immunoassay (MIA) and ELISA. Collected stool samples were cultured for the isolation of *Salmonella* and *Shigella sp*. Recombinant proteins of Ebola, Sudan, Marburg and Lassa viruses were expressed using Drosophila S2 cells and used to develop a MIA for viral hemorrhagic fevers (VHF). MIA was pre-validated using human or humanized monoclonal antibodies, and 408 previously collected plasma samples were screened for the presence of IgG antibodies for hemorrhagic fever viruses.

Results: Although malaria was the main cause of febrile illnesses in Cameroon, the accuracy of clinical diagnosis of malaria was poor. That is, 38% of the patients clinically diagnosed as having malaria had fever caused by other pathogens, including acute respiratory tract infections, typhoid fever, amebiasis, toxoplasmosis, shigellosis, dengue fever, West Nile fever, and chikungunya virus infection. We developed and pre-validated a multiplex MIA for the diagnosis of VHF, and identified samples (5/408) suspected to be positive for Ebola, Lassa and Marburg viruses.

Conclusion: Collectively, our data provide the first evidence of several pathogens causing febrile illnesses in Cameroon and may provide the basis for developing an algorithm for the management of febrile illnesses. This study also reports for the first time the development of a MIA for the diagnosis of VHF.

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ABBREVIATIONS

ARI	Acute respiratory infection
AMT	Antimalarial treatment
BSA	Bovine serum albumin
CRP	C-reactive protein
CHIKV	Chikungunya virus
CBC	Complete blood count
CCHF	Crimean Congo hemorrhagic fever
DDx	Differential diagnosis
DENV	Dengue virus
EHEC	Enterohemorrhagic *E. coli*
EVD	Ebola virus disease
EBOV	Ebola virus
FN	False negative
FP	False positive
GP	Glycoprotein
GPC	Glycoprotein complex
HCW	Health care workers
HAI	Hemagglutination inhibition
HFV	Hemorrhagic fever virus
HRP	Histidine rich protein
Hgb	Hemoglobin
HSV	Herpes simplex virus
HumAb	human monoclonal antibody
IAC	Immunoaffinity chromatography
ITN	Insecticide-treated nets
JEV	Japanese encephalitis virus
IFA	Immune-fluorescence assay
IMCI	Integrated management of childhood illnesses
LAMP	loop-mediated isothermal amplification
LASV	Lass virus
LHF	Lassa hemorrhagic fever
MP+	Malaria positive

MP-	Malaria negative
MIA	Microsphere immune assay
MFI	Median florescence intensity
MARV	Marburg virus
MHF	Marburg hemorrhagic fever
MSP	Merozoite surface protein
mAb	Monoclonal antibody
NSAIDS	Nonsteroidal anti-inflammatory drugs
NP	Nasopharyngeal swab
NPV	Negative predictive value
NP	Nucleo protein
POCT	Point-of-care test
Pf	*Plasmodium falciparum*
Pv	*Plasmodium vivax*
PRNT	Plaque reduction neutralization test
PCR	polymerase chain reaction
pLDH	*Plasmodium* lactate dehydrogenase
PPV	Positive predictive value
PIV	Parainfluenza virus
PCT	Procalcitonin
PE	Phycoerythrin
RDT	Rapid diagnostic test
RSV	Respiratory syncytia virus
RBC	Red blood cell
RT-PCR	Reverse transcriptase-PCR
RVF	Rift Valley fever
SS	Salmonella-Shigella
SIRS	systemic inflammatory response syndrome
SUDV	Sudan virus
TFM	Thick-film microscopy
TP	True positive
TN	True negative
TSI	Triple sugar iron
UTI	Urinary tract infection

URI	Upper respiratory infection
VHF	Viral hemorrhagic fever
VP	Viral protein
WHO	World Health Organization
WBC	White blood cell
WNV	West Nile virus
YF	Yellow fever

Chapter 1

Hidden Dengue Burden in the Shadow of Malaria in Africa

Hidden Dengue Burden in the Shadow of Malaria in Africa

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Abstract

Lack of effective surveillance dictates that most febrile illnesses in Africa are due to malaria. Dengue is an important but under-recognized cause of febrile illness in Africa. The mosquito species responsible for transmission of dengue virus have been well established in Africa. The most recent global estimate of dengue indicates that Africa's dengue burden represents 16% (16 million) of the global total, representing a larger burden than previously estimated. Between 2011 and 2016, locally acquired dengue virus infection has been reported in 20 African countries. Out of 26,563 serum samples tested from suspected dengue patients, 5,781 (22%) were positive for dengue virus. Since most febrile illnesses in Africa are presumed to be caused by malaria, dengue is thus hidden and overlooked due to failure to consider dengue in the differential diagnosis of febrile illnesses. Moreover, in areas of Africa where malaria and dengue vectors co-exist, co-infections of dengue and malaria cannot be ruled out. Co-infection of dengue and malaria poses challenges in diagnosis due to the similar clinical presentation. The focus of this review is to understand the burden of dengue in Africa, the possibility of dengue and malaria co-infection in Africa and other geographical regions, which may form the basis to further develop policies for systematic testing for malaria parasite and dengue virus in febrile patients in endemic regions of Africa.

Background

Infectious diseases continue to emerge and re-emerge due to globalization and climate change, posing daunting challenges for diagnostics and clinical management (1). Febrile illnesses are known to be a common cause of hospital admissions in most African countries (2) and pose a diagnostic and therapeutic challenge to health care workers (HCW) in resource-limited areas. Diagnosis of febrile illnesses in Africa mostly focuses on confirming or ruling out malaria (3) since malaria is considered a major health threat. Because of the high prevalence of malaria in Africa, other febrile illnesses such as respiratory infections, dengue, leptospirosis, typhoid fever, and sepsis are often misdiagnosed as malaria. Epidemiological data on the etiologic agents of febrile illnesses in Africa are few and far between. Also, some studies have documented high rates of viral infections in febrile patients from various tropical countries (1, 4-6). Mapping the etiology of non-malaria febrile illnesses demonstrates a heterogeneous distribution depending on the study area and population (4-10).

Differential diagnosis of febrile illnesses based on clinical criteria alone cannot be accurate because clinical signs and symptoms overlap. For appropriate management of febrile illnesses, timely laboratory diagnosis of the etiologic agent is required. The recent outbreak of Ebola virus disease in West Africa shows that prompt and accurate diagnosis is important to curb the spread of an infectious agent, and a single outbreak anywhere can lead to a global health emergency (11). Several point-of-care tests (POCTs) are being developed to test for febrile illnesses in the tropics so as to improve patient care and disease surveillance (12). However, with the diverse range of pathogens causing febrile illnesses in the tropics, a strong will is needed to carry out a panel of tests for each patient, taking into consideration the financial cost and required technical expertise (3). The objective of this review is to understand the burden of dengue in Africa, and the possibility of dengue and malaria co-infection in Africa and other regions of the world.

Methods

Review of literature was conducted using PubMed. Search terms included "dengue in Africa," "dengue and malaria co-infection," "dengue and malaria concurrent infection," "malaria over-diagnosis in Africa," and "non-malaria fevers" without any date filter. Additionally, review of the literature was conducted using search terms "dengue (in each African country)" for articles between 2011 to 2016. We also searched the online abstract books of the American Society of

Tropical Medicine and Hygiene (2013) and the American Society for Virology (2015) meetings. Additional data was obtained by searching the WHO Digital Library. We excluded duplicate articles. Similarly, after reviewing the abstracts of various publications and reports, we excluded those that were not relevant to the topic based on a review of the full text.

Beyond malaria: non-malaria febrile illnesses in the tropics

Febrile illnesses are caused by diverse pathogens with signs and symptoms that overlap, making differential diagnosis challenging. The prevalence of these pathogens may vary depending on the geographical region and season. Schoepp and colleagues recently reported that 25% of Lassa-virus negative and malaria-negative samples submitted to the Lassa virus diagnostic laboratory in Sierra Leone had detectable IgM to dengue (2.4%), West Nile (1.2%), yellow fever (2.5%), Rift Valley fever (2%), chikungunya (2%), Ebola (8.2%) and Marburg viruses (3.2%) (13). These pathogens are known to cause febrile illnesses with overlapping signs and symptoms that could mimic those of malaria. Even though treatment information was not published, it would not be surprising that some of these patients might have been treated for malaria. Therefore, to treat non-malaria fevers appropriately, pathogens that cause febrile illnesses need to be identified. Moreover, some patients may be co-infected with malaria parasites and other pathogens that cause febrile illnesses, such as dengue (14) and chikungunya viruses and various bacteria (15, 16), further confounding the situation. Although the particular pathogen may not be identified, knowing the pathogen category - virus, parasite, fungi or bacterium- would be useful in deciding treatment options (17). Although data on the epidemiology and characterization of non-malaria febrile illnesses in Africa is scanty, studies in Southeast Asia, South America and Africa have reported dengue, chikungunya, Japanese encephalitis, HIV, respiratory tract infections, leptospirosis, rickettsiosis, typhoid fever, pneumonia, blood stream infections, sepsis, scrub typhoid, enteric infection, and brucellosis infection in febrile patients (1, 4-8, 10, 16, 18-22). Therefore, more studies are warranted in malaria-endemic countries to elucidate the geographical and age distribution of non-malarial febrile illnesses, to guide policy makers and clinicians on the best course of action to take in treating febrile patients who test negative for malaria.

Diagnostic challenges of non-malaria febrile illnesses in the tropics

Correct diagnosis of non-malaria febrile illnesses is only possible with the use of laboratory tests. However, once malaria is excluded as the cause of the febrile illness, there are very few diagnostic tools available to guide the management of non-malaria febrile illnesses. In the absence of accurate and readily available diagnostic tests, febrile illness-associated diseases

will continue to go undiagnosed leading to poor health outcomes. Deaths from some of these diseases can easily be prevented if specific and sensitive diagnostic tools are readily made available in resource-poor areas. Based on the successful contribution of rapid diagnostic tests (RDTs) in malaria diagnosis and treatment, several POCT could be developed for non-malaria febrile illnesses. With the myriad of pathogens that can cause non-malaria febrile illnesses in the tropics, it is challenging to know the number of diagnostic tests that may be sufficient for the differential diagnosis of febrile illnesses. However, with the availability of epidemiological data on the distribution of various etiologic agents of febrile illnesses in tropical countries, diagnostic tests could be selected by the disease prevalence in a particular region. Most RDTs for non-malaria febrile illnesses detect just a single etiologic agent (23), which means that several laboratory tests might be needed for each febrile patient. Despite the urgency, there is no multiplex POCT that can be used to diagnose multiple etiologies of febrile illnesses in the tropics. Using multiple single POCT to test for non-malaria febrile illnesses will be cost-prohibitive as compared to using multiplex POCT. Thus, the availability of a multiplex POCT for tropical fevers will enable differential diagnosis of febrile illnesses thereby improving patient care. These POCT could be used to test for antigens, antibodies, and biomarkers.

Most RDTs for non-malarial febrile illnesses currently rely on the detection of host antibodies. However, the sensitivity and specificity of host-antibody detection tests are both inherently limited. For example, dengue RDT performance is still not reliable due to complex reactivity with the four serotypes of dengue. Moreover, prolonged antibody responses to dengue virus preclude the use of serological RDTs for monitoring response to treatment. Considering these limitations, RDT for dengue and other non-malaria febrile illnesses will rely on combined use of antibody and antigen-capture tests.

Until diagnostic tests for febrile illnesses become widely available in most tropical countries, there is the need to continuously improve clinical diagnosis. Clinical diagnosis is the first step and fundamental principle in the diagnosis of any disease. Continuous training and refresher courses for HCW on syndromic approaches to tropical fevers needs to be reinforced. For example, it is well documented that among dengue patients in Africa, hemorrhagic manifestation is very low, which in other parts of the world is used as one key clinical criteria for the diagnosis of dengue. Ultimately, integration of POCT into a validated syndromic approach to tropical fevers is urgently needed. Related research priorities are to determine the etiologic agents of febrile illnesses in the tropics; to determine how combinations of RDTs could be used to

improve health outcomes.

Distribution of *Aedes sp.* in Africa

Aedes aegypti and *Aedes albopictus*, which are the most important vectors for the transmission of dengue virus, have been reported in Africa (24-26). *Aedes aegypti* originated from Africa but is now found in tropical and sub-tropical regions of the world due to increased international travel and trade development (27). *Aedes aegypti* mosquitos are found in most countries in Africa and occur in a broad range of environments, from sylvan to urban. However, data from Algeria, Tunisia, Western Sahara and Libya (Northern Africa) is not available (24, 25). Other mosquitoes species reported in Africa that could potentially spread dengue virus include *Aedes africanus*, and *Aedes luteocephalus* (28). *Aedes albopictus* has been reported in several countries in Central Africa with high enough levels compatible with dengue virus transmission (25). The international trade especially in used car tires, which easily accumulate rainwater, has aided the geographical expansion of the *Aedes* mosquito. In September 2015, *Aedes albopictus* was recorded for the first time in Morocco (29). A total of 46 African countries has reported the presence of *Aedes aegypti* (28) with the potential to trigger local dengue virus transmission.

The hidden burden of dengue in Africa

Dengue is a mosquito-borne viral disease transmitted by female *Aedes aegypti* and *Aedes albopictus mosquitoes.* There are four serotypes of dengue virus (DENV-1, -2, -3 and -4). Infection with one serotype confers life-long immunity against that serotype. However, persons infected subsequently with a different DENV serotype may experience antibody-dependent enhancement (ADE), in which antibodies from the previous infection cannot neutralize the virus but form a complex with the virus which may lead to severe dengue disease (30). Infection with DENV can lead to dengue fever or severe dengue (dengue hemorrhagic fever and dengue shock syndrome). There is no specific treatment or the United States Food and Drug Administration-approved vaccine for dengue, but early detection and appropriate medical care can reduce the case fatality.

Dengue is an important but unrecognized cause of febrile illness in Africa. The epidemiology of dengue in Africa is poorly understood due to lack of active surveillance systems and weak, passive surveillance (diseases notification). Passive surveillance is the platform for any country to start surveillance activities. However, in African countries passive surveillance has several limitations, including underreporting and reflects the HCW knowledge, attitudes and practices.

The most recent global estimate of dengue indicates that Africa's dengue (symptomatic) burden represents 16% (16 million) of the global total, representing a larger burden than previously estimated (31). With the increase in the use of tires and disposable containers, international travel and trade, open-water storage, lack of window screens, combined with the explosion in urban populations in most African countries, the incidence of dengue in Africa are expected to increase in the absence of effective control measures (32). Recent data suggest that the predicted risk of dengue in Africa is much more widespread than previously reported with movement between populations being an important facilitator of spread (31).

Available data suggest that dengue is probably endemic in most African countries (28, 31) (Figure 1). Between 1960–2010, transmission of dengue has been reported in 34 African countries with 22 of them reporting local transmission (20 with laboratory confirmed cases and 2 with clinical cases only) whereas in 12 countries dengue was only diagnosed in returning travelers who had visited these countries from regions non-endemic to dengue (24). Results from a 12-year (1998–2010) surveillance of dengue among French military in Africa demonstrated additional locations for circulating DENV-1 (Cameroon, Djibouti, Gabon and Mayotte) and DENV-3 (Comoros) (33). Between 2011–2016, there have been published reports of locally acquired dengue infection in 20 African countries (Table 1). Out of 26,563 serum samples tested, reviewed from 37 publications from Africa (2011–2016), 5,781 (22%) samples were positive for dengue by serology (IgM, IgG, PRNT, IFA) and RT-PCR. Within the last five years, dengue outbreaks have been reported in Angola (2013), Burkina Faso (2013), Mozambique (2014), Kenya (2013) and Tanzania (2014). All four DENV serotypes have now been documented to be circulating in Africa with DENV-2 being predominant (Table 1).

Clinical features of severe dengue that are more distinct and can be used for differential diagnosis are infrequently reported in Africa. There were six cases of severe dengue reported in Senegal during the 2009 dengue outbreak with one death (34). Meanwhile, in Angola, 11 deaths were recorded during the 2013 dengue outbreak (35). Several reasons can be advanced for the rare occurrence of severe dengue in Africa. Firstly, signs and symptoms of dengue mimic other infectious causes of febrile illnesses in the tropics. Second, most HCW are unfamiliar with dengue and do not consider dengue during differential diagnosis of febrile patients even with bleeding manifestations. Third, a genetic protective variant has been proposed to be partially responsible for the low incidence of severe dengue in Africans (36). However, with limited data from Africa, the true burden of dengue in Africa remains uncertain.

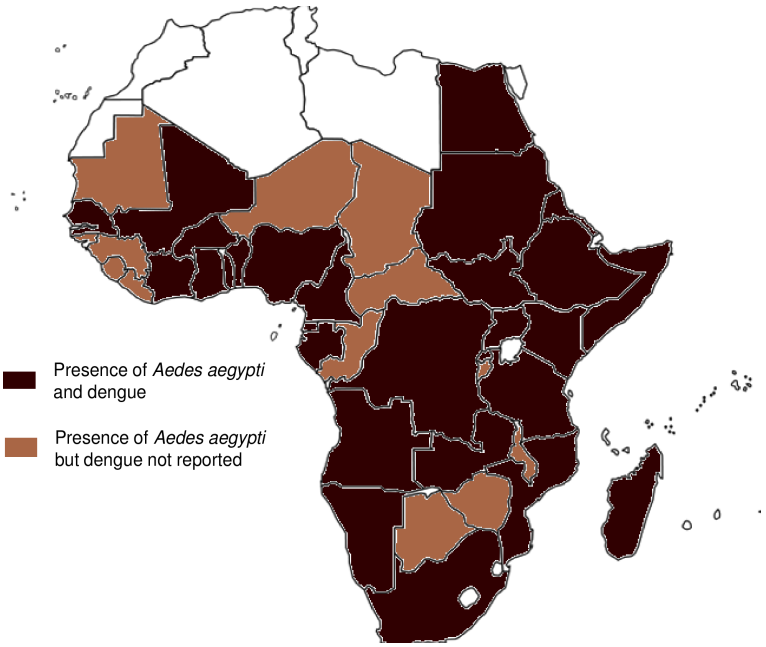

Fig 1. Dengue and *Aedes aegypti* in Africa (1960-2011) (24).

Dark color represents countries in which dengue (including dengue in travelers) and *Aedes aegypti* have been reported. Light color represents countries in which *Aedes aegypti* have been reported, but dengue was not reported. White indicates countries in which data are not available.

Table 1. African countries with published reports of local transmission of dengue (2011–2016).

Country (reference)	Total number of samples tested	Number positive (%)*	DENV serotypes
Angola (35, 37)**	1,214	811 (67)	1
Burkina Faso (38-40)**	653	95 (15)	2, 3, 4
Cameroon (41)	2,030	666 (33)	1, 2
Comoros (42)	400	303 (76)	1, 2, 3, 4
Cote d'Ivoire(43, 44)	812	10 (1)	3
Djibouti(45, 46)	1,265	322 (25)	1, 2, 3
Gabon (47)	4,287	376 (9)	2
Ghana (48)	218	54 (25)	NA
Kenya (49-52)**	4,720	1,493 (32)	1, 2, 3
Madagascar (53)	1,244	97 (8)	NA
Mozambique (54)**	193	100 (52)	2
Namibia (55)	312	25 (8)	NA
Nigeria (56-59)	943	389 (41)	NA
Sao Tome and Principe (60)	78	28 (36)	NA
Senegal (34)	696	196 (28)	3
Sierra Leone (61-63)	446	142 (32)	1, 2, 3, 4
South Sudan (64)	632	8 (1)	2, 3
Sudan (65)	615	170 (28)	NA
Tanzania (66-70)**	2,181	347 (16)	2
Zambia (71)	3,624	149 (4)	NA
Total	**26,563**	**5,781 (22)**	NA

* Includes IgM, IgG, PRNT, IFA and RT-PCR positive samples
** Countries reporting dengue outbreaks
NA – Data not available

Malaria in Africa

Distribution of malaria vectors in Africa

Malaria is a mosquito-borne disease caused by five species of malaria parasites (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malaria*, *Plasmodium ovale and Plasmodium knowlesi*). Female *Anopheles* mosquitoes transmit malaria parasites. Of the 140 species of *Anopheles* mosquitoes known to transmit malaria, 20 are reported in Africa (72). Malaria is characterized by its biological diversity that is determined by the vector species present in a given region (73). The most widespread and potent human malaria vectors in sub-Saharan Africa belong to the *Anopheles gambiae* complex (*Anopheles gambiae, Anopheles funestus, Anopheles arabiensis, Anopheles nili and Anopheles moucheti*) and are responsible for over 95% overall transmission (72, 74). *Anopheles gambiae* is widely recognized as the most important of these vectors especially in lowlands, and commonly associated with *Anopheles funestus* known to be responsible for intense seasonal malaria transmission (73). However, the

transmission pattern of malaria in Africa is highly variable due to differences in ecological conditions, mosquito feeding behavior and longevity of the different malaria vectors (75, 76).

The burden of malaria in Africa

The African region is most affected by malaria with about 90% of all malaria deaths in children occurring in sub-Sahara Africa (77). Malaria affects the lives of almost everyone in sub-Saharan Africa in part, because, most at risk groups live in areas with stable malaria transmission. Young children and pregnant women are the most susceptible group. Malaria in pregnancy can cause anemia, low birth weight and other related complications (78). The World malaria report estimates 212 million new cases of malaria worldwide in 2015 with the WHO Africa Regions accounting for 90% (79). In 2015 there were an estimated 429,000 deaths attributed to malaria worldwide. Most of these deaths (92%) occurred in the African region. The high malaria transmission and mortality rate can be attributed to the fact that the most effective malaria vector (*Anopheles gambiae*) is wide spread in Africa and the majority of infections are caused by the most dangerous *Plasmodium* species (*Plasmodium falciparum*), respectively. In five African countries (Algeria, Morocco, Tunisia, Libya and Egypt), malaria has been well controlled (77).

Malaria over-diagnosis

The advent and widespread use of malaria RDTs present HCW the challenge on an appropriate course of action to take when malaria RDT results are negative (Figure 2) (17). Studies have documented the widespread use of antimalarial drugs to treat febrile patients who are negative for malaria (80, 81). Most clinical decisions on the treatment of malaria are not based on the parasite guidelines proposed by WHO but rather based on non-specific signs and symptoms such as fever, headache and joint pains that overlap with other diseases including dengue. Moreover, the World Malaria Report estimates that in 2015 only 51% of children with a fever who sought care at a public health facility in 22 African countries received a malaria diagnostic test (79). Given the present scenario, there is the likelihood that malaria over-diagnosis could mask other causes of fever including dengue virus and also puts evolutionary pressure on malaria parasites that can enhance the development of drug resistance. Therefore, educational activities should be focused on training of HCW to treat the only parasite confirmed malaria patients with antimalarial drugs and to further investigate negative malaria patients for other causes of fever.

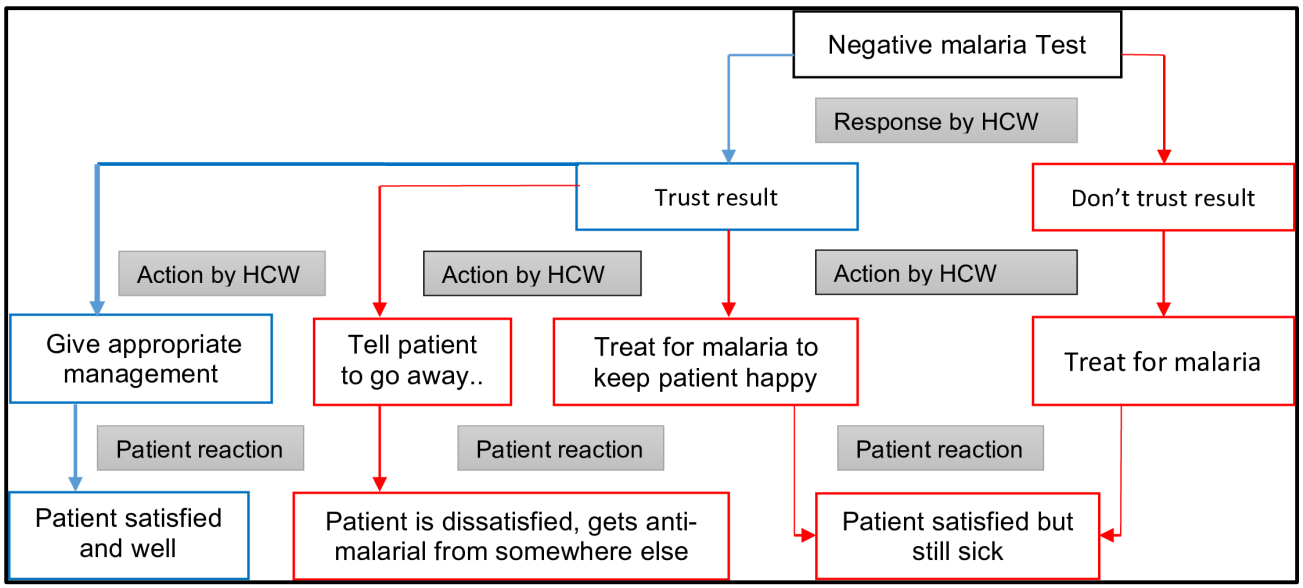

Fig 2. The course of action by health care workers when the result of a malaria RDT is negative per WHO recommendation (17).

Blue indicates recommended actions while red indicates not recommended actions/behaviors

Dengue and malaria co-infection in Africa

Dengue and malaria co-infection refers to the simultaneous existence of both malaria and dengue in a patient. The worry of any co-infection is that it challenges the appropriate clinical management of the patient. Thus, misdiagnosis of co-infection may make clinical management difficult or worsen patient outcome. The mosquito species responsible for transmission of dengue and malaria have been well established in Africa (24, 28). Areas in Africa where malaria and dengue vectors co-exist, co-infections of dengue and malaria cannot be ruled out (Figure 3). Thus, the concept that dengue occurs in urban areas and malaria in rural areas may be contradicted by the fact that in a given country, co-infections may occur due to an overlap between the two vectors. A study in French Guiana showed dengue virus to have spread to malaria-endemic rural areas (82). The first published case report of dengue and malaria co-infection occurred in a patient returning to France after visiting 3 African countries (Guinea, Senegal, and Sierra Leone) for 18 days (83). France is not known to be endemic for either dengue or malaria; this patient was therefore infected while visiting these African countries.

Although malaria and dengue co-infections have been documented in Asia and Latin America, there is limited information on dengue and malaria co-infection in Africa. This is because, in Africa, malaria is generally ascribed to most febrile illnesses. More so, febrile patients show

symptoms, which are common to both malaria and dengue making it difficult to diagnose co-infections clinically (84). Also, malaria can be easily diagnosed in its acute febrile phase, and once malaria is confirmed, other possible causes of fever such as dengue are not considered. This implies that dengue and malaria co-infection will be treated as malaria mono-infection.

There have been reports of dengue and malaria co-infection in Africa (Table 1). A recent study in Ghana investigated archived blood samples from children with laboratory-confirmed malaria for possible exposure to dengue. Anti-dengue virus-specific IgM antibodies were detected in 3.2% of the children, indicating probable co-infection, whereas IgG antibodies were detected in 21.6% of the children, indicating previous exposure (85). In a similar study in Nigeria, 18 of 310 febrile patients were co-infected with dengue and malaria (86). Moreover, during the 2014 dengue outbreak in Tanzania, 4% of confirmed dengue cases were co-infected with malaria (67). However, results from these studies should be interpreted with caution (Table 1). Dengue infection and disease are different because not all infections are clinically symptomatic. Detection of anti-dengue IgM or IgG antibodies in an individual is evidence of current or past infection, respectively, but not necessarily disease (illness). While it is important to highlight co-infection, it is equally important not to overestimate co-illness of dengue and malaria. For example, a patient with fever positive for malaria by blood smear and having evidence of dengue IgM antibodies maybe co-infected with dengue and malaria but only malaria is causing the illness. Out of the seven studies reporting dengue and malaria co-infection in Africa only two studies tested for dengue antigens (Table 2). The rest used ELISA or RDT to test for dengue IgM/IgG antibodies, which may only indicate previous or probable dengue infection in malaria positive patients. However, in studies where dengue antigens were detected, the prevalence of co-infection with malaria was 4% and 1.4%, respectively (Table 2). Even though the prevalence of dengue and malaria co-infection was low in these studies, it is not possible to draw conclusions on the degree of co-infections in Africa based on two studies carried out in the same country (Tanzania). Moreover, once a patient is confirmed to be malaria positive, clinicians don't think of the possibility of co-infection, leading to underreporting of malaria co-infections. Thus, more studies on dengue-malaria co-infection and detection of dengue are warranted to elucidate the burden of dengue infection and disease (asymptomatic and symptomatic) in Africa. Such information will help health authorities plan for improving medical care for both diseases.

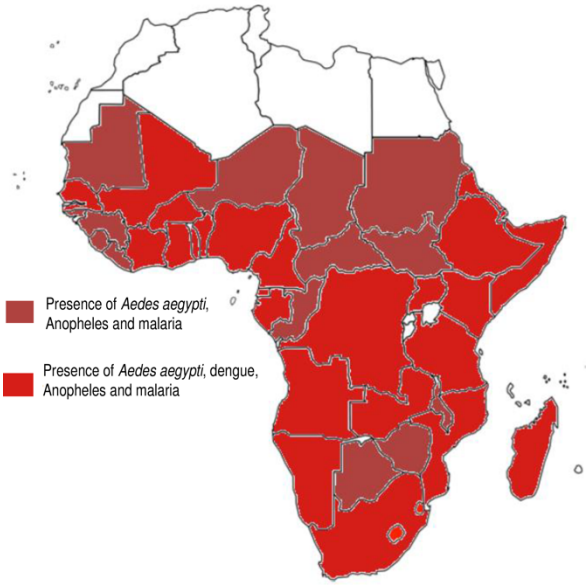

Fig 3. Dengue and malaria risk areas in Africa**.**

Clinical parameters and implications of dengue and malaria co-infection

Taking into consideration that the incubation period of malaria, 7-30 days (87), is longer than that of dengue, 4-7 days (88), concurrent clinical presentation of both infections is possible (Table 3). Co-infection of dengue and malaria leads to an overlap in their clinical presentation, which can pose a diagnostic challenge to HCW. Recently, >50 mg/L C-reactive protein (CRP) has been demonstrated to be a simple and sensitive tool to discriminate malaria from dengue (84). Unfortunately, CRP is non-specific for a particular disease. CRP levels are known to rise in response to inflammation. Thus, systematic testing for malaria parasite and dengue virus in febrile patients living in areas where both infections are present is ideal since their clinical parameters overlap. Treatment for both infections is different. While fluid replacement therapy is given to dengue patients, malaria patients are treated with antimalarial drugs. Therefore, a delay in treatment or not treating one of the dual infections may lead to serious consequences. A recent study suggests an increase in disease severity in dengue and malaria co-infections compared to mono-infections, with co-infected patients presented with anemia and severe thrombocytopenia more frequently than patients with single infections (89).

Table 3. Comparison of clinical and laboratory parameters between dengue and malaria.

Parameter	Dengue	Malaria	Malaria and dengue co-infection
Fever	Present	Present	Present
Incubation period	4-7 days	7-30 days	Variable
Vomiting	Present	Present	Present (90)*
Headache	Present	Present	Present
Myalgia	Present	Present	Present (90)
Thrombocytopenia	Moderate to severe	Moderate to severe	Severe (89, 91) (90)
Anemia	Negative	Possible	Possible (89, 91)
Hematocrit	High	Usually normal	High to moderate (90)
Rash	Present	Absent	Possible
Bleeding manifestations	Common	Rare	Possible (90)
Shock	Possible	Possible	Possible
CRP	Usually <50 mg/L (84)	Usually >50 mg/L (84)	Usually >50 mg/L (89)
Circulatory collapse	Possible	Possible	Possible
Severe disease outcome	Dengue shock syndrome	Cerebral malaria, severe anemia	More severe than mono infection (89, 91)
Tourniquet test	Positive	Negative	Positive
Malaria parasite	Absent	Present	Present
Fatigue	Yes	Yes	Yes
Treatment	Fluid therapy	Antimalarial	Antimalarial plus fluid therapy

*References are cited in parenthesis

Dengue and malaria co-infection in other regions of the world

There are several case reports of dengue and malaria co-infections mostly from Southeast Asia (91-98). The incidence of dengue and malaria co-infection varies in different geographical regions. A recent study in India showed dengue and malaria co-infection in 7.4% (27/367) of febrile patients (99). Similarly, in Kolkata, India, 7.6% (46/605) of dengue patients were co-infected with malaria (100). In French Guiana, 104 dengue and malaria co-infections were reported in a retrospective study between 2004-2010 (89). A separate study in French Guiana confirmed dengue and malaria co-infection in 17 of 1,723 febrile patients (82). In the Brazilian Amazon region, dengue and malaria co-infection were reported in 2 of 11 patients (101). Meanwhile, two separate studies from Pakistan reported 23.2% and 33% of dengue-positive patients co-infected with malaria (90, 102). A study in Jamaica reported 2.4% concurrent dengue and malaria co-infection (103). In a study investigating causes of febrile illnesses in Cambodia, dengue virus was frequently observed in co-infections (32/56, 57%), especially with

malaria parasite (n=27) (104). Similarly, in a prevalence study in Bangladesh, 5 (1%) concurrent cases of dengue and malaria were recorded (105). Based on these published results (Table 2), it can be assumed that dengue and malaria co-infection is common and may occur more frequently than reported.

Conclusions

Dengue virus infection in Africa is a reality but is being masked by malaria over-diagnosis and mistreatment. There is limited data on the prevalence of dengue in Africa, which makes the burden prediction uncertain. Because local transmission of dengue virus is evident in most malaria-endemic countries in Africa, dengue and malaria co-infection is plausible. Thus, special attention should be given to the possibility of malaria and dengue co-infection, which can be more severe than mono-infection. Since the clinical signs and symptoms of dengue and malaria overlap, it will be difficult to diagnose both infections clinically. Therefore, we recommend that dengue should be included in the differential diagnosis of febrile illnesses in Africa and the possibility of malaria co-infection(s) with other tropical febrile illnesses (including dengue) should be kept in mind by HCW attending to febrile patients in Africa or individuals returning from Africa. Also, HCW should ensure that patients with fever are investigated for other causes of febrile illnesses beyond malaria and prompt treatment to avert serious complications. Development of a geographically relevant multiplex point-of-care test for common tropical fevers will enable timely and accurate differential diagnosis of febrile illnesses with overlapping signs and symptoms, thereby improving patient care.

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Table 2. Selected publications on dengue and malaria co-infection.

Country (ref)	Year of sample collection	Study design	Sample size	Number of malaria and dengue co-infection	Malaria parasite species/ method of detection	Dengue serotype/ method of detection	Remarks
Brazil (93)	2012	Prospective	ND	11	Pv/ Microscopy& PCR	2, 3, 4/ PCR	One patient was infected with *Pv*, DENV-3 & DENV-4.
Brazil (101)	2003-2005	Prospective	111	2	Pv/ Microscopy	2/ PCR	Dengue and malaria co-infection should be common in places where both diseases co-exist.
Brazil (106)	2009-2011	Cross sectional	1578	44	Pv/ Microscopy, PCR	2, 4/ ELISA, PCR	Higher chance of bleeding and severe disease in co-infected patients
Bangladesh (105)	2007-2010	Cross sectional	659	5	Pf, Pv/ Microscopy, RDT and PCR	NR/ ELISA	High rate of malaria co-infections with other pathogens also recorded
Bangladesh (107)	2012	Cross sectional	720	1	Pv/ microscopy, RDT	NR, ELISA	
Cambodia (104)	2008-2010	Prospective observational	1193	27	Pf and Pv/ RDT, PCR	1, 2, 3, 4/ RT-PCR	A majority of malaria negative patients did not receive appropriate diagnosis and treatment.
East Timor (108)	2006	Case report	1	1	Pf/ RDT, Microscopy	NR/ IgM/IgG RDT	This case study shows that, it is difficult differentiating dengue from malaria purely on

							clinical grounds
French Guiana (89)	2004-2010	Retrospective	312	104	Pf and Pv/ Microscopy	1, 2, 3/ ELISA, PCR	Co-infection more severe than mono-infections with increased risk of anemia and thrombocytopenia.
French Guiana (82)	2004-2005	Retrospective	1723	17	Pf and Pv/ Microscopy	1, 3/ ELISA, PCR, culture	Diagnosis of one of the dual infections should not rule out the other.
France (83)	2004	Case report	1	1	Pf/ Microscopy	3/ ELISA, RT-PCR	Patient returned to France from an 18-day trip to Senegal, Guinea and Sierra Leone.
Ghana (85)	Archival samples	Retrospective	218	3.2%(IgM) 21.6% (IgG)	Pf/ Microscopy	NR/ ELISA	High rate of clinical diagnosis of malaria in Ghana
India (99)	2012	Cross sectional	367	27	Pf and Pv/ Microscopy	NR/ ELISA	Clinical presentation of co-infection were more like dengue than malaria
India (100)	2005-2010	Prospective	605	46	Pf and Pv/ Microscopy	NR/ ELISA	Possible risk of dengue and malaria co-infection should be kept in mind in endemic areas.
India (109)	2011-2012	Cross sectional	300	1	Pv/ Microscopy	NR/ ELISA	Study was conducted in pregnant women
India (110)	2013	Case report	1	1	Pf and Pv/ Microscopy, RDT	NR/ ELISA	
India (111)	2006	Case study	1	1	Pv/ Microscopy	NR/ ELISA	
India (112)	2006	Case study	1	1	Pv/ Microscopy	2/ ELISA, virus isolation	

India (98)	2013	Case report	1	1	Pv/ Microscopy	NR/ ELISA	
India (96)	2006	Case report	1	1	Pf/ Microscopy	NR/ ELISA	
India (92)	2007	Case report	1	1	Pf and Pv/ Microscopy, RDT	NR/ ELISA	
India (113)	2009	Case report	1	1	Pv/ RDT, Microscopy	NR/ ELISA	Patient was co-infected with dengue, leptospirosis and hepatitis E virus
India (94)	2012	Case report	1	1	Pv and Pf/ RDT, Microscopy	NR/ ELISA	Patient responded well to treatment
India (91)	2013	Case report	1	1	Pf/ RDT, Microscopy	NR/ RDT for (NS1, IgM, IgG), ELISA	Patient had cerebral malaria and dengue co-infection with anemia and thrombocytopenia.
India (114)	2012-2013	Retrospective	298	9	Pf and Pv/ Microscopy	NR/ ELISA	
India (115)	2014	Case reports	3	3	Pv/ Microscopy, RDT	NR/ ELISA	All cases were children
India (116)	2014	Cross sectional	300	1	NR/ NR	NR/ ELISA	Intrauterine death of fetus in the pregnant woman co-infected
India (117)		Cross sectional	1980	22	Pf and Pv/ Microscopy, RDT	NR/ RDT, ELISA, PCR	
Indonesia (118)	2008	Case report	1	1	Pf/ RDT, Microscopy	NR/ ELISA	Patient also suffered from acute renal failure
Malaysia (119)	2013	Case report	1	1	Pv/ Microscopy	NR/ ELISA	Patient also co-infected with leptospirosis
Nigeria (120)	2014	Cross sectional	60	1	Pf/ RDT, PCR	NR/ RDT	
Nigeria (121)	2014	Case report	1	1	Pf/ Microscopy	NR/ PCR, ELISA	Patient also co-infected with chikungunya virus

Pakistan (90)	2012	Cross sectional	856	17	Pf and Pv/ Microscopy	NR/ ELISA, PCR	For most clinical features, co-infection was similar to mono-infection
Peru (122)	2002-2011	Retrospective		17	Pf and Pv/ Microscopy, PCR	1, 3/ Culture, PCR	Co-infected group had similar findings to dengue mono infected group
Tanzania (123)	2013	Cross sectional	364	8	NR/ Microscopy	2/ ELISA, RT-PCR	Study conducted in children
Tanzania (124)	2014	Cross sectional	483	7	NR/ RDT	2/ ELISA, RT-PCR	Dengue out break
Thailand (97)	2014	Case report	1	1	Pf/ Microscopy	NR/ ELISA, IgM, NS1	Early recognition and treatment of co-infection can avert potential complications.
Singapore (125)	2012	Case report	1	1	Pf/ Microscopy	NR/ ELISA	Patient also suffered from rhabdomyosis and acute renal failure
Senegal (126)	2009-2013	Cross sectional	13845	1	ND/ Microscopy, RDT	NR/ ELISA, PCR	Co-infection of malaria and other arboviruses also reported.
USA (95)	2003	Case report	1	1	Pv/ Microscopy	NR/ ELISA	Patient returned to the US after visiting India for 3 months and was not on any chemoprophylaxis

Pf = *Plasmodium falciparum*
Pv = *Plasmodium vivax*
NR = not reported
RDT = rapid diagnostic test

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Chapter 2

Dissertation Scope

Background

Currently, the World Health Organization (WHO) recommends limiting anti-malarial treatment to individuals with positive malaria test results. However, significant over-diagnosis of malaria is evident in most malaria endemic countries. Most febrile patients diagnosed and treated as malaria have underlying non-malarial causes. Under the current practices, there is a real potential of missing other pathogens that cause fever. In the absence of diagnostic tests, health care workers in tropical countries are posed with a daunting task of identifying a particular cause of febrile illness especially during the early stages of infection in which fever is the only symptom. Even when a patient presents with fever plus other focal signs and symptoms that can assist in differential diagnosis, clinical diagnosis is still challenging because some focal signs and symptoms are nonspecific (Table 1). For example, a patient with fever and other focal signs and symptoms such as diarrhea and/or bloody stool may be infected with *Entamoeba histolytica*, *Shigella spp* or enterohemorrhagic *E. coli* (EHEC) making a differential diagnosis and clinical management challenging. Likewise, a patient with fever and signs of hemorrhage may be infected with dengue, Ebola, Lassa or Marburg viruses, which require different treatment approaches and preventive measures (Table 1). Therefore, laboratory diagnosis remains the best tool to rule out or confirm a diagnosis of febrile illnesses. Thus, profiling pathogens that cause febrile illnesses is essential to provide local epidemiologic data that can be used to develop diagnostic tools, optimize clinical care and develop effective prevention strategies in communities and hospitals. However, there remains a large *gap* in our understanding of pathogens, other than malaria, which causes febrile illnesses in malaria-endemic countries. The paucity of reliable data is in part due to the lack of diagnostic tools, active surveillance systems and the misplaced emphasis on malaria as the sole cause of the fever.

Evidence regarding etiologic agents of febrile illnesses in the tropics is variable. Available data shows that in children less than five years of age most fever episodes are associated with acute respiratory infections, and a high proportion of infectious etiologies are due to viruses. However, in older children and adults, non-malaria febrile illness has been shown to be associated with immunocompromised status due to HIV infection (1). Moreover, specific infections such as brucellosis, leptospirosis, scrub typhus and rickettsiosis have been shown to be more prevalent in older children and adults in part due to environmental and occupational exposure to pathogens and vectors in livestock or forest. Although there are some similarities in the incidence of disease and prevalence of various etiologic agents of febrile illnesses, available

data show a wide variation of different fever causing pathogens due to factors like geographical area, setting (urban or rural), age, season (dry or wet) and immune status of the patients. For example, dengue is prevalent in studies conducted in Asia but relatively less common in Africa, while malaria is more common in studies undertaken in Africa than Asia (Table 2). Thus, more studies are needed on the causes of febrile illnesses in different geographical settings because if the local epidemiology is well understood, patients' age and geographic settings can help direct the appropriate diagnostic approach and treatment.

Table 1: Transmission, clinical features and diagnosis of febrile illnesses in the tropics

Disease/Pathogens* and Mode of Transmission	Clinical Features	Laboratory Diagnosis	Management
Parasites			
Malaria/*Plasmodium spp* Anopheles mosquito	General: fever, chills, sweats, headaches, nausea and vomiting. Focal**: none Complications: cerebral malaria, severe anemia Differential diagnosis (DDx): typhoid fever, dengue, leptospirosis, influenza, meningitis, viral hemorrhagic fevers, gastroenteritis etc.	Microscopy: identification of blood stage parasites (gold standard). RDT: detects parasite-specific protein (HRP) or enzymes (LDH, aldolase) Molecular (PCR, LAMP): detects parasite DNA	Artemisinin combination therapy is used for uncomplicated malaria while IV quinine can be used for severe malaria.
Amebiasis/*Entamoeba histolytica* Fecal-oral	General: stomach pain and fever. Focal: bloody stool Complications: Rarely, E. histolytica invades the liver and forms an abscess and spread to other parts of the body. DDx: salmonellosis, shigellosis, EHEC, EIEC, viral hepatitis, campylobacter infection	Microscopic examination of stool (gold standard). Antigen detection using ELISA Serology for extra intestinal diagnosis (can't distinguish past from present infection)	Tinidazole used for intestinal and extraintestinal amebiasis while metronidazole is the mainstay of therapy for invasive amebiasis.
Toxoplasmosis/ Toxoplasma gondii Foodborne, animal-to-human and vertical	General: flu like symptoms in healthy people. In immune compromised individuals; fever, headache, nausea, and poor coordination. Focal: confusion, seizures, visual changes Complications: encephalitis DDx: CMV, HSV, ocular syphilis, WNV, listeriosis, toxocariasis	Serology: combination of IgM and IgG avidity. Direct observation of the parasite in stained tissue sections, CSF, or other biopsy material. PCR to detect the parasite's DNA in the amniotic fluid.	Current drugs act against the tachyzoite. Pyrimetamine is the most effective and given in combination with sulfadzine or clindamycin.
Bacteria			
Salmonellosis/*Salmonella spp* Fecal-oral	General: constipation, fever, relative bradycardia, dry cough Focal: rose spots, splenomegaly, diarrhea Complications: sepsis, perforation of Peyer's	Blood culture (early during infection), stool culture (later during infection), bone marrow culture (most sensitive)	Antibiotic treatment (Azithromycin and ceftriaxone). Fluid and electrolyte

Disease/Pathogens* and Mode of Transmission	Clinical Features	Laboratory Diagnosis	Management
	patches DDx: *Campylobacter* infection, *E. coli*, listeriosis, shigellosis,	Widal test (low specificity). PCR-based multiplex panel for enteric bacteria is available.	replacement in severe cases.
Leptospirosis/*Leptospira interrogans* Urine of infected animals (dogs, cats, horses, pigs, rodents) which can get into water or soil	General: flu-like illness, myalgia, tachycardia Focal: transient petechial eruption, nonpurulent conjunctival discharge and conjunctivitis Complications: meningitis, renal failure, myocarditis, pulmonary hemorrhage, shock DDx: dengue, VHF, chikungunya, malaria, rickettsial infection, enteric fever	Microscopic agglutination testing. Dark-field microscopy of blood (not usually available). Culture of leptospires from bodily fluids (takes several weeks) Serology (ELISA) is usually positive after 5-6 days of illness. RDT (IgM/IgG) available DNA PCR of blood, urine, CSF, tissue	• Antibiotic treatment (Doxycycline) • Antibiotic treatment not necessary for mild diseases. • Severe cases: I.V. penicillin G and supportive therapy
Shigellosis/*Shigella spp* Fecal-oral	General: fever, and abdominal pain Focal: acute bloody diarrhea Complications: hemolytic uremic syndrome, bacteremia DDx: Amebiasis, salmonellosis, EHEC, *Campylobacter* infection, *Clostridium difficile* colitis	Stool culture Serological grouping using different antisera. PCR-based multiplex panel for enteric bacteria is available.	Antibiotic treatment Oral rehydration
Viruses			
Dengue fever, dengue hemorrhagic fever /dengue virus Aedes mosquito	General: fever, headache, eye pain, joint pain, muscle pain Focal: mild bleeding manifestation (e.g., nose or gum bleed, petechiae, or easy bruising), maculopapular rash, positive tourniquet test Complications: hemorrhagic fever and shock DDx: influenza, malaria, typhoid fever, leptospirosis, chikungunya, rubella, meningococcemia.	Virus isolation, PCR and serology. DENV can be detected in the blood during the first 5 days of symptoms. Dengue NS1 antigen can be detected in serum as early as one day post onset of symptoms and up to 18 days. PRNT can be used for confirmation and	No specific antiviral treatment available. Supportive therapy with analgesics, fluid replacement and bed rest may suffice. Aspirin, corticosteroids and NSAIDs should be avoided.

Disease/Pathogens* and Mode of Transmission	Clinical Features	Laboratory Diagnosis	Management
		when specific serological diagnosis is required.	
WNV encephalitis/ West Nile virus Culex mosquito, blood transfusion, organ transplant and mother to child.	General: headache, body aches, joint pains, vomiting, Focal: rash (erythematous or maculopapular) Complications: encephalitis, meningitis DDx: bacterial and viral meningitis, leptospirosis, toxoplasmosis, herpes simplex	Blood and CSF can be used for testing. Viral culture and test to detect RNA can be done on specimens collected early in the course of illness. WNV-specific IgM antibodies can be detected 3 to 8 days after onset of illness and may persist for up to 90 days. PRNT can be used to confirm acute infection.	No specific antiviral treatment available. Supportive therapy is given.
Chikungunya/ Chikungunya virus Aedes mosquito	General: fever and joint pain, headache, muscle pain, joint swelling Focal: multiple joint pain that may continue for weeks to months, rash Complications: incapacitation of the patient DDx: malaria, dengue, leptospirosis, GAS, rickettsial infection	Molecular testing best within 7 days of onset of signs and symptoms. Antibodies (IgM) detectable 4 days after onset of symptoms. PRNT used for confirmation.	No specific antiviral. Severe arthralgia may be managed with NSAIDs (once dengue is excluded) and physiotherapy
Flu, pneumonia/ Influenza viruses Droplets generated when infected people sneeze, cough, or talk. Touching contaminated objects and surfaces	General: Fever, cough, sore throat, body aches, headaches, tiredness, pharyngitis, myalgia Focal: runny or stuffy nose plus cough Complications: pneumonia, bronchitis, sinus and ear infections. DDx: RSV, adenoviruses, enteroviruses, and paramyxoviruses. Early stages of flavivirus infections	Specimens include NP, throat and nasal swabs and blood. Diagnostics include: RDT (antigen detection), molecular (nucleic acid detection), IFA, cell culture and serological testing of paired sera.	Bed rest and supportive therapy. The following used for chemoprophylaxis and treatment: Oseltamivir, Amantadine, Rimantadine, Peramivir and Zanamivir
Bronchiolitis/RSV Respiratory droplets. Infected people can be contagious for 3 to 8 days	General: flu like symptoms, wheezing Focal: none Complications: bronchiolitis and pneumonia in children <1 year DDx: neonatal sepsis, croup, asthma, bronchiolitis, other respiratory viruses	Viral culture, antigen detection, PCR and serological testing. A multiplex respiratory panel diagnostic test is available.	Supportive care is the mainstay of therapy. Ribavirin: for high risk patients (transplant recipients). Palivizumab: is given

Disease/Pathogens* and Mode of Transmission	Clinical Features	Laboratory Diagnosis	Management
			as prophylaxis for children at risk for severe disease
Adenovirus Aerosolized droplets, fecal-oral route and direct inoculation to the conjunctiva.	General: flu-like symptoms, Focal: conjunctivitis, gastroenteritis, diarrhea. Complications: rarely cause serious illnesses or death DDx: influenza, URTIs, bacterial pneumonia and pharyngitis, community acquired pneumonia	Specimens: NP, stool, conjunctival secretions and blood. Culture (specimen should be collected early in clinical course) Serology (A 4-fold rise in acute titers to convalescent titers) Antigen test (indirect IFA) PCR (high specificity)	Presently, the use of antivirals remains a matter of debate. Supportive and symptomatic treatment is the mainstay. Fortunately, most infections are self-limiting
Ebola virus disease/Ebola virus Blood/bodily fluids, objects (needles), infected fruit bats or primates and possibly contact with semen from a man who has recovered from Ebola.	General: fever, headache, muscle pain, weakness, fatigue, vomiting, stomach pain Focal: bleeding through mucosa and orifices, diarrhea Complications: multiple organ failure, shock DDx: malaria, typhoid and other viral hemorrhagic fevers (VHF)	Within a few days after onset of symptoms: antigen-capture and IgM ELISA, PCR and virus isolation. Later in the course of disease or after recovery: IgM and IgG antibodies.	No specific therapy currently available that has demonstrated efficacy. Supportive treatment with attention to intravascular volume, nutrition, electrolytes, replacement of coagulation factors is beneficial to the patient.
Marburg hemorrhagic fever/Marburg virus Transmission is similar to Ebola virus. Reservoir is the Egyptian fruit bat, *Rousettus aegyptiacus*.	General: fever, chills, headache, myalgia, nausea, vomiting, chest pain, sore throat, abdominal pain. Focal: hemorrhage, maculopapular rash (most prominent on the trunk) Complications: multiple organ failure, shock DDx: malaria, typhoid, other VHF.	Similar to Ebola virus	Similar to Ebola virus
Lassa hemorrhagic	General: fever, malaise, body weakness,	Virus can be cultured in 7-10	Supportive care

Disease/Pathogens* and Mode of Transmission	Clinical Features	Laboratory Diagnosis	Management
fever/Lassa virus Contact with urine and droppings of infected *Mastomys* rodents	vomiting, headache, pain in the chest and back, and abdomen Focal: facial swelling, hemorrhage Complications: deafness, shock DDx: malaria, typhoid, leptospirosis, Rift valley fever, Q fever, influenza, other VHF	days following symptoms. RT-PCR can be used during early stage of the disease. ELISA can be used to detect IgM/IgG and antigens.	including fluid and electrolytic balance, blood pressure monitoring can be lifesaving. Ribavirin is used in Lassa fever

*Only pathogens covered in this dissertation are included

**Signs and symptoms which may sometimes help in differential diagnosis

LAMP: loop-mediated isothermal amplification, **PRNT**: plaque reduction neutralization test, **NP**: nasopharyngeal swab, **IFA**: immune-fluorescence assay, **HAI**: hemagglutination inhibition, **NSAIDS:** nonsteroidal anti-inflammatory drugs, **DDx**: differential diagnosis, **VHF**: viral hemorrhagic fever

Table 2: Summary of most common diagnosis of febrile illnesses in the tropics

Most common diagnosis	Study design	Setting	Remarks	Year of study (reference)
Africa				
• 62% ARI (5% chest X-ray-confirmed pneumonia) • 11.9% nasopharyngeal viral infection • 10.5% malaria • 10.3% gastroenteritis • 5.9% UTI • 3.7% typhoid fever • 1.5% skin/mucosal infections • 0.2% meningitis	N = 1,005 (< 10 years with fever) Computer algorithm generated diagnosis using history, physical, and wide array of lab investigations	Tanzania: one urban and one rural outpatient clinic	• 3.2% undiagnosed cases. • Multiple diagnoses in 22.6% of the study participants	2014 (2)
• 10% chikungunya virus • 7.7% leptospirosis • 7.4% spotted fever rickettsial disease • 3.4% bacteremia • 2.6% Q fever • 2% brucellosis • 1.3% malaria • 0.9% fungemia	N = 467 (ages 2 months to 13 years). Diagnoses by case definitions and convalescent serum at 4 to 6 weeks post discharge	Tanzania: In-patients	• 64% undiagnosed cases. • Limited viral testing. • High prevalence of zoonoses; consider different empiric antibiotic regimens	2013 (3)
• 62% malaria • 7% clinical pneumonia Serologically diagnosed: • 5.8% typhoid • 5.1% typhus • 2.6% brucellosis	N = 653 (ages 3–17 years) with acute fever or fever within the past three days	Ethiopia: Four outpatient clinics in Gojjam zone	Limited viral testing	2009(4)

Most common diagnosis	Study design	Setting	Remarks	Year of study (reference)
• 65% ARIs: (54% viral, 12% bacterial, 18% unknown) • 26% watery diarrhea • 5% skin infections • 2% bloody diarrhea • 0.2% malaria	N = 677 cases, 200 controls (ages 2–59 months) Diagnoses by IMCI classifications plus laboratory investigations	Zanzibar, Tanzania	Of the viral ARIs most common PCR results: 16% RSV 9% influenza (A/B) 9% rhinovirus	2013 (1)
• 93% ARIs as follows: 47%URI, 29% common cold, 12% pharyngitis, 4% pneumonia and 1% otitis media. • 10% diarrhea • 8% skin infections • 2% urinary tract infections	N = 1,602 (<10 years with fever in last 24 hours). Clinical diagnoses for RDT or Microscopy negative for malaria per local clinical guidelines	Uganda, study clinic within a referral third level hospital	15% unknown diagnosis. Limited testing for bacterial illnesses such as typhoid	2007 (5)
Southeast Asia				
• 16.2% dengue virus • 7.8% scrub typhus 6.3% culture-proven bloodstream infection, including ○ 1.8% *Salmonella typhi* ○ 1.1% *Streptococcus pneumoniae* ○ 0.7% *Escherichia coli* ○ 0.6% *Haemophilus influenzae* ○ 0.5% *Staphylococcus aureus* ○ 0.5% *Burkholderia pseudomallei* • 5.8% Japanese encephalitis virus	N = 1180 (<16 years with fever). Demographic, clinical, laboratory and outcome data were comprehensively analyzed.	Cambodia, study at a Children's hospital in Siem Reap province.	Identified a microbiological cause of fever in almost 50% of episodes. 18.8% had two pathogens, 3.7% had three pathogens, and 0.3% had four pathogens causing fever	2013 (6)

Most common diagnosis	Study design	Setting	Remarks	Year of study (reference)
Top five diagnoses when only one etiological agent per patient were: • 8% dengue • 7% scrub typhus • 6% Japanese encephalitis virus • 6% leptospirosis and 2% bacteremia Six months influenza testing with 32% positive.	N = 1,938 (ages 5 months to 49 years) with fever	Laos, two provincial hospitals	Multiple diagnosis: 5% had >1 pathogen, 5% had two pathogens and <1% had 3 pathogens. 59% of participants without a diagnosis.	2013 (7)
• 47% ARI • 23% diarrhea or dysentery • 17% enteric fever • 2% bacteremia • 0.5% UTI • 0.4% malaria	N = 1,248 febrile episodes (all ages) Case definition and laboratory investigations	Pakistan, small peripheral clinic	High proportion of enteric disease	2013 (1)

*Only studies that included at least one or more respiratory, enteric and systemic pathogens are included
ARI: acute respiratory infection, UTI: urinary tract infection, URI: upper respiratory infection, IMCI: integrated management of childhood illnesses

Long-term goal, objective and hypothesis

Our **long-term goal** is to develop rapid, sensitive and specific tools for diagnosis of tropical febrile illnesses. The **primary objective** of this research is to profile the infectious etiologies of febrile illnesses in Cameroon. Our **central hypothesis** is that approximately 50% of febrile illnesses attributed to and treated as malaria in Cameroon are caused by pathogens other than the malaria parasite. The **rationale** is that to treat non-malaria febrile illnesses appropriately, pathogens that cause fever must be identified. Moreover, some patients may be co-infected with malaria and other pathogens that cause fever. We **expect** to profile bacteria, parasites, and viruses that cause febrile illnesses, which will form the basis for the development of new clinical management strategies and better diagnostics, as well as vaccines and other preventive modalities. These fundamental data will have a significant **impact** on public health and medicine and will assist policy makers to allocate resources to support surveillance systems to monitor the prevalence and trends of febrile diseases.

Specific Aims

Specific Aims 1. To evaluate the accuracy of clinical diagnosis, thick-film microscopy (TFM), rapid diagnostic test (RDT) and polymerase chain reaction (PCR), in the diagnosis of malaria

Hypothesis: Clinical diagnosis of malaria is the least accurate method for diagnosing malaria.
Rationale: Prompt and accurate diagnosis of malaria is not only an essential component of malaria treatment, control and elimination but also valuable in the identification of malaria-negative patients, for which further investigations need to be sought and appropriate treatment administered. The performance of malaria test methods may vary from one region to another and likely to be influenced by the level of malaria endemicity and competency of the technicians. Therefore, active monitoring of the performance of various diagnostic methods for malaria at the country level is important to guide policy on the diagnostic methods to pursue for malaria diagnosis and elimination.
Approach: This aim will focus on conducting a comprehensive evaluation of the three commonly used diagnostic test methods for malaria (clinical diagnosis, TFM, and RDT), concurrently with PCR, in samples collected from febrile Cameroonian patients with suspected malaria. Laboratory test results will be compared to the patient health records and clinical diagnosis.

Specific Aim 2. To assess the prevalence of infectious causes of febrile illnesses in Cameroon

Hypothesis: Approximately 50% of febrile illnesses on the African continent are due to viral infections, and the remainders are due to bacteria and parasites.

Rationale: There is a paucity of data on the causes of febrile illnesses in Cameroon, therefore this aim will focus on screening patient-derived biological samples for the presence of pathogens.

Approach: We will test sera from febrile patients for antibodies against *Toxoplasma gondii, Salmonella typhi, Shigella* sp., *Entamoeba histolytica*, *Leptospira interrogans*, West Nile virus (WNV), chikungunya virus (CHIKV), influenza A/B viruses, respiratory syncytial virus (RSV), adenovirus, parainfluenza virus types 1, 2, 3 and 4, using Luminex® assay, ELISA, RDT and PRNT**.** Procalcitonin levels will be measured using ELISA in plasma samples of patients who meet sepsis criteria.

Specific Aim 3. To determine the prevalence of viral hemorrhagic fevers (VHF) among febrile Cameroonian patients

Hypothesis: Cameroonians are infected with viruses causing hemorrhagic fevers, such as dengue, Ebola, Marburg and Lassa viruses.

Rationale: The scarcity of reliable data on the epidemiology of VHF in Cameroon and most African countries is in part due to lack of diagnostic tools and active surveillance. The highly infectious and diverse etiologies of VHF underscore the need to develop diagnostic tools that are sensitive and can be multiplexed to concurrently diagnose multiple VHF causing viruses.

Approach: We will test serum samples from febrile patients for antibodies against Ebola, Marburg, and Lassa viruses, using newly developed Luminex®-based assays.

Significance

Evaluating the performance of various malaria diagnostic methods

As malaria-endemic countries move toward malaria elimination, there is a need for rapid and accurate diagnosis of malaria since the performance of available malaria test methods varies from one region to another and is influenced by the level of malaria endemicity and competency of the technicians. In the proposed study, we will compare the accuracy of clinical diagnosis, rapid diagnostic test (RDT), thick-film microscopy (TFM) and PCR in the diagnosis of malaria in Cameroon. Active monitoring of the performance of various malaria diagnostic methods at the country level is necessary to guide policy for malaria diagnosis. Moreover, accurate diagnosis of

malaria is not only ***significant*** for malaria treatment and elimination but also to identify other pathogens affecting malaria-negative febrile patients to provide effective and efficient clinical management of patients.

Profiling etiologies of febrile illnesses

Health care workers (HCWs) in malaria-endemic countries are always faced with the daunting challenge of the proper course of action to take when a patient with fever tests negative for malaria. To treat non-malaria fevers appropriately, pathogens that cause febrile illnesses must be identified. Febrile illnesses are caused by diverse pathogens with signs and symptoms that overlap, making a differential diagnosis based on clinical criteria alone challenging (Table 1). The prevalence of these pathogens may vary depending on the geographical region, age and season. Although a specific pathogen may not be identified, knowing the category (virus, parasite, fungi and bacteria) would be useful in deciding treatment options. Moreover, recent outbreaks of febrile illnesses, including EVD, have taught us that routine surveillance of some febrile illnesses, including viral hemorrhagic fevers, is critical. Therefore, the ***significance*** of this study is its focus on identifying infectious etiologies of febrile illnesses in Cameroon.

This study will thus fill ***gaps in knowledge*** and will provide information that can be used by HCWs to improve diagnosis and management of febrile illnesses, thereby improving disease outcomes. Moreover, data from this study can contribute to the development of algorithms in the management of febrile illnesses in the tropics and the development of diagnostic tools.

Developing accurate diagnostics for febrile illnesses

Correct diagnosis of febrile illnesses is only possible with the use of laboratory tests. In the absence of accurate and available diagnostic tests, the health and economic burden of these diseases will continue to rise. Deaths from some febrile illnesses can easily be prevented if accurate diagnostic tools are made available. With the myriad of pathogens that can cause febrile illnesses in the tropics, it is challenging to use a battery of single tests to systematically test for every pathogen. Therefore, this proposal will also focus on developing a multiplex immunoassay that will be utilized for the surveillance of hemorrhagic fever viruses, which, in the future, will include other pathogens, such as hantavirus, Zika virus and CHIKV. Thus, the availability of a multiplex diagnostic platform for diagnosis of tropical fevers will ***significantly*** enable rapid and accurate differential diagnosis of febrile illnesses, thereby improving patient

care and breaking the pathogen transmission cycle leading to the early identification, isolation, and treatment of affected patients.

Innovation

The proposed research is innovative as it represents the first study to profile pathogens that cause febrile illnesses in Cameroon, including hemorrhagic fever viruses. An innovative multiplex microsphere immunoassay, using Luminex® technology, which incorporates recombinant antigens for simultaneous detection of Ebola, Marburg, Lassa and dengue viruses is the first of its kind to be developed and used for surveillance in Cameroon. Data obtained from this study can be used to develop algorithms for the management of febrile illnesses.

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Chapter 3

Flipping the Switch to Accurately Diagnose Malaria: A Comparison of Clinical Diagnosis, Thick-Film Microscopy, Rapid Diagnostic Test, and PCR in the Diagnosis of Malaria in Cameroon

Flipping the Switch to Accurately Diagnose Malaria: A Comparison of Clinical Diagnosis, Thick-Film Microscopy, Rapid Diagnostic Test, and PCR in the Diagnosis of Malaria in Cameroon

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Abstract

Accurate diagnosis of malaria is important for effective disease management and control. In Cameroon, presumptive clinical diagnosis, rapid diagnostic test (RDT) and thick-film microscopy (TFM) are commonly used in malaria diagnosis. However, these methods lack sensitivity to detect low parasitemia. PCR, on the other hand, enhances the detection of sub-microscopic parasitemia making it a much-needed tool for malaria diagnosis and elimination. In this study, we compared the accuracy of clinical diagnosis, RDT, and TFM against PCR in the diagnosis of malaria. Blood samples, collected from 551 febrile patients from Cameroon between February 2014 and February 2015, were tested for malaria by RDT, TFM, and PCR. The hospital records of participants were reviewed at the end of their hospital visit for clinical diagnosis of malaria. TFM-, RDT- and PCR-based prevalence of malaria was 31%, 45%, and 54%, respectively. However, 92% of participants were presumed to have malaria based on fever, of which 38% were negative by PCR. PCR detected 23% and 12% more malaria infections than TFM and RDT, respectively. The sensitivity of TFM, RDT, and clinical diagnosis was 57%, 78% and 100%, respectively; the specificity was 99%, 94%, and 17%, respectively; the positive predictive value was 99%, 94%, and 59%, respectively; the negative predictive value was 66%, 78%, and 100%, respectively. Thus, 38% of the participants clinically diagnosed as having malaria, had fever caused by other pathogens. A total of 128 individuals with sub-microscopic infections were identified by PCR in the study population. The data suggest that PCR may be the best tool for the accurate diagnosis and control of malaria since the presence of sub-microscopic malaria infections may be a potential hindrance towards malaria elimination. The development of a rapid and sensitive PCR-based test to diagnose malaria could flip the switch to accurate diagnosis, control and elimination of malaria in Cameroon and around the globe.

Introduction

Malaria remains a major public health threat, particularly in sub-Saharan Africa, where about 191 million new infections and 395,000 deaths were reported in 2015 (1). The World Health Organization (WHO) now recommends a confirmatory diagnosis of malaria before initiation of treatment, partly influenced by the fear of the development of drug resistance and to enable the identification of malaria-negative patients, for which further investigations need to be sought and appropriate treatment provided (2). Accurate diagnosis of malaria is thus vital for effective management and control of malaria while avoiding the wrong use of antimalarial drugs. In most malaria-endemic countries, malaria diagnosis depends mainly on clinical evidence, and in some cases, thick film microscopy (TFM) and rapid diagnostic test (RDT) kits may be used for laboratory confirmation.

The traditional practice by health care workers (HCW) in malaria-endemic countries has been to diagnose malaria based on a history of fever (3-6). Clinical diagnosis even though imprecise remains the mainstay for therapeutic care for the majority of febrile patients in malaria-endemic countries. The specificity of clinical diagnosis of malaria is reduced by the overlap of malaria symptoms with other tropical diseases, such as typhoid fever, respiratory tract infections, bacterial disease and viral infections. The accuracy of clinical diagnosis may vary with the level of endemicity, malaria transmission season and age group. A high-quality microscopy service for malaria diagnosis is not widely available, especially in rural areas where diagnostic and treatment services are required. Moreover, malaria microscopy is complex, which includes different species and blood stages of the *Plasmodium* parasite, and requires a competent microscopist who often is overworked. Also, the presence of sub-microscopic parasitemia greatly reduces the sensitivity of malaria diagnosis by TFM. Unlike TFM, RDT detects malaria antigens, not malaria parasites, which gives it an added advantage in its ability to diagnose malaria in patients with low-grade parasitemia below the detection limit of TFM. However, the specificity of the commonly used RDT that detects histidine rich protein (HRP) of *P. falciparum*, is limited when the parasite is cleared and antigens remain in circulation for about 28 days (false positive) (2).

As malaria-endemic countries move towards malaria elimination, there is a need for rapid and accurate diagnostic tools for malaria, which are capable of detecting low-grade parasitemia. In this study, we compared the performance of clinical diagnosis, RDT, TFM and PCR in the diagnosis of malaria in Cameroon. Active monitoring of the performance of various diagnostic methods for malaria at the country level is necessary to guide policy on the diagnostic methods to use for malaria diagnosis and elimination.

Methods

Study area

This study was conducted in three regions of Cameroon, Far North, Center and North West with varied climatic conditions and altitudes (Table1). In the Far North Region, we conducted the study in Maroua in October 2014. In the Center Region, the study was conducted in Nkolbisson (a neighborhood in Yaoundé) from February 2014 to April 2014. In the North West Region, the study was carried out in Bamenda in February 2015.

Study design

A cross-sectional study was conducted in selected health care facilities in Maroua, Nkolbisson, and Bamenda (Figure 1). Inclusion criteria were age >6 months and axillary temperature >37.5^{o}C at the time of recruitment or fever within 24 hours preceding recruitment. A written informed consent was obtained from all study participants $\geq$18 years of age. Parents or legal guardians of children <18 years gave a written informed consent on behalf of their children.

Ethical considerations

Ethical approvals were obtained from the Committee on Human Subjects of the University of Hawaii (protocol number CHS 21724) and the National Research Ethics Committee of the Ministry of Public Health Cameroon (protocol number 2014/04/442/CE/CNERSH/SP). Administrative approvals were obtained from the Minister of Public Health Cameroon and the Directors of the various Health Institutions.

Study procedure

We provided an easy-to-read questionnaire for the collection of demographic and clinical data. After obtaining informed consent from all patients, the research or clinic staff took axillary temperature readings and conducted a physical examination with a clinical note of the reported signs and symptoms. Venous blood, 2–5 mL, was collected from each participant, dispensed into EDTA tube and stored in cold boxes until transported to the research laboratory where they were stored at 4–8^{o}C. RDT and TFM for malaria were conducted for all participants, and both results were presented to the consulting HCW. At the end of their hospital visit, an exit survey was conducted for previously consented patients to verify if the consulting HCW adhered to the malaria test results or prescribed antimalarial drugs to malaria-negative patients. When exit survey was not possible, we consulted the hospital records for such information.

Laboratory investigations

Malaria RDT: Approximately five μL of blood was used to conduct malaria RDT using the Ag Pf/Pan kit (Standard Diagnostic Inc., South Korea), following the manufacturer's instructions. This RDT is a qualitative immunochromatographic test that detects *P. falciparum* HRP-II and *Plasmodium* lactate dehydrogenase (pLDH), which is a glycolytic enzyme common to *P. falciparum, P. ovale, P. vivax* and *P. malariae*.

Malaria TFM: Thick blood films were prepared and stained using 10% Giemsa for 15 minutes. A slide was considered positive if at least one asexual blood stage of malaria parasite was visualized. Parasitemia was determined by counting the number of parasites against 200 white blood cells and assuming that each subject had 8,000 WBCs/μL of blood. Two readings were conducted for each slide and discrepancies were resolved by the third reading by an independent technician.

Malaria PCR: DNA was extracted from 200 μL of whole blood by spin-column technique (Macherey-Nagel, Germany) following the manufacturer's instructions. Detection of malaria parasite DNA was based on nested PCR amplification of the 18s rRNA gene in a reaction that used 2 μL of the extracted DNA, 10 μL of GoTaq polymerase and master mixes (Promega, USA), 0.25 μM each of upstream and downstream primers, and 6 μL of buffer, in a total reaction volume of 20 μL. The first PCR encompassed genus-specific primers and the second nested PCR run encompassed the species-specific primers for *P. falciparum, P. malariae and P. ovalae,* as previously described (7). The amplicon size of the products was estimated after electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized on a UV transilluminator.

Statistical analysis

Data were entered into Microsoft Office Excel and analyzed using StatPlus 5.9.80 (AnalystSoft Inc., Walnut, CA) and Prism 6.0 (Graphpad Software, San Diego, CA) for descriptive statistics. Diagnostic test performance for clinical diagnosis, RDT, and TFM for the diagnosis of malaria, was analyzed using MedCalc 16.8 (Ostend, Belgium). Descriptive statistics are represented as frequencies and medians. Sensitivity, specificity, positive and negative predictive values, accuracy and percentage of agreement (*kappa* value) were calculated with confidence intervals by age groups. Multivariable logistic regression was conducted to identify correct diagnosis comparing other test methods (RDT and TFM) to PCR as the gold standard. We also conducted

linear mixed models treating multiple test methods performed on the same patients to identify the difference in proportions by test methods and age groups. PCR test was used as a reference standard for all the analyses. P <0.05 was considered statistically significant. These analyses were conducted using SAS 9.4 (SAS Institute, Cary, NC).

Results

General characteristics of the study population and study sites environmental factors

Of the 551 febrile patients, recruited from selected health care facilities in Cameroon (Figure 1) between February 2014 and March 2015, 57% were from Nkolbisson, 23% from Maroua and 20% from Bamenda. The overall distribution of males was 48%, and females were 52% in the study population. The majority of the study participants were in the age-group of 0-5 years, 81 (65%) in Maroua, and 180 (57%) in Nkolbisson. However, 69 (61%) of the study participants in Bamenda were greater than 17 years of age (Table 1).

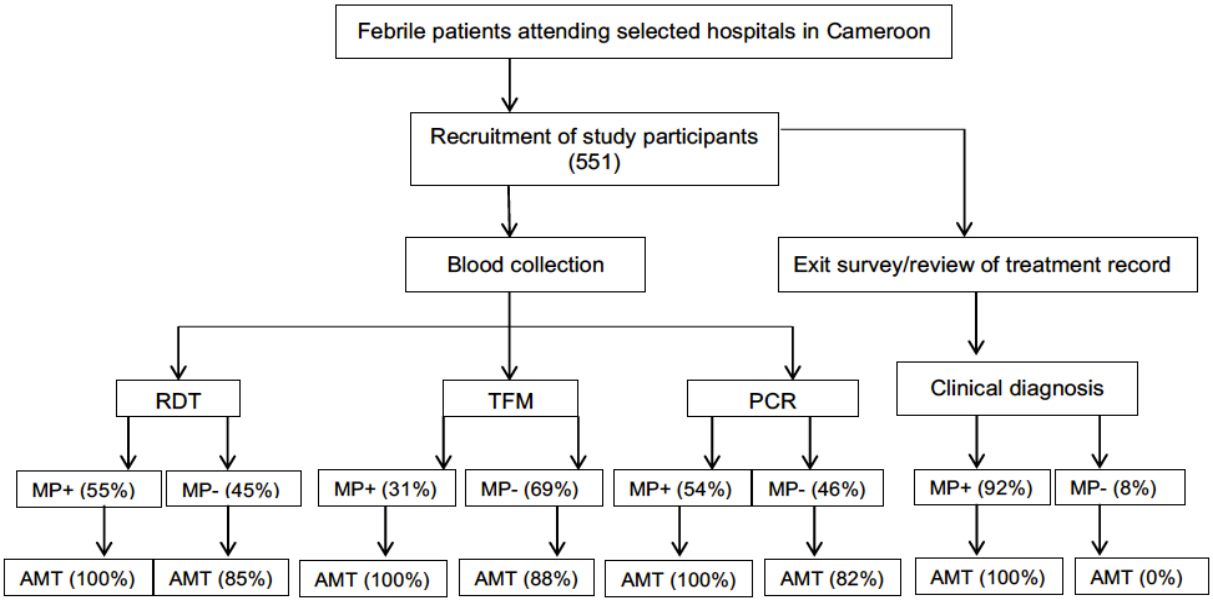

Figure 1: Flow chart of recruited patients, malaria test methods and antimalarial treatment by health care workers
RDT, rapid diagnostic test; TFM, thick film microscopy; PCR, polymerase chain reaction; MP+, malaria positive; MP-, malaria negative; AMT, antimalarial treatment

Table 1: General characteristics of study population, study sites and environmental factors

Characteristics	Study sites			Total n (%)
	Maroua n (%)	**Nkolbisson n (%)**	**Bamenda n (%)**	
Gender				
Male	64 (52)	168 (53)	33 (29)	265 (48)
Female	60 (48)	147 (47)	79 (71)	286 (52)
Total	124	315	112	551
Age group (years)				
0-5	81 (65)	180 (57)	34 (30)	295 (54)
6-10	15 (12)	86 (27)	5 (4)	106 (19)
11-16	9 (7)	49 (16)	4 (4)	62 (11)
>17	19 (15)	0 (0)	69 (61)	88 (16)
Environmental factors (8)	**Maroua**	**Nkolbisson**	**Bamenda**	
Climate	Sahelian	Tropical savanna	Tropical	
Average annual temperature	28.3 ^{o}C	23.7^{o}C	21.5 ^{o}C	
Average annual rainfall	794 mm	1,643 mm	2,145 mm	
Elevation	384 m	750 m	1,614 m	
Weather condition at time of specimen collection	End of rainy season	Rainy season	Dry season	

High malaria prevalence in Maroua and Nkolbisson

The overall prevalence of malaria by TFM, RDT, and PCR in the general population was 31%, 45%, and 54%, respectively. However, 92% (507/551) of the participants were diagnosed clinically for malaria, of which 38% (209/551) were negative for malaria by PCR (Table 2). The prevalence of malaria by PCR in Maroua, Nkolbisson, and Bamenda was 57%, 70% and 5%, respectively.

Table 2: Malaria prevalence stratified by test method and study site

Test Method	Study sites						Total (n=551)	
	Maroua (n=123)		**Nkolbisson**(n=351)		**Bamenda**(n=112)			
	MP+	P (%)	MP+	P (%)	MP+	P (%)	MP+	P (%)
TFM	32	26	135	43	5	4	172	31
RDT	67	54	175	55	7	6	249	45
PCR	71	57	221	70	6	5	298	54
Clinical diagnosis	120	97	301	96	86	77	507	92

TFM, thick film microscopy; RDT, rapid diagnostic test; PCR, polymerase chain reaction, MP+, malaria positive; P; prevalence

Differences in the proportion of diagnosed malaria cases by test method and age group

In participants younger than 17 years of age, malaria prevalence was higher by PCR, TFM, RDT and clinical diagnosis, as compared to those older than 17 years of age. There was variation in the age-specific prevalence of malaria by all test methods (Figure 2). Specifically, the difference between the age-specific prevalence of malaria by PCR and RDT was 8%, 3%, 2% and 1% for the age group 0-5, 6-10, 11-16 and >17 years, respectively. Meanwhile, the difference between the age-specific prevalence of malaria by PCR and TFM was 18%, 27%, 24% and 13% for the age group 0-5, 6-10, 11-16 and >17 years, respectively. Clinical diagnosis of malaria was common irrespective of the age-group. Across different age groups, the age-specific prevalence of malaria by RDT was closer to that of PCR (Figure 2).

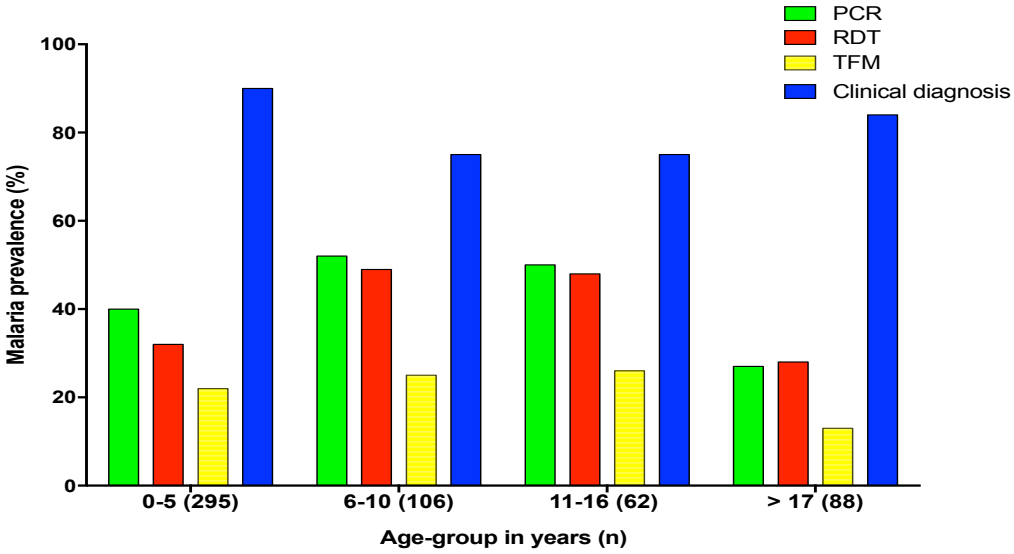

Figure 2: Age-specific prevalence of malaria by test method

Age-group, gender and study site did not influence the ability to correctly diagnose malaria

To determine if there was a difference in correctly diagnosing malaria across different age-groups, gender and study sites, a multivariable logistic regression was conducted to identify correctly diagnosed malaria cases by comparing the various test methods, RDT vs. PCR and TFM vs. PCR. There was no significant difference in accurately diagnosing malaria by RDT between those aged >5 years and <5 years ($p = 0.12$), males and females ($p = 0.41$) and, between study sites with high malaria transmission intensity ($p = 0.84$) (Table 3). Similarly, there was no significant difference in correctly diagnosing malaria by TFM in the age group >5 years vs. <5 years ($p = 0.36$), males and females ($p = 0.11$) and, between study sites with high malaria transmission intensity ($p = 0.15$). Those living in Bamenda were 11 and 68 times more

likely to be correctly diagnosed having Malaria by RDT and TFM, respectively, compared to those residing in Maroua after adjusting for age-group and gender.

Table 3. Multivariate logistic regression analysis of correctly diagnosing malaria by RDT and TFM in different age-groups, gender and study sites

Test method	Variable	Odds ratio (95% CI)	p-value
RDT	Age-group (6-16 vs. 0-5 years)	1.52 (0.89-2.67)	0.12
	Gender (Female vs Male)	0.81(0.49-1.34)	0.41
	Region (Maroua** vs Nkolbisson**) (Maroua vs Bamenda*)	0.94 (0.53-1.68) 10.61 (2.96-38.04)	0.84 0.0003
TFM	Age-group (6-16 vs 0-5 years)	0.81 (0.52-1.26)	0.36
	Gender (Female vs Male)	1.41 (0.92-2.16)	0.11
	Region (Maroua vs. Nkolbisson) (Maroua vs Bamenda)	0.70 (0.43-1.14) 67.63 (8.87- 515.5)	0.1 <0.0001

** High malaria transmission intensity study site
* Low malaria transmission intensity study site

Substantial number of sub-microscopic infections detected by PCR and RDT

Sub-microscopic *P. falciparum* infection was defined as, i) positive by PCR but negative by microscopy, ii) positive by PCR but negative by RDT, and iii) positive by RDT but negative by microscopy. A total of 128 (23%) febrile patients were positive for *P. falciparum* by PCR but negative by TFM (Table 4). Meanwhile, 65 (12%) febrile patients were positive for *P. falciparum* by PCR and negative by RDT. RDT detected 84 (15%) malaria infections that were missed by microscopy. In general, sub-microscopic *P. falciparum* infections were more common in the younger age-group.

Table 4: Prevalence of sub-microscopic *P. falciparum* infection by age-group

Age-group (years)	Test characteristic		
	PCR (+), TFM (-) n (%)	PCR (+), RDT (-) n (%)	RDT (+), TFM (-) n (%)
0-5 (n=259)	72 (24)	45 (15)	37 (13)
6-10 (n=106)	34 (32)	12 (11)	27 (25)
11-16 (n=62)	16 (26)	5 (8)	13 (21)
> 17 (n=88)	6 (7)	3(3)	7 (8)
Total (n=551)	128 (23)	65 (12)	84 (15)

PCR (+), TFM (-) = PCR positive but TFM negative
PCR (+), RDT (-) = PCR positive but RDT negative
RDT (+), TFM (-) = RDT positive but TFM negative

Comparison of diagnostic accuracy of clinical diagnosis, RDT, and TFM

According to the reference method, PCR, 298 participants were positive for malaria while 253 participants were negative for malaria (Table 5). RDT correctly identified 234 (79%) infections (true positive), microscopy correctly identified 170 (57%) infections whereas clinical diagnosis identified all 298 (100%) infections. However, there were 209 (83%) false positive clinical diagnosis, 15 (6%) by RDT and 2 (0.8%) by TFM. The sensitivity of TFM, RDT, and clinical diagnosis was 57%, 78%, and 100%, respectively, the specificity was 99%, 94%, and 17%, respectively, positive predictive value was 99%, 94%, and 59%, respectively, and negative predictive value was 66%, 78%, and 100%, respectively. The clinical diagnosis had a "poor" agreement (kappa 0.18), malaria RDT had a "good" agreement (kappa 0.71), and malaria microscopy had a "moderate" agreement (kappa 0.54) when compared to the reference method, malaria PCR. In general, the accuracy of clinical diagnosis was 62%, RDT 85%, and TFM 76%. However, the error rate of clinical diagnosis was 34%, RDT was 14%, and TFM was 23% (Table 5).

Table 5: Diagnostic test performance of clinical diagnosis, RDT, and TFM in the diagnosis of malaria with PCR as reference method

Test characteristic	Clinical diagnosis	RDT	TFM
TP (PCR=298)	298	234	170
FP (PCR negative)	209	15	2
TN (PCR=253)	44	237	251
FN (PCR positive)	0	65	128
Sensitivity [95%CI]	100% [99-100]	78% [73-82]	57% [51 to 63]
Specificity [95%CI]	17% [13-23]	94% [90-97]	99% [97 to 99]
PPV [95%CI]	59% [54-63]	94% [90-96]	99% [96 to 99]
NPP [95%CI]	100% [92-100]	78% [73-83]	66% [61 to 71]
Accuracy [95%CI]	62% [58-66]	85% [82-88]	76% [73 to 80]
Kappa value [95%CI]	0.18 [0.14-0.24]	0.71 [0.65-0.77]	0.54 [0.48-0.60]
Misclassification rate	34%	14%	23%

TP, True positive; FP, False positive; TN, True negative; FN, False negative; PPV, Positive predictive value; NPV, Negative predictive value

Discussion

Accurate and prompt diagnosis of malaria is the only way to effectively treat, manage and eventually eliminate the disease. In this study, we investigated the diagnostic accuracy of clinical diagnosis, TFM, and RDT in the diagnosis of malaria using PCR as the reference standard. We demonstrate a high prevalence of clinical diagnosis of malaria among febrile patients in all the study sites, which led to the indiscriminate use of antimalarial drugs. RDT for malaria performed best as compared to TFM and clinical diagnosis. However, PCR detected several malaria infections that were missed by TFM and RDT.

Heterogeneous distribution of malaria

This study has reported an overall malaria prevalence of 54% by PCR. The prevalence of malaria by PCR was 1.2 and 1.7-fold higher than that determined by RDT and TFM, respectively. In a previous study in Ethiopia, the PCR-based prevalence of malaria was 3.3- and 5.6-fold greater than that determined by RDT and TFM, respectively (9). PCR, a very sensitive method, can detect parasite burden as low as 0.002 parasites/μL (10).

The prevalence of malaria was high in Nkolbisson and Maroua but low in Bamenda. Differences in climatic variables (temperature, relative humidity, rainfall), altitude, vector population, transmission dynamics and human behavior, have been shown to affect the prevalence of malaria in a given region (11-15). In Nkolbisson, the study was conducted during the rainy season, and in Maroua, it was at the end of the rainy season. Meanwhile, in Bamenda, the study was conducted during the dry season. Also, Maroua and Nkolbisson are at a low altitude, 384 m and 760 m, respectively, as compared to Bamenda, situated at 1,614 m. A previous study in Tanzania reported malaria prevalence proportions of 79-90%, 27-46% and 8-16% in low, intermediate and high altitudes, respectively (16). The climatic variables described above could have accounted for the different distribution of malaria reported throughout Cameroon. Results of this study provide information on the prevalence of malaria in three climatically different regions of Cameroon, which is important to guide malaria control interventions.

Sub-microscopic symptomatic malaria infections

We demonstrate that TFM missed 23% of PCR-positive malaria infections. In a meta-analysis, microscopy missed about 50% of PCR-positive malaria infections (17). False-negative microscopy results are known to increase as parasite density decreases(18). Moreover, the detection threshold of Giemsa-stained TFM varies considerably between 50–500 parasites/μL of

blood. RDT missed 12% of the malaria infections that were positive by PCR. Several factors have been demonstrated to affect the sensitivity of RDTs that detect HRP-II including, an inherent limitation of the device, mutation or deletion of the gene encoding the HRP-II and environmental factors including temperature(18, 19). However, RDT detected 15% of malaria infections that were negative by TFM. Unlike microscopy, RDT detects antigens, not parasites, which gives it an added advantage over microscopy in its ability to diagnose malaria in patients with low-grade parasitemia below the detection limit of microscopy. Because PCR detected 23% and 12% additional malaria infections as compared to TFM and RDT, respectively, the utility of TFM and RDT to accurately diagnose malaria, especially for the purpose of malaria elimination, warrants further investigation.

Performance of clinical diagnosis

The WHO currently recommends antimalarial treatment only for laboratory confirmed malaria cases (20). However, there was poor adherence to the WHO's recommendation in our study. Even in Bamenda, where only six patients were positive for malaria, 86 patients were treated for malaria. Elsewhere, high rates of clinical diagnosis and overtreatment of malaria have been reported with economic consequences (21-24). As expected, the sensitivity of clinical diagnosis of malaria in this study was high (100%). This is consistent with a recent study in Tanzania in which the sensitivity of clinical diagnosis of malaria was 97% (25). However, a clinical malaria diagnosis could predict the presence of malaria parasite in only 59% of our study participants. Hence, clinical diagnosis cannot be relied upon as a "rule in" test for malaria because of overlapping malaria symptoms with other tropical febrile illnesses. Moreover, the specificity of clinical diagnosis in the present study was low (17%) with 209 false positive results. Therefore, 209 patients were erroneously treated for malaria. Clinical malaria diagnosis could predict the absence of malaria parasite in a high percentage of patients making it a useful "rule out" test for malaria diagnosis.

Performance of malaria RDT

The accuracy of malaria RDT was good as compared to PCR. We reported a sensitivity and specificity of 78% and 94%, which is consistent with a recent study in Kenya (10) that evaluated the same RDT used in this study. Malaria RDT could predict the presence of malaria parasite in 94% of the study participants making it a good "rule in" test for malaria. It has been suggested that the specificity of HRP-II tests is reduced in high malaria transmission settings (26). In our study, there was a decrease in the specificity of malaria RDT when comparing Maroua (81%

specificity), located in a high transmission area, with results from Bamenda (98% specificity), characterized as a low transmission area (data not shown). Consequently, those living in Bamenda were more likely to be correctly diagnosed having malaria by RDT compared to those living in Maroua. A combination of long-lasting HRP-II antigens after parasite clearance and a high frequency of malaria infections may lead to persistent antigenemia in people living in high transmission settings. Malaria RDT was the most accurate method of malaria diagnosis in our study population compared to TFM and clinical diagnosis. Therefore, malaria RDT can be used to improve the quality of care by ensuring appropriate treatment of confirmed malaria cases while avoiding indiscriminate administration of anti-malarial drugs for malaria-negative patients.

Performance of TFM

The accuracy of TFM in this study was moderate, compared to PCR. The sensitivity and specificity of TFM were 57% and 99%, respectively. The observed lower sensitivity of TFM could be due to submicroscopic malaria infection in the study population. The two false-positive malaria cases diagnosed by TFM could be due to artifacts mistook for malaria as a result of poor blood film preparation. TFM has been shown to miss a substantial amount of malaria infections in low transmission areas (17). However, we found that those living in Bamenda (low transmission area) were more likely to be correctly diagnosed as having Malaria by TFM compared to those residing in Maroua (high transmission area). Nevertheless, only five patients were positive for malaria in Bamenda. Therefore, it may be premature to draw a conclusion due to the small sample size. Overall, TFM could predict the presence of malaria parasite in 99% of the study participants making TFM a good "rule in" test for malaria.

Conclusions

The accuracy of clinical diagnosis of malaria was poor, leading to the administration of antimalarial treatment to a large percentage of febrile malaria-negative patients without treating the true underlying cause of their fever. The diagnostic performance of RDT was superior to that of TFM and clinical diagnosis. Although TFM and RDT gave moderate and good results, respectively, they are not adequate when precise laboratory diagnostic data are needed to monitor antimalarial treatment. PCR permitted the detection of sub-microscopic parasitemia, making it a valuable tool for malaria diagnosis, control, and elimination. The development and standardization of a rapid and sensitive PCR-based test capable of detecting sub-microscopic malaria infection are urgently needed for the global elimination of malaria. Furthermore,

continual training and proficiency testing should be instituted for laboratory technicians on malaria microscopy and post-market surveillance to assure the quality of malaria RDT.

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Chapter 4

Infectious Etiologies of Febrile Illnesses in Cameroon

Infectious Etiologies of Febrile Illnesses in Cameroon

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Abstract

Fever is a common cause of patients seeking treatment in healthcare facilities in most tropical countries and poses a diagnostic and therapeutic challenge to healthcare workers in limited resource areas. Diagnosis of febrile illnesses in most malaria endemic countries mainly focuses on confirming or ruling out malaria. Thus, healthcare workers are often faced with the challenge of the course of action to take in treating febrile patients negative for malaria. The lack of information on the specific etiologic agents of non-malaria febrile illnesses prevents effective treatment and management of otherwise often treatable diseases. Despite their importance, there is no published data on the epidemiology of non-malaria febrile illnesses in Cameroon, and their true burden remains unknown. In this study, we sought to identify pathogens that cause febrile illnesses in Cameroon. We recruited 551 febrile patients from three different geographical regions of Cameroon. Blood and stool specimens were collected, and rapid diagnostic test, ELISA, microsphere immunoassay, microscopy, culture, and PCR were conducted to identify various etiologic agents of febrile illnesses. Of the 551 participants, 45% had malaria, 41.5% had one or more acute respiratory viral infections, 3% had typhoid fever, 2% had acute toxoplasmosis, 0.9% had probable dengue, 0.7% had evidence of West Nile virus infection, and 1.3% had chikungunya virus infection. Meanwhile, 10.8% and 5% of the stool samples were positive for *Salmonella* and *Shigella,* respectively. We identified 137 patients with febrile illnesses other than malaria. Of these, 44.5% were co-infected with malaria. Furthermore, 91% participants were presumed to have malaria based on fever, of which 41% were negative for malaria by PCR. Our results show evidence of non-malaria febrile illnesses in Cameroon, which should be considered by clinicians in the differential diagnosis of febrile illnesses. However, lack of access to diagnostic tests for febrile illnesses impedes precise diagnosis and clinical management of patients.

Introduction

Fever is a common cause of patients seeking treatment in healthcare establishments in most tropical countries and poses a diagnostic and therapeutic challenge to health care workers (HCW) in limited resource areas (1). Diagnosis of febrile illnesses in malaria-endemic countries mostly focuses on confirming or ruling out malaria (2) as malaria is considered a major health threat. Studies have documented the use of antimalarial drugs to treat patients who are negative for malaria (3-5). Because of the high rate of malaria over-diagnosis in malaria-endemic countries, other febrile illnesses such as respiratory tract infections (RTI), dengue, leptospirosis, typhoid fever, and sepsis are often misdiagnosed as malaria. HCW in most malaria-endemic countries are often faced with a difficult challenge on the course of action to take when a patient is negative for malaria, especially as epidemiologic data on the etiologic agents of febrile illnesses is scanty. The lack of information on the specific etiologic agents of non-malarial febrile illnesses prevents effective treatment and management of otherwise often treatable diseases. Moreover, in regions with high malaria prevalence, the possibility of malaria co-infection with other pathogens that cause fever cannot be ruled out (1). This is particularly important because once malaria is confirmed clinicians do not readily suspect co-infection.

Studies in Laos, Tanzania, and Kenya have identified other causes of fever apart from malaria including acute RTI, and enteric and blood stream infections (6-10). To date, there are no studies from Cameroon investigating if malaria is solely responsible for the periodic outbreak of febrile illnesses. Moreover, there have been reports of periodic outbreaks of febrile illnesses in the Far North region of Cameroon, which have been attributed to malaria (11). The current study sought to identify pathogens that cause febrile illnesses in three regions of Cameroon and the prevalence of co-infection with malaria. An understanding of the prevalence and distribution of fever-causing pathogens is the first step for effective disease management thereby positively impacting morbidity and mortality due to febrile illnesses. This study will thus provide local epidemiologic data that can assist HCW in Cameroon to improve diagnosis, management, and control of febrile illnesses, thereby improving disease outcomes. Furthermore, data from this study can contribute to the identification of research priorities, the development of algorithms in the management of febrile illnesses in Cameroon and in the development of diagnostic tools.

Materials and Methods

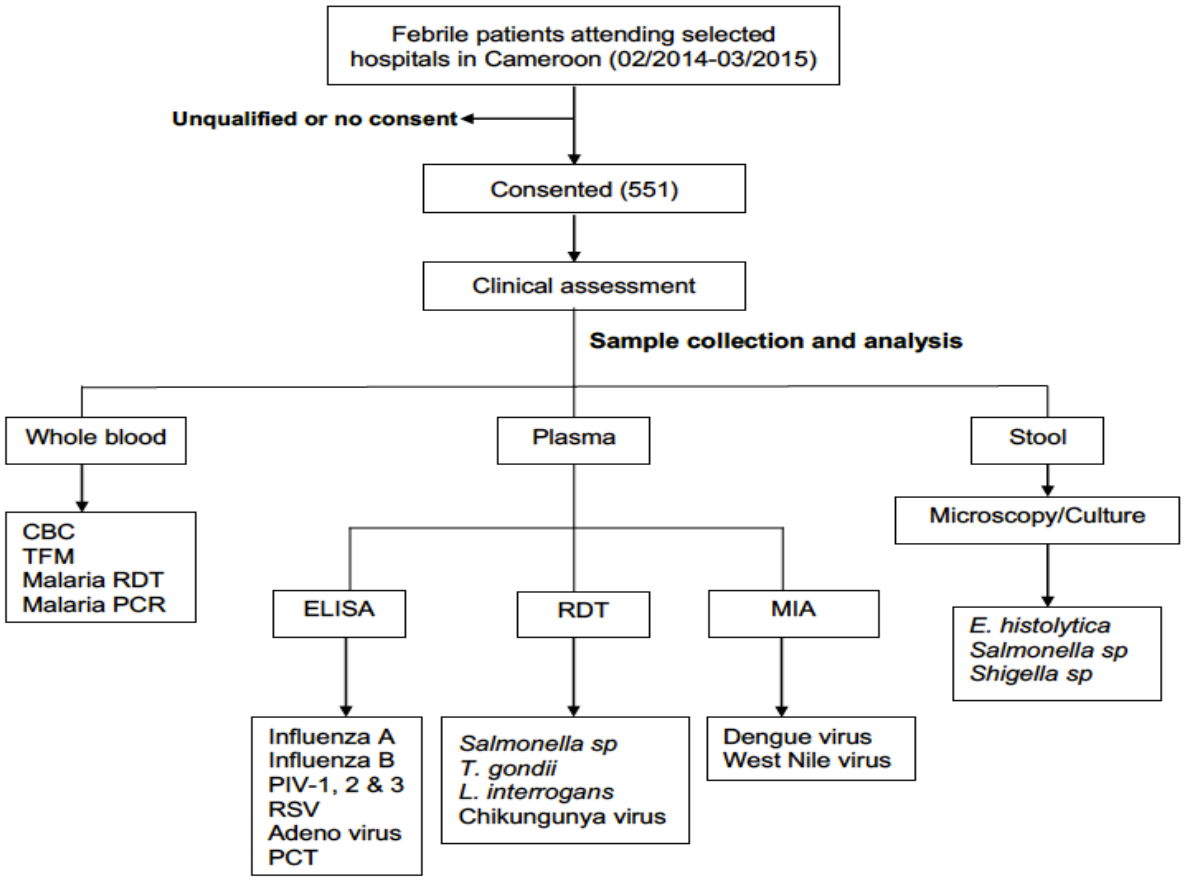

Figure 1: Flow chart depicting enrollment, sample collection and laboratory assays
CBC, complete blood count; TFM, thick film microscopy; RDT, rapid diagnostic test; MIA, microsphere immune assay; PRNT, plaque-reduction neutralization test; PIV, parainfluenza virus; RSV, respiratory syncytia virus; PCT, procalcitonin

Study Setting and Justification

This study was conducted in three regions of Cameroon.

Maroua: In the Far North region, the study was conducted in Maroua in October 2014. The climate in Maroua is hot and semi-arid, with a rainy season from May to September. We included Maroua in this study because of the yearly outbreak of febrile illnesses that occurs between August and October. In October 2013, more than 600 people were reported to have succumbed to malaria within one month in Maroua (12). It was important for our study to confirm if similar febrile illnesses in Maroua are due to malaria or other pathogens.

Nkolbisson: In the Center region, the study was conducted in Nkolbisson from February 2014 to April 2014. We included Nkolbisson because it is holoendemic for malaria and we wanted to investigate if other febrile illnesses co-exist with malaria either as mono or co-infections.

Bamenda: In the North-West region, we conducted the study in Bamenda in February 2015. There are two seasons in Bamenda: A rainy season runs from April to October and a dry season from November to March. We included Bamenda because the prevalence of malaria in Bamenda is generally low and thus it is important to identify the pathogens that cause febrile illnesses in this region.

Recruitment of study participants and sample collection:

We conducted a cross-sectional study in which 551 febrile patients were recruited between February 2014 and March 2015. Inclusion criteria were age >6 months and axillary temperature >37.5^{o}C at the time of recruitment or complaint of fever within 24 hours preceding recruitment. Children <6 months and individuals with readily identifiable causes of fever such as a dental abscess and deep wound were excluded. The research or clinic staff took axillary temperature readings and collected clinical data of the reported signs and symptoms. Venous blood (2–5 mL) was collected into an EDTA tube from each patient and stored in cold boxes until transported to the research laboratory where they were stored at 4–8^{o}C. Stool samples were collected from participants with diarrhea.

Ethical considerations

Signed informed consent was obtained from all study participants. Parents or legal guardians of children <18 years gave a written informed consent on behalf of their children. Ethical approvals were obtained from the Committee on Human Subjects of the University of Hawaii (protocol number CHS 21724) and the National Research Ethics Committee of the Ministry of Public Health Cameroon (protocol number 2014/04/442/CE/CNERSH/SP). Administrative approvals were obtained from the Minister of Public Health Cameroon and the Directors of the various Health Institutions.

Laboratory Procedures

Complete blood count

Complete blood count was conducted using URIT 3200 automated hematology analyzer (URIT Medical, China) following the manufacturer's instructions. The analyzer displayed complete blood count results including white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hgb) and platelets. Anemia was classified as mild (Hgb 10-11.9 g/dL), moderate (Hgb 7-9.9 g/dL), and severe (Hgb <7 g/dL).

Diagnosis of malaria

All participants were screened on-site for malaria parasite by RDT, which detects the presence of *P. falciparum* HRP-II and *Plasmodium* lactate dehydrogenase (pLDH), which is a glycolytic enzyme common to *P. falciparum, P. ovale, P. vivax* and *P. malariae*. Approximately 5 µL of blood was tested using the malaria Ag Pf/Pan (Standard Diagnostic Inc. South Korea) RDT kit following the manufacturer's instructions.

Diagnosis of enteric infections

Stool samples were collected from febrile patients with diarrhea.

Microscopy: A wet mount of stool sample was prepared and analyzed for the presence of *Entamoeba histolytica* cyst.

Isolation of *Salmonella* and *Shigella*: Stool samples for the isolation of *Salmonella*, were enriched in Selenite F broth overnight and a loop-full was streaked the next day on Salmonella-Shigella (SS) agar and incubated aerobically at 37^{o}C overnight. For the isolation of *Shigella*, a loop-full of stool samples was streaked within 1 hour of the collection on SS-agar and incubated aerobically at 37^{o}C overnight. Suspected colonies of *Salmonella* and *Shigella* were streaked on nutrient agar and incubated aerobically at 37^{o}C overnight to obtain pure colonies. Pure colonies were subjected to Gram staining and biochemical tests including triple sugar iron (TSI) agar, motility, citrate utilization, indole and urea hydrolysis. *Salmonella species* were identified as colorless colonies on SS-agar, Gram-negative bacilli, TSI-alkaline slant and acid butt, H_2S positive, indole negative, motile and citrate negative. *Shigella species* were identified as colorless colonies on SS-agar, Gram-negative bacilli, TSI-alkaline slant and acid butt, no H_2S, indole negative, non-motile and citrate negative.

Microsphere Immunoassay (MIA) for dengue virus (DENV) and West Nile virus (WNV) infection

Coupling of microspheres: Magnetic carboxylated microspheres (MagPlexTM-C) and the amine coupling kit were purchased from Luminex Corporation (Austin, TX, USA). Ten μg of each protein (DENV-2, DENV-NS1, and WNV-E protein) was conjugated to the surface of 1.25 X 10^6 beads using a two-step carbodiimide process recommended by Luminex Corporation. The antigen-conjugated microspheres were stored in 250 mL of PBN buffer (Sigma-Aldrich) at 4^{o}C for future use.

MIA test: MIA test was conducted, using the protocol described previously by our group (13). Briefly, plasma samples were diluted 1:100 using PBS-1%BSA. For IgM measurement, GullSORB^{TM} was added to the plasma samples to remove human IgG. Fifty μL of bead dilution (1:200) was added into each 96-well followed by the addition of 50 μL of diluted plasma samples. The plate was incubated at room temperature (RT) for 30 minutes on a plate shaker. The beads were washed twice with PBS-1%BSA, and 50 μL of diluted (1:250) anti-human IgG-phycoerythrin (PE) or anti-human IgM-PE in PBS-1% BSA (Jackson Immunoresearch, West Grove, PA) was added to the corresponding wells and incubated at RT for 45 minutes in the dark. After washing the beads twice with PBS-1%BSA, 100 μL of PBS-1%BSA was added into each well and read using the Luminex 100^{TM} system.

Cut-off determination: We determined the cut-off median florescence intensity (MFI) for each antigen by running 20 negative control samples and calculating the mean +3 standard deviation. Cut off MFI were 185, 318 and 201 for WNV E protein, DENV NS1, and DENV-2 antigens, respectively. Samples with MFI greater than the cut-off were considered positive for the respective antigens.

Diagnosis of acute respiratory viral infections

We did a clinical assessment of our study participants to identify those with signs and symptoms consistent with respiratory tract infections (RTI). Plasma samples from these participants were tested for the presence of IgM antibodies to influenza A virus, influenza B virus, parainfluenza virus types 1, 2 and 3, and adenovirus using commercial ELISA kit (GenWay Biotech, Inc., San Diego USA). Briefly, 100 μL each of the 1:100 diluted samples and the ready-to-use standards and controls were added to microtiter strip wells pre-coated with antigens. After washing the wells, horseradish peroxidase (HRP) labeled anti-human IgM conjugate was added. Tetramethylbenzidine (TMB) substrate was added to visualize the immune complex at 450 nm. Four control sera included in each kit were treated the same way as the test samples to

determine cut-off concentration. The calculated absorption for the patient sera were compared with the absorption value for the cut-off standard for each pathogen. If the value of the sample was higher, it was considered positive as recommended by the manufacturer.

Diagnosis of typhoid/paratyphoid fever, toxoplasmosis, leptospirosis and chikungunya

We used the onsite typhoid/paratyphoid IgG/IgM, *Toxoplasma* IgG/IgM, *Leptospira* IgG/IgM and chikungunya IgM rapid test kits (CTK Biotech, San Diego USA), which is a lateral flow immunoassay that can simultaneously detect and differentiate IgG and IgM antibodies to *Salmonella typhi*/*paratyphi*, *Toxoplasma gondii* and *Leptospira interrogans* and IgM antibodies to chikungunya virus. Briefly, one drop of plasma was added to the sample well, immediately followed by one drop of sample diluent. Results were read within 15 minutes. The appearance of a burgundy colored band in the C (control) line and the M or G lines was considered to be positive for IgM and IgG antibodies respectively. Meanwhile, the absence of a colored band in both the G and M lines but present in the C line was considered negative. Study participants positive for IgM were considered to have a recent infection while those positive for IgG were considered to have a past infection.

Diagnosis of sepsis

We selected plasma samples of participants that were negative for all pathogens tested who met systemic inflammatory response syndrome (SIRS) criteria (temperature >38.5^{o}C or <36^{o}C and WBC >12,000 cells/mm^{3} or <4,000 cells/mm^{3}). We measured the levels of procalcitonin (PCT), which is a protein produced by the body in response to a bacterial infection, using human procalcitonin ELISA kit (Thermo Scientific, Frederick, MD, USA), following the manufacturer's instructions. We used PCT cut-off levels established by Thermo Fisher Scientific for their FDA-cleared B·R·A·H·M·S PCT-assay to classify sepsis as detailed in Table 1 (14).

Table 1: Reference values of PCT

PCT level (μg/L)	**Interpretation**
<0.05	Healthy individuals (sepsis unlikely)
0.05 - <0.5	Systemic infection (sepsis) not likely but local bacterial infection possible
≥0.5 - <2	Systemic infection (sepsis) possible with moderate risk of progression to severe sepsis
≥2 - <10	Systemic infection (sepsis) likely with high risk of progression to severe sepsis
≥10	Severe bacterial sepsis or septic shock

Statistical Analysis

All data are presented as proportions for categorical variables and mean and standard deviation for continuous variables. Demographic and clinical characteristics of patients stratified by location were investigated using chi-square tests or Fisher's exact tests and one-way analysis of variance based on the type of variable.

Multivariable logistic regression models were conducted to predict the association between clinical signs and symptoms, and infectious etiologies, while controlling for age, gender and other clinical characteristics. The results are presented as the odds ratios (ORs) and 95% confidence intervals (CIs). A p-value <0.05 was considered statistically significant. To confirm diagnostic accuracy of predictors (signs and symptoms) for each type of infection, concordance (or c) statistic (equivalence of area under the receiver operating characteristic (ROC) curve) of predicted values was calculated. A c-statistic value of 0.5 indicates the model is no better than random chance, a value higher than 0.7 indicates moderately accurate whereas 0.8 indicates strong accuracy. All analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC).

Results

Demographic and clinical characteristics of study participants

Of the 551 febrile patients recruited for this study, 57.1% were from Nkolbisson, 22.5% from Maroua and 20.3% from Bamenda. Men (48.1%) and women (51.9%) were about equal in proportion, while children less than five years were the majority (53.5%). Headache was the most common symptom reported (36.8%) followed by cough (28.6%), abdominal pain (26%) and vomiting (22.1%). Joint pain, rhinorrhea, and diarrhea, each was often reported (>14%). The mean body temperature, hemoglobin concentration and WBC count were 38.3^{o}C, 11.7 g/dL, and 8,700 cells/mm^{3}, respectively. We found severe and moderate anemia in 34 (10.2%) and 91 (16.5%) study participants, respectively. Of the severe and moderate anemia cases, 65% and 75% were positive for malaria, respectively. The proportion of severe anemia cases was highest in Maroua (13.9%), followed by Nkolbisson (8.8%) and none in Bamenda (Table 2).

Table 2. Demographic and clinical characteristics of study participants

Characteristics	Study sites n (%)			Total (n=551)
	Bamenda (n=112)	Maroua (n=124)	Nkolbisson (n=315)	
Gender				
Male	33 (29.5)	64 (51.6)	168 (53.3)	265 (48.1)
Female	79 (70.5)	60 (48.4)	147 (46.7)	286 (51.9)
Age Group (years)				
0-5	34 (30.4)	81 (65.3)	180 (57.1)	295 (53.5)
6-10	5 (4.5)	15 (12.1)	86 (27.3)	106 (19.2)
11-16	4 (3.6)	9 (7.3)	49 (15.6)	62 (11.3)
17 and above	69 (61.6)	19 (15.3)	0 (0)	88 (16.0)
Major Clinical Symptoms				
Joint pain	56 (51.9)	10 (10.5)	6 (1.9)	72 (13.9)
Headache	62 (57.4)	50 (52.1)	78 (25.0)	190 (36.8)
Dyspnea	24 (22.2)	20 (21.1)	2 (0.6)	46 (8.9)
Rhinorrhea	41 (37.9)	33 (34.4)	27 (8.6)	101 (19.5)
Cough	39 (36.1)	45 (46.9)	64 (20.5)	148 (28.6)
Abdominal pain	7 (6.5)	10 (8.1)	125 (39.8)	142 (26.0)
Diarrhea	21 (19.4)	19 (20.0)	33 (10.5)	73 (14.1)
Vomiting	17 (15.7)	32 (33.7)	65 (20.7)	114 (22.1)
Convulsion	1 (0.9)	4 (3.2)	2 (0.6)	7 (1.3)
Other parameters				
Mean body temperature (^{o}C) (Mean ± SD)	37.5 ± 1.0	38.6 ± 1.0	38.4 ± 1.3	38.3 ± 1.2
Mean Hgb conc. (g/dl) (Mean ± SD)	15.3 ± 3.2	9.4 ± 2.3	11.0 ± 2.9	11.7 ± 3.5
Severe anemia	0 (0)	12 (13.9)	22 (8.8)	34 (10.2)
Mean WBC count (cells/mm^{3}) (Mean ± SD)	8.9 ± 5.2	10.2 ± 7.6	8.2 ± 4.	8.7 ± 5.3
Clinical diagnosis of malaria	86 (76.8)	120 (96.8)	301 (95.6)	507 (92.0)

Causes of fever

Malaria was diagnosed in 45% of the study participants. Malaria prevalence was high in Nkolbisson (55%) and Maroua (54%) but low in Bamenda (5%) (Table 3). The prevalence of malaria was highest in children <5 years old (49%) and decreased with increasing age (Figure 2). We found evidence of recent toxoplasmosis in 2% of the participants and evidence of past infection in 23% of study participants. Overall 3% of the participants had recent typhoid or paratyphoid fever while 3.3% had a past infection. *Entamoeba histolytica* was detected in 27% of the stool samples collected from participants who had diarrhea while *Salmonella* and *Shigella sp* were grown in culture from 10.8% and 5% of the stool samples, respectively. *Entamoeba histolytica* was identified in participants from both Maroua and Bamenda. Positive culture for *Salmonella* and *Shigella species* was limited to participants from Bamenda.

Table 3. Infectious causes of febrile illnesses

Pathogens	**All**	**Bamenda**	**Maroua**	**Nkolbisson**
Malaria parasite	551 (45)	112 (6)	124 (54)	315 (55)
Toxoplasma gondii-IgM	551 (2)	112 (1.8)	124 (0)	315 (3.2)
Toxoplasma gondii-IgG	551 (23)	112 (5.3)	124 (5.6)	315 (37)
*Entamoeba histolytica**	37 (27)	20 (10)	17 (47)	NT
Salmonella sp-IgM	551 (3)	112 (0.9)	124 (1.6)	315 (4)
Salmonella sp-IgG	551 (3.3)	112 (0.9)	124 (3.2)	315 (4)
*Shigella sp** (culture)	37 (5)	20 (10)	17 (0)	NT
*Salmonella sp** (culture)	37 (10.8)	20 (20)	17 (0)	NT
Influenza A virus-IgM**	162 (8)	44 (16)	43 (7)	75 (4)
Influenza B virus-IgM**	162 (8)	44 (16)	43 (9)	75 (2.5)
PIV 1, 2 and 3-IgM**	162 (7)	44 (9)	43 (14)	75 (1.3)
RSV-IgM**	162 (18)	44 (18)	43 (18)	75 (16.5)
Adenovirus-IgM**	162 (8.6)	44 (11)	43 (14)	75 (4)
Chikungunya virus-IgM	236 (1.3)	112 (1.9)	124 (0.8)	NT
Dengue virus-IgG	551 (3.4)	112 (7)	124 (3.2)	315 (1.6)
Dengue virus-IgM	551 (0.9)	112 (0)	124 (0)	315 (1.5)
West Nile virus-IgG	551 (12)	112 (4.5)	124 (17)	315 (12)
West Nile virus-IgM	551 (0.7)	112 (1.8)	124 (0)	315 (0.6)

Values represent total number of samples tested (% positive)
*Only samples from febrile patients with diarrhea were tested.
**Only samples from febrile patients with signs and symptoms consistent with respiratory infection were tested
NT= not tested

RTIs were more common in Bamenda and Maroua, as compared to Nkolbisson. RSV was the most common respiratory virus in all study sites (Table 3). The prevalence of respiratory viral infections was highest in children less than five years (57%) of age (Figure 2A). RSV was the

most common respiratory pathogen in children less than five years of age, while in adults 7influenza A virus was the most common pathogen (Figure 2B). We found 0.9% probable DENV, 1.3% acute chikungunya virus and 0.7% recent WNV infections, and evidence of prior WNV infection in 12% of the population. Prior WNV infection was most common in Maroua.

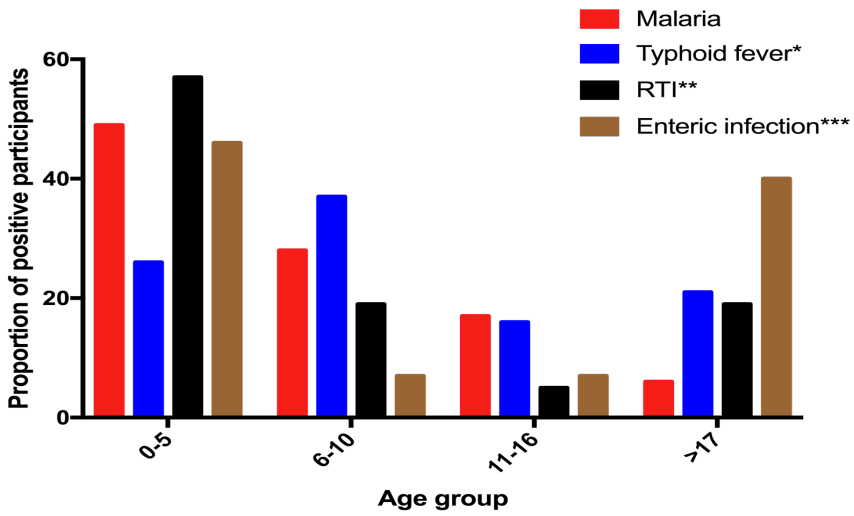

Figure 2A. Distribution of common causes of fever according to age group
*IgM positive, ** Influenza A and B, parainfluenza, adeno and RSV
*** *E. histolytica*, *Salmonella* and *Shigella species*

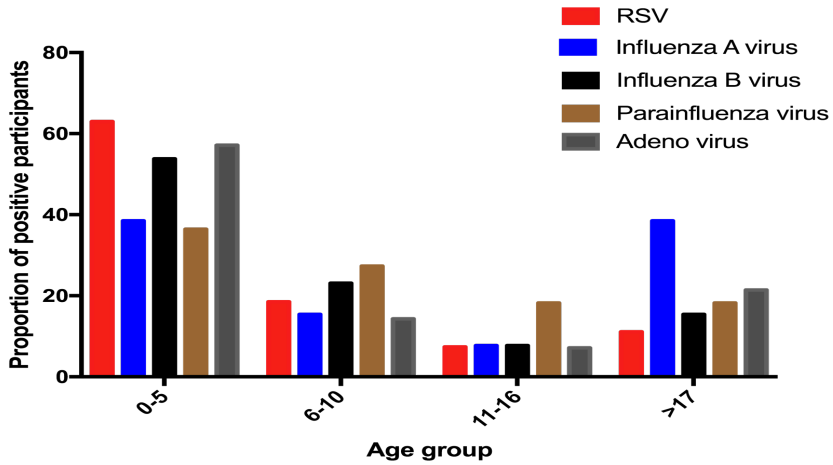

Figure 2B. Distribution of acute respiratory tract infections according to age group

We measured PCT levels in plasma samples from 42 study participants who were negative for all pathogens tested in this study and met SIRS criteria. We were able to identify eight individuals with suspected local bacterial infection, eight with suspected systemic infection (sepsis) and one with severe bacterial sepsis (septic shock). The remaining 25 individuals had PCT levels that were unlikely to be associated with systemic infection (sepsis). PCT levels were undetectable in all healthy control samples (Table 4).

Table 4. Assessment of sepsis risk in individuals without a diagnosis meeting SIRS criteria

Category	n	PCT levels (ng/mL) (Mean ± SD)	Body temp. (^{o}C) (Mean ± SD)	WBC count (mm^{3}) (Mean ± SD)
Suspected local bacterial infection	8	0.23 ± 0.09	39 ± 0.99	12.3 ± 6.59
Systemic infection (sepsis) possible	4	0.95 ± 0.23	37.46 ± 1.7	27.3 ± 3.25
Systemic infection (sepsis) likely	4	2.7 ± 0.46	38.7 ± 1.21	15.26 ± 2.03
Severe bacterial sepsis	1	16.09	40.5	22.6
Sepsis unlikely	25	0	39.2 ± 0.7	16.1 ± 4.01
Healthy controls	10	0	37 ± 0.4	10 ± 1.2

Malaria co-infections

There were 137 patients with febrile illnesses other than malaria. Of these, 44.5% were co-infected with malaria. Apart from *Shigella sp*., we observed malaria parasite co-infections with all the other pathogens. Of those that were positive for typhoid, 79% (15/19) also had malaria. Meanwhile, 83% (10/12) of those positive for toxoplasmosis had malaria (Table 5).

Table 5. Co-infections in malaria-positive individuals

Pathogen	n	Malaria, n (%)	
		Positive	Negative
Toxoplasma gondii-IgM	12	10 (83)	2 (17)
Salmonella sp-IgM	19	15 (79)	4 (21)
*Entamoeba histolytica**	10	3 (30)	7 (70)
Salmonella sp (culture)	4	0 (0)	4 (100)
Shigella sp (culture)	2	0 (0)	2 (100)
Influenza A virus-IgM	13	3 (23)	10 (87)
Influenza B virus-IgM	13	3 (23)	10 (87)
PIV 1, 2 & 3-IgM	11	6 (54)	5 (46)
RSV-IgM	27	10 (37)	17 (63)
Adenovirus-IgM	14	6 (43)	8 (57)
Chikungunya virus-IgM	3	1 (33)	2 (67)
Dengue virus-IgM	5	2 (40)	3 (60)
West Nile virus-IgM	4	2 (50)	2 (50)

* Microscopic examination for cysts in stool samples

Predictors associated with detection of infectious etiologies

We used a prediction model to identify signs and symptoms that were significantly associated with the detection of infectious etiologies of febrile illnesses in patients with evidence of one pathogen only. Patients with asthenia, headache and vomiting were 4.0, 2.5 and 1.8 times respectively, at higher odds to be malaria positive compared to those without these symptoms. Meanwhile, those with vomiting and abdominal pain were 3.5 and 4.5 times respectively, at

higher odds to have typhoid fever compared to those with no vomiting or abdominal pain. Our model showed a high prediction power (Table 6).

Table 6. Multivariate logistic regression analysis of predictors significantly associated with detection of infectious etiologies in febrile patients with evidence for one pathogen

Category	Subcategory	OR (95% CI)	p-value
Malaria*			
Asthenia	Yes	4.01 (1.57-10.2)	0.004
Headache	Yes	2.48 (1.52-4.03)	0.000
Vomiting	Yes	1.76 (1.06-2.94)	0.030
Typhoid**			
Vomiting	Yes	3.45 (1.20-9.89)	0.021
Abdominal pain	Yes	4.52 (1.53-13.3)	0.006

*C-statistic for malaria = 0.793, 95% CI=0.755 – 0.831
**C-statistic for typhoid = 0.832, 95% CI=0.741 - 0.923

Discussion

Cameroon is known to be one of the malaria-endemic countries in sub-Saharan Africa (15). However, there is a lack of information on the prevalence of non-malarial febrile illnesses with similar clinical presentation. The purpose of this study was to identify infectious causes of febrile illnesses in Cameroon to improve awareness amongst HCW of the diseases in the country. This study revealed that different etiologic agents of febrile illness, including malaria parasite, *Salmonella sp*, *Shigella sp*, *Entamoeba histolytica*, respiratory viruses, DENV, chikungunya virus and WNV, are present in Cameroon.

Malaria is the main cause of febrile illnesses in Cameroon

We identified pathogens that cause febrile illnesses in 67% of the study participants. Our findings convincingly demonstrate that malaria is a significant cause of febrile illnesses in Cameroon with a heterogeneous distribution. This is in sharp contrast with recent studies from Tanzania and Kenya in which, up to 65% of febrile illnesses were due to acute RTI with few malaria cases (7, 16, 17). Effective malaria control measures in these countries may have been responsible for the low malaria prevalence. Our data also suggest that periodic outbreaks of febrile illnesses in Maroua, the Far North region of Cameroon, are due to seasonal malaria transmission. Further, this study corroborates with reported outbreaks of febrile illnesses in Maroua (11, 18).

Seasonal factors determine the heterogeneity in the etiologies of febrile illnesses

More than half of the study participants in Maroua and Nkolbisson had malaria. However, in Bamenda malaria prevalence was low. Instead, RTI were more common in Bamenda. In general, respiratory viral infections were more common in Bamenda and Maroua as compared to Nkolbisson. These variations in the distribution of infectious etiologies could be due to the difference in climatic conditions at the various study regions. In Nkolbisson, we carried out the study during the rainy season, while in Maroua samples were collected during the transition period between the rainy and the dry season, and in Bamenda, it was during the dry season. The seasonality, distribution, and prevalence of malaria and other vector-borne diseases are known to be influenced by climatic factors including temperature, humidity and rainfall (19). RTI have also been shown to be associated with climatic conditions including temperature and vary by season, with increasing incidence during the cold season (20).

Unwarranted use of antimalarial drugs to treat febrile illnesses

Most of the study participants in Bamenda who were negative for malaria had evidence of acute RTI and therefore did not require antimalarial treatment but rather an antibiotic treatment for those with bacterial complications. Unfortunately, 92% of the study participants in the present study were clinically diagnosed with malaria and were prescribed antimalarial drugs. Thus, the systematic provision of antimalarial treatment in patients without malaria as observed in the present study is clinically detrimental for the patient. Such unwarranted treatment leads to the development of drug resistance and exposes the patient to drug-associated adverse events. Furthermore, when we conducted multivariable logistic regression models to predict the association between clinical signs and symptoms, and infectious etiologies, we found that very few signs and symptoms could predict the cause of fever in our study population. Therefore, laboratory diagnosis remains the best tool to rule out or confirm a diagnosis for febrile illnesses.

Few patients were found to be acutely infected with *Toxoplasma gondii* (2%), and viruses such as dengue (0.9%), West Nile (0.7%) and chikungunya (1.3%). Most patients infected with these pathogens are asymptomatic or suffer a mild febrile illness, which may not warrant seeking medical care. Because this was a hospital-based study, it is possible that we missed cases of dengue, West Nile and chikungunya viral infections in the community. Previous studies in similar settings also recorded few or no cases of acute DENV, WNV and CHIKV infections (6, 7, 21, 22). Meanwhile, dengue and leptospirosis were common in most studies conducted in South East Asia (9, 10, 23, 24). However, on the basis of serodiagnostic tests for *Toxoplasma gondii*, DENV, and WNV, our results show that transmission of these pathogens occurred in all three regions of Cameroon.

Malaria and co-infection with other pathogens causing febrile illnesses

As evident from this study and other studies conducted in similar settings (7, 9), HCW should be aware of malaria co-infections when managing febrile illnesses, in particular for patients with persistent symptoms who are administered antimalarial treatment. It is a common practice that once malaria is confirmed, co-infection with other febrile illnesses is overlooked by HCW, which may cause unrecognized deaths. The most common malaria co-infections in this study were typhoid, RTI, and toxoplasmosis. A previous study in a different region of Cameroon reported malaria and typhoid co-infection prevalence of 6.7% (25). Co-infection of malaria and typhoid fever can occur due to overlapping factors in the same area including poor water quality, hygiene, ecology, an abundance of vector and high malaria transmission. The presence of co-

infection makes optimal clinical management of a patient very challenging. Hence, if co-infection is misdiagnosed, it can lead to a complicated patient outcome.

Predominance of febrile illnesses in children under five years of age

The preponderance of febrile illnesses among children 0-5 years' old observed in this study, can be attributed to the general susceptibility of this age group to infectious diseases. Children under five years of age are particularly vulnerable to malaria due to their poorly developed immune system to fight the infection (26). In 2015, children under five years of age accounted for approximately 70% of malaria deaths globally (15). Moreover, in our study seven children with malaria had convulsions, while 43 had severe anemia due to malaria. Also, the incidence of respiratory viral diseases is highest among infants and young children who suffer an average of 6-8 infections per year, with some developing otitis media or pneumonia, caused by the viral or bacterial infections leading to inflammation of the ear drum, which may require antibiotic treatment (27-29).

PCT as a surrogate for blood culture in diagnosing sepsis in febrile patients

Thirty-three (33%) of patients with febrile illness were not diagnosed for any pathogen. This could be due to other important causes of fever not covered in this study including pneumonia, bacterial and localized infections, and sepsis. In order to determine if some of the participants negative for all pathogens tested in this study had a localized infection or sepsis, we measured PCT levels in blood samples for those who met SIRS criteria. Sepsis is a potentially fatal febrile illness, and early diagnosis and treatment are critical to improving outcome. Blood culture, which is considered to be the gold standard for the diagnosis of sepsis is too slow and limited by false negative results because microbial organisms are found late during the disease (30). PCT, which is currently an FDA-approved test to aid in sepsis diagnosis, has been shown to have a unique kinetics that allows it to be used for early detection of sepsis. Further, PCT can distinguish between sepsis and host response to inflammation and guide choice of antibiotic therapy (31-33). By measuring PCT levels, we were able to identify patients with suspected localized bacterial infection, sepsis, and septic shock. Our data clearly show that apart from malaria, some febrile patients had sepsis. Therefore, if PCT is made available as a POCT in Cameroon and other tropical countries, it will support informed decision on the initiation and duration of antibiotic therapy for patients with sepsis. This will improve patient care while decreasing antibiotic and antimalarial misuse and resistance. Moreover, the use of PCT levels to treat patients with antibiotics was demonstrated to decrease the length of hospital stay (34).

Limitations

Our pilot study has important limitations. Several potential pathogens that can cause febrile illnesses were not assessed due to logistical and funding constraints. Also, the study design did not permit the collection of convalescent-phase samples. However, IgM seropositivity can provide an indication of recent infection. The presence of co-infection is more certain if the pathogens are grown in culture or shown by a specific antigen test. Hence, assays based on detection of IgM antibodies are at risk of being interpreted as a mixed infection when they represent the previous infection. Procalcitonin is not fully specific for sepsis with elevated levels being found after trauma, surgery, in those with autoimmune disease, or in severe cases of malaria. However, patients who have undergone recent surgery or trauma were excluded from this study.

Conclusions

This study for the first time reports an extensive list of medically important pathogens that cause febrile illnesses in Cameroon, which should be considered by HCW in the differential diagnosis of patients presenting with fever**.** Taken together, our results demonstrate that, the distribution of febrile illnesses in Cameroon varies by location and age with the possibility of co-infections. These data warrant formulation of algorithms to manage febrile illnesses. Moreover, heterogeneous distribution of pathogens, age, and the possibility of co-infection should be taken into consideration for developing effective algorithms. Also, monitoring of febrile illnesses is warranted as disease pattern may change over time. However, lack of easy access to diagnostic tests for febrile illnesses impedes precise diagnosis and clinical management of patients.

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Chapter 5

Development of a Microsphere Immunoassay Incoporating Recombinant Protein Antigens for Detection of Viral Hemorrhagic Fevers in Cameroon

Development of a Microsphere Immunoassay Incoporating Recombinant Protein Antigens for Detection of Viral Hemorrhagic Fevers in Cameroon

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Abstract

Viral hemorrhagic fevers (VHF) are caused by several viruses, some of which are highly contagious and can lead to potentially lethal diseases. Early onset of VHF is characterized by non-specific signs and symptoms and can be confused with other tropical febrile illnesses. Cameroon shares borders with countries that have reported sporadic cases or outbreaks of VHF. However, data on the presence of VHF in Cameroon is scanty. It is plausible that VHF reported in neighboring countries also occur in Cameroon. The scarcity of reliable data on the epidemiology of VHF in most African countries is in part due to the lack of diagnostic tools and active surveillance. Therefore, the development of rapid, specific and sensitive diagnostic assays for use in surveillance and outbreak investigations of VHF is important even for countries without reported cases. In this study, we expressed recombinant proteins of Ebola (EBOV), Sudan (SUDV), Marburg (MARV) and Lassa (LASV) viruses and used them to develop a multiplex microsphere immunoassay (MIA) for the simultaneous identification of hemorrhagic fever virus (HFV) IgG-specific antibodies in plasma samples collected from febrile patients, greater than 6 months old in Cameroon between February 2014 and March 2015. Using human or humanized monoclonal antibodies for LASV, MARV, SUDV and EBOV, our pre-validation experiments showed that the assay was highly sensitive, specific and reproducible. Of the 408 Cameroonian plasma samples tested, 20 were suspected to be positive for one or more VHF. This study reports for the first time, the development of a high-throughput multiplex laboratory assay for the serological detection of IgG antibodies against LASV, MARV, SUDV and EBOV. Our results suggest that VHF may be occurring unrecognized in Cameroon.

Introduction

Viral hemorrhagic fevers (VHF) are a group of acute viral diseases associated with fever, vascular leakage, bleeding, organ failure and shock. Clinically, VHF are not easily distinguished from other tropical febrile illnesses, such as malaria, and therefore are often misdiagnosed (1). In Africa, there are many species of animals, mosquitoes and ticks that serve as natural reservoirs or vectors for viruses that cause haemorrhagic fevers with reported outbreaks in several African countries (2).

Currently, confirmation of infection in the acute phase of VHF is achieved by detection of viral nucleic acid, viral antigen, and infectious virus, using reverse transcriptase-PCR (RT-PCR), antigen-capture ELISA, or virus culture, respectively. (3). RT-PCR and antigen-capture assay takes 4–6 hours, whereas virus culture takes 2–5 days or longer, and all three assays require relatively large volumes of blood (0.2 to 1.0 mL) and high biocontainment infrastructure. The highly infectious and diverse etiologies of VHF underscore the need to develop diagnostic tools that are rapid, sensitive, specific, require small sample volume, and can be multiplexed to concurrently diagnose multiple VHF. Multiplex assays have the advantage of being able to detect different hemorrhagic fever viruses (HFV) simultaneously within a few hours. This could allow early triaging, quarantine and discharge of patients with other common infectious diseases, such as malaria held in “transit” center, thereby reducing the potential for nosocomial transmission. The development and use of a multiplex immunoassay will therefore be ideal for diagnosis and surveillance of VHF. Moreover, since the genetic material from HFV does not remain detectable in the bloodstream for very long, antibody detection methods are indispensable, especially for non-viremic patients and in retrospective epidemiological studies and surveillance.

The requirement of a BSL-4 laboratory to work with most HFV and the associated safety concerns makes recombinant antigens a valuable alternative to the use of inactivated virus antigens. The use of recombinant DNA technology will enable the production of antigens that are: of consistent quality, highly purified (well defined composition), safe for the manipulator and environment, and less costly than products from natural sources (4). Because recombinant viral antigens contain the exact viral amino acid sequence, it means that they contain a region that can be recognized by antibodies produced by different individuals, reducing the number of false-negative results.

Several VHF outbreaks, such as Ebola virus disease (EVD), Crimean Congo hemorrhagic fever (CCHF), Rift Valley fever (RVF), Marburg hemorrhagic fever (MHF), Lassa hemorrhagic fever (LHF) and yellow fever (YF), have been reported in Africa (2, 5-8). Cameroon shares its border with countries that have reported sporadic cases or outbreaks of VHF, including Nigeria (LHF, EVD and CCHF), Gabon (EVD), Central Africa Republic (LHF) and Republic of Congo (EVD) (2, 8). Even though YF is endemic in Cameroon (6), whether other VHF are also present is not known. Moreover, it is plausible that HFV reported in neighboring countries also cause infection and disease in Cameroon. The scarcity of reliable data on the epidemiology of VHF in Cameroon and most African countries is in part due to the lack of diagnostic tools and active surveillance. As international trade and travel increases, there is the potential that VHF could be introduced from endemic countries into regions that are virus free as was seen with the recent outbreak of EVD in several West African countries. Therefore, the development of diagnostic assays for use in surveillance and outbreak investigations of VHF is important even for countries without reported cases.

In this study, we used LUMINEX® technology to develop a multiplex microsphere immunoassay (MIA) for the simultaneous detection of specific antibodies against Lassa (LASV), Ebola (EBOV), Sudan (SUDV) and Marburg viruses (MARV) in serum samples collected from febrile patients in Cameroon. This is the first, high-throughput multiplex laboratory serodiagnostic assay for these viruses.

Methodology

Clinical samples: Archival plasma samples collected from febrile patients in Cameroon between February 2014 and March 2015 were tested. Inclusion criteria were age >6 months and axillary temperature >37.5^{o}C at time of recruitment or report of fever within 24 hours preceding recruitment.

Ethical considerations: Ethical approvals for the use of archival samples were obtained from the Committee on Human Studies of the University of Hawaii (protocol number 2016-30564) and from the National Research Ethics Committee of the Ministry of Public Health Cameroon (protocol number 2016/06/783/CE/CNERSH/SP).

Expression and purification of EBOV, SUDV, MARV and LASV recombinant proteins

Gene and primer design

The gene sequence of EBOV-GP, EBOV-VP40 and EBOV-NP (Mayinga strain), SUDV-GP (Boniface strain), MARV-GP (Angola strain) and LASV-GPC (Josiah strain) were obtained from NCBI GenBank. C-terminally truncated versions of the various glycoproteins (devoid of membrane anchor regions) were designed with the help of Clone Manager Professional 9 (Scientific & Educational Software, Denver, CO). PCR primers for molecular cloning were designed to consist of a hybridization sequence of about 18 to 21 base pairs. Appropriate restriction sites were added to the 5'- and 3'-ends of the PCR product using forward and reverse primers, respectively, with about 4–6 extra base pairs upstream of each restriction site to improve cutting efficiency.

Cloning

PCR cloning was performed using the previously designed primers to make copies of the gene of interest. PCR products were isolated using a PCR clean up kit (Macherey-Nagel, Bethlehem, PA). Restriction digests with appropriate enzymes were conducted for PCR products (insert) and expression plasmid (pMT/Bip; Invitrogen, Carlsbad, CA). The backbone of the expression plasmid and gene of interest were then isolated by gel purification. The insert (gene of interest) was ligated into 50 ng of the expression plasmid using quick ligation kit (NEB, Ipswich, MA) with a plasmid to insert ratio of 3:1. Chemically competent DH-5α E. coli cells were transformed using 1-5 μL of the ligation product and incubated overnight at 37^{o}C. Colony PCR was performed to screen E. coli cells for successful ligation/transformation. An individual bacterial colony containing the desirable expression plasmid was then scaled-up using a 50 mL shaker

flask. The expression plasmid was purified using a commercial plasmid DNA purification kit (Macherey-Nagel, Bethlehem, PA) and verified by restriction digest and sequencing prior to further use.

Expression and purification

$1x10^6$ cells/mL of *Drosophila* S2 cells adapted to ExCell 420 (Sigma, St. Louis, MO) were seeded in a 6-well plate. The S2 cells were co-transfected the next day with 2 μg of expression plasmids for EBOV-GP, EBOV-VP40 and EBOV-NP, SUDV-GP, MARV-GP or LASV-GPC and selectable marker plasmid pCoHygro (Invitrogen, Carlsbad, CA) using lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) or the calcium phosphate precipitation method (using 20 μg plasmid + 1 μg pCoHygro). Hygromycin B was added to the media the next day for lipofectamine and 3 days after transfection for calcium phosphate to start selection of stable transformants. After establishing stably transformed S2 cell lines, cultures were scaled up to 1–5 L scale using a Wave bioreactor (GE Healthcare, Aurora, OH) and protein expression was induced by addition of 200 μM $CuSO_4$ to the culture medium. Protein expression was verified by SDS-PAGE and Western Blot (probed with EBOV, SUDV, MARV and LASV GP-reactive monoclonal antibodies). S2 cells were harvested from the Wave bioreactor and proteins were purified using immunoaffinity chromatography (IAC). For this, monoclonal antibodies specific for the individual proteins were coupled onto HiTrap columns prepacked with NHS-activated Sepharose (GE Healthcare, Aurora, OH). These antigen-specific IAC columns were then used for protein purification.

Microsphere Immunoassay(MIA) for VHF

Assay Design: We adapted xMAP Technology to design an indirect MIA requiring both a target protein and an anti-human IgG antibody.

Coupling of microspheres: Ten μg of each protein (EBOV-GP, EBOV-NP, MARV-GP, LASV-GPC, SUDV-GP, MSP-1, HSV-1) were conjugated to the surface of 1.25 X 10^6 beads using a two-step carbodiimide process recommended by Luminex Corporation. Magnetic carboxylated microspheres (MagPlexTM-C) and the amine coupling kit were obtained from Luminex Corporation (Austin, TX, USA). The antigen-conjugated microspheres were stored in 250 μL of PBN buffer (Sigma Aldrich) at 4°C until use.

MIA test: The MIA testing was conducted using the protocol previously described by our group (9). Briefly, plasma samples were diluted 1:100 using PBS with 1% BSA. Fifty μL of bead dilution (1:200) was added into each well of a 96-well plate followed by the addition of 50 μL of

diluted plasma samples. The plate was incubated at room temperature (RT) for 30 minutes on a plate shaker. The beads were washed twice with PBS-1%BSA using a magnetic plate holder. Finally, 50 μL of 1:250 dilution of goat anti-human IgG phycoerythrin (Jackson Immunoresearch, West Grove, PA) was added into the wells and incubated at RT for 45 minutes in dark. After washing the beads twice with PBS-1%BSA, supernatants were removed and 100 μL of sheath fluid was added into each well and read using the Luminex 200™ system.

Controls: Ten controls were included on each plate and treated the same way as the test samples. Positive controls consisted of EBOV, MARV and SUDV humanized monoclonal antibodies (HMB) (MAPP Biopharmaceutical, San Diego, CA), and LASV human monoclonal antibody (HumAb) (Zalgen Labs, German Town, MD) and an irradiated serum sample from a rhesus macaque experimentally infected with EBOV.

As internal controls, we used a subunit of *Plasmodium falciparum* merozoite surface protein (MSP-1) containing the 19K C-terminal sequence of MSP-1, kindly provided by Dr. George Hui of the University of Hawaii and human herpes simplex virus (HSV-1) inactivated antigens (Meridian Life Science Inc, Memphis, TN). MSP-1 was used as internal control because blood samples for this study were collected from Cameroon, which is endemic for malaria. Thus, most of the participants are expected to have preexisting antibodies to MSP-1 due to malaria parasite infection (10). HSV-1 is a highly contagious virus that commonly causes infections throughout the world with prevalence of about 87% in Africa (11). Therefore, most of our study participants are expected to have antibodies to HSV-1. For negative controls, we determined buffer background and samples collected from individuals living in the United States with no travel history to regions endemic for any of the HFV covered in this study (US controls).

Cut-off determination: We determined the cut-off median florescence intensity (MFI) for each antigen by conducting MIA on 32 US-control serum samples and calculating the mean +3 standard deviations. Samples with MFI greater than the cut-off were considered positive for the respective antigens.

Pre-validation studies

Limit of detection: To determine the limit of detection we tested 10-fold serial dilutions of EBOV, MARV, LASV and SUDV human or humanized monoclonal antibodies from 1 to 10,000 ng/mL. The MIA was conducted using 50 μL of each mAb dilution and 50 μL of diluted antigen-conjugated microspheres as described previously. We included PBS-1%BSA as blank.

Linearity: The linearity for each analyte (EBOV-GP, MARV-GP, LASV-GPC, SUDV-GP) was assessed by testing several different dilutions of EBOV, MARV, LASV and SUDV mAbs.

Standard curves were generated for each analyte by plotting the various antibody dilutions against the MFI.

Specificity: To evaluate the specificity or cross reactivity of the assay, we used 1,000 ng/mL of LASV mAb, 10,000 ng/mL of each EBOV, MARV, LASV and SUDV mAbs, and a dengue virus (DENV)-IgG positive serum sample. Each monoclonal antibody and DENV-IgG positive control were tested against a panel of 8 different antigen-conjugated microspheres including: EBOV-GP, EBOV-NP, MARV-GP, LASV-GPC, SUDV-GP, MSP-1, HSV-1, PBS and BSA.

Repeatability: To evaluate the repeatability of the assay, 1,500 ng/mL of LASV mAb, 2,000 ng/mL each of MARV and EBOV mAb, and cocktail (1500 ng/mL of LASV+ 2000 ng/mL each of MARV and EBOV) mAbs were tested 13 times by the same technician on the same plate and on the same day.

Reproducibility (intra-assay variability): To evaluate the reproducibility of the assay 1,000 ng/mL of LASV mAbs, 10,000 ng/mL each of MARV and EBOV mAbs, and cocktail (300 ng/mL of LASV+ 3,000 ng/mL each of MARV and EBOV) mAbs were prepared and stored at -80^{0}C in aliquots. Each antibody was tested 2–3 times daily on different plates by two different technicians for a period of six days. Each test was performed in duplicate.

Statistical analysis

Data analysis was conducted using Graph Pad Prism version 7 and Microsoft Excel 2016. Coefficient of variation (CV) was calculated for repeatability and reproducibility. The correlation between antibody concentration and MFI was determined by calculating the correlation coefficient (R^2).

Results

Recombinant proteins are immunoreactive

Recombinant proteins were expressed using S2 cells expression system and purified using immunoaffinity chromatography. Figure 1 shows purified recombinant glycoproteins of EBOV, MARV, SUDV and LASV. Western blots were probed with different mAbs. The recombinant proteins are immunoreactive with little or no cross reactivity.

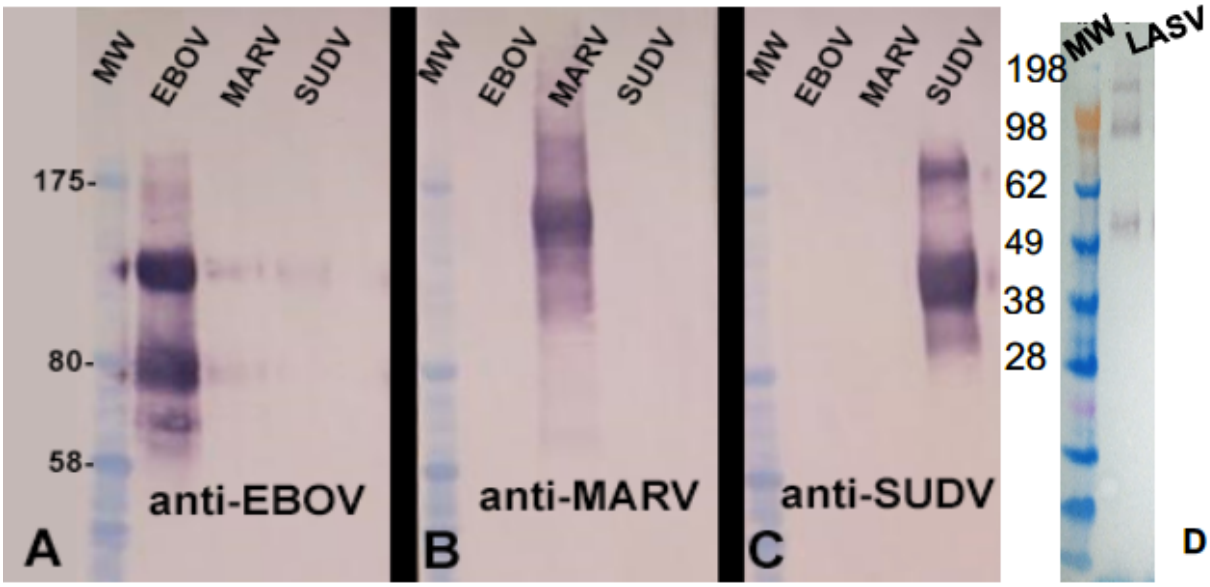

Figure 1: SDS-PAGE analysis of purified (A) EBOV, (B) MARV, (C) SUDV and (D) LASV recombinant glycoproteins.

MIA for VHF is highly sensitive

The limit of detection of the MIA was determined by testing serial dilutions of EBOV, MARV, LASV and SUDV mAb with microspheres conjugated with EBOV-GP, LASV-GPC, MARV-GP and SUDV-GP. The MFI of LASV-GPC conjugated microsphere was 315 at 10 ng/mL of LASV-GP1 mAB and increased to 13,082 at 10,000 ng/mL of LASV-GP1 mAb (Fig. 2A). Only background MFI was observed for EBOV-GP conjugated microsphere at 10 ng/mL of EBOV mAb concentration and increased to 7,294 at 10,000 ng/mL concentration of EBOV mAb (Fig. 2A). Meanwhile, lower MFI were observed for MARV-GP conjugated beads, when 100 ng/mL of MARV mAb was used (Fig. 2C), and for SUDV-GP conjugated beads, when 1,000 ng/mL of SUDV mAb was used (Fig. 2D), but increased at 10,000 ng/mL of mAb concentration.

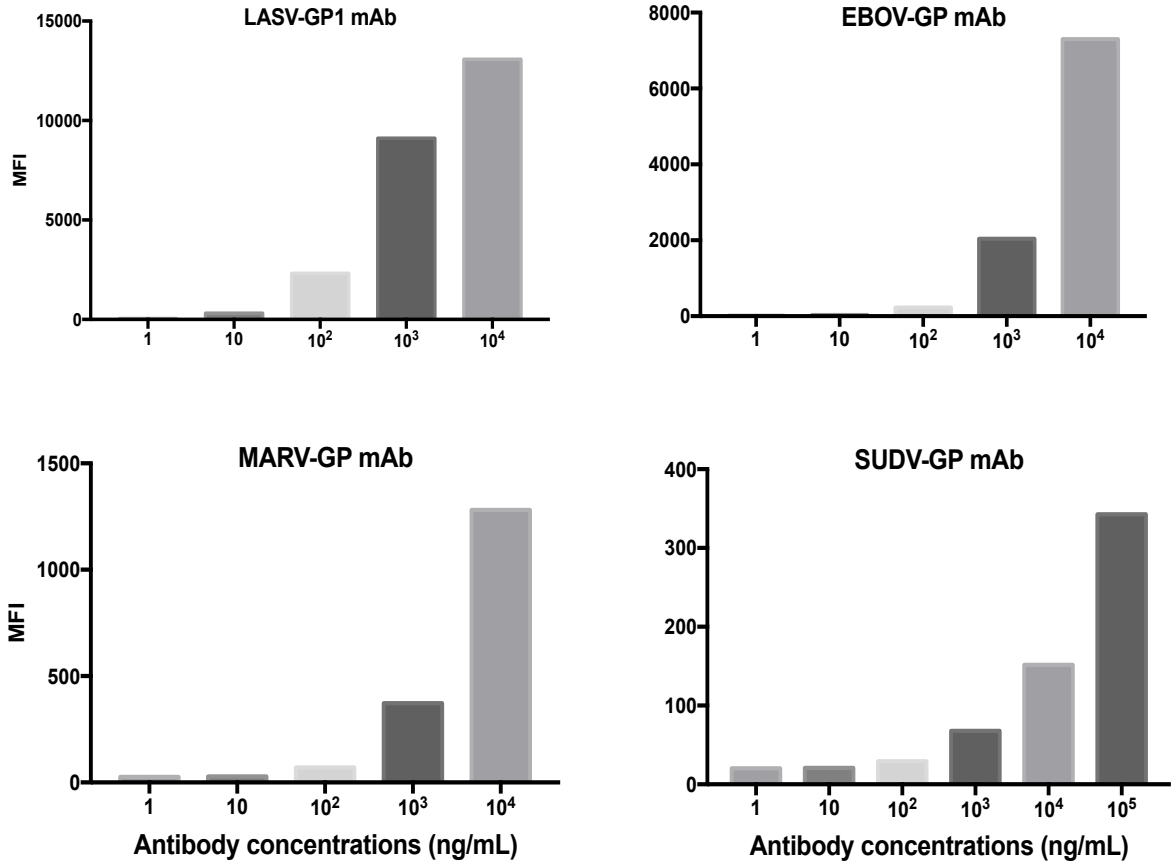

Figure 2: Limit of detection of MIA for VHF. Microsphere-conjugated beads of (A) LASV-GPC, (B) EBOV-GP, (C) MARV-GP and (D) SUDV-GP were tested with different concentrations of human or humanized monoclonal antibodies. Values represent the mean of each duplicate sample.

Very strong correlation was observed between HFV mAB and MFI

To determine the correlation between HFV mAb and MFI, several dilutions of each HFV mAb were tested and standard curves were generated. As observed in Fig. 3A-D, there was a very strong correlation between antibody concentration and MFI for all four mAb. However, SUDV mAb was discontinued from further testing due to low MFI resulting most likely from the heavy chain structure of the recombinant mAb.

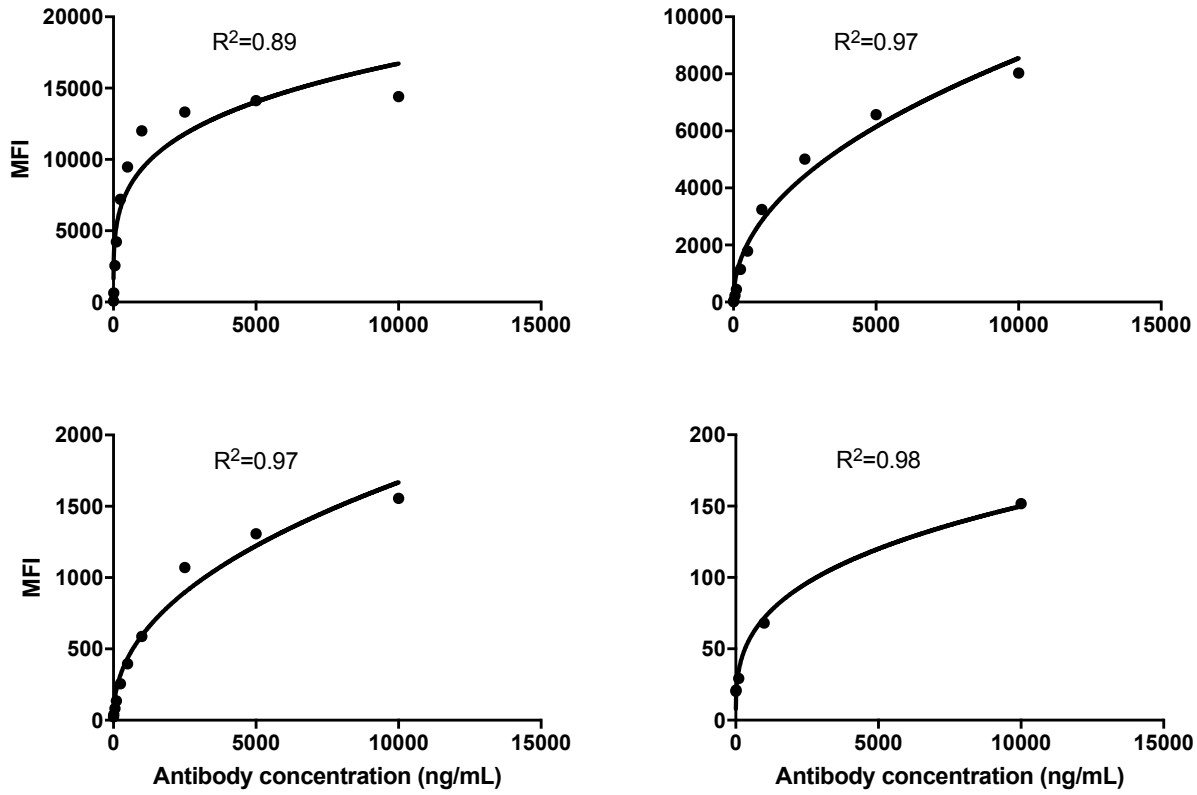

Figure 3: Correlation between concentration of HFV monoclonal antibodies and MFI.

Limited cross reactivity to EBOV, LASV and MARV monoclonal antibodies

To evaluate the specificity of the MIA, seven microspheres coupled with four HFV recombinant glycoproteins (EBOV, LASV, MARV and SUDV) and three control antigens (MSP1, HSV and BSA) were mixed together and detected using monoclonal antibodies for each HFV recombinant protein. As shown in Fig. 4A-C, EBOV, LASV and MARV monoclonal antibodies had no apparent cross-reactivity with the other antigens. In a separate experiment in which dengue virus (DENV) IgG-positive control serum (confirmed by PRNT) was used, only background MFI were observed (Fig. 4D). SUDV GP exhibited the highest background MFI with DENV IgG positive control, indicating possible cross reactivity.

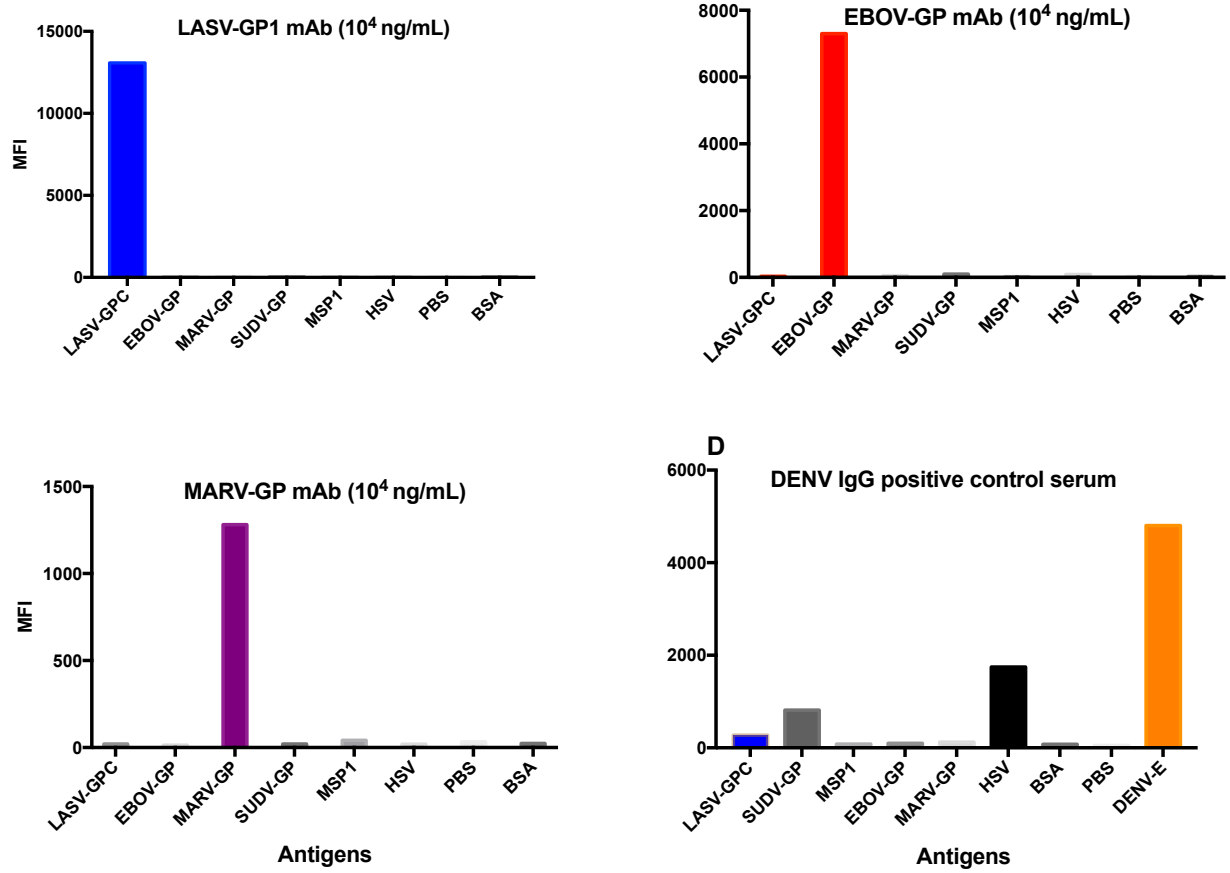

Figure 4: Specificity of seven-plexed MIA**.** Seven microspheres coupled with different antigens were mixed and tested with monoclonal antibodies to (A) LASV, (B), EBOV, and (C) MARV. Microspheres were tested with (D) DENV IgG-positive serum sample.

MIA for VHF is highly reproducible

The precision of the MIA was expressed by evaluating the repeatability and reproducibility and by calculating the CV. After each monoclonal antibody was tested 13 times for repeatability, the CV for LASV-GPC, EBOV-GP, MARV-GP and BSA conjugated microspheres was 3.1%, 5.7%, 7.4%, and 3.7% respectively. Meanwhile, the CV for EBOV positive control NHP sample was 2.7% (Fig. 5A). The day-to-day variability of the assay conducted by two individuals on 13 different plates was 6.9%, 8.8%, 11.8%, 7.4% and 5.9% for LASV-GPC, EBOV-GP, MARV-GP, BSA conjugated microspheres, respectively and 5.9% for EBOV positive control NHP sample (Fig. 5B). Repeatability and reproducibility were not assessed for SUDV due to limited antibody availability and low MFI readings.

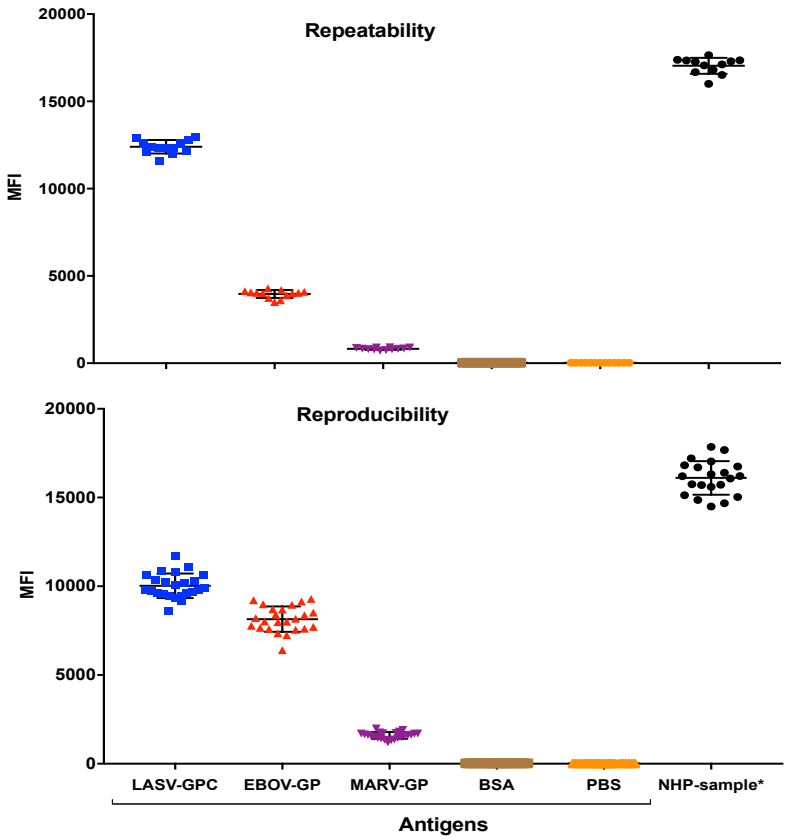

Figure 5: Repeatability and reproducibility of VHF MIA. The same concentration of each monoclonal antibody was measured 13 times on one 96-well plate for (A) repeatability and (B) reproducibility. * Irradiated serum sample from a rhesus macaque that was previously infected with EBOV

MIA can simultaneously detect multiple HFV antibodies

To determine the ability of the MIA to detect multiple infections in a single individual, a cocktail of EBOV, LASV and MARV monoclonal antibodies was tested. As shown in Fig. 6A and 6B, the MIA was able to detect and differentiate between the individual monoclonal antibodies of the cocktail. The results were repeatable (Fig. 6A) and reproducible (Fig. 6B). The inter- and intra-assay CV was similar to that observed when individual antibodies were used (Fig. 5 and Fig. 6). Specifically, the intra-assay (repeatability) CV for LASV, EBOV and MARV was 2.1, 2.2 and 6.2, respectively, while the day-to-day variability was 6.6, 6.8 and 7, respectively, for LASV, EBOV and MARV.

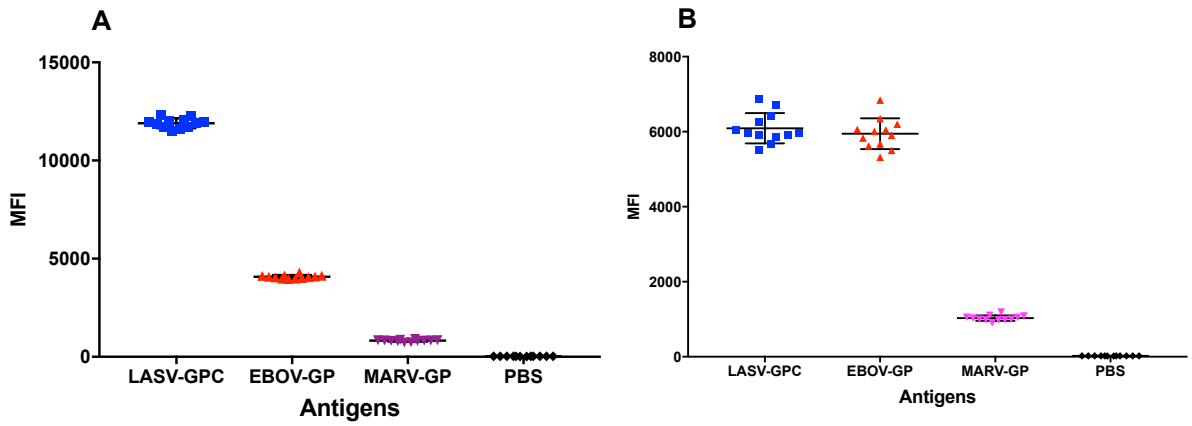

Figure 6: Detection of multiple HFV antibodies. A cocktail containing three monoclonal antibodies was tested with microspheres conjugated with various HFV antigens and evaluated for (A) repeatability and (B) reproducibility).

Evidence of silently circulating HFV in Cameroon

To determine if Cameroonians have been previously infected with HFV, 408 plasma samples collected from three regions in Cameroon were tested using the MIA. PBS and BSA were used as negative control while MSP-1 and HSV-1 microsphere conjugated beads were used as internal controls. As expected, most of the samples had high MFI to MSP-1 and HSV-1. Of the 408 samples tested, samples 3, 4 and 2 had MFI above the cut-off for LASV, MARV and EBOV, respectively (Table 1). However, there were 11 other samples that had MFI higher than the cut-off for all the microspheres conjugated with HFV antigen. Such samples were considered suspected VHF-positive; however, the suspected infecting virus could not be determined. An example of such a sample can be found in Table 1 (sample ID, HF10).

Table 1: MFI of suspected VHF-positive plasma samples

ID (Age in Years)	MFI of HFV antigens							Comments
	MSP-1	LASV GPC	MARV GP	SUDV GP	EBOV GP	EBOV NP	EBOV VP40	
HF1 (52)	2570	2934*	218	512	42.5	90	102	LASV
HF2 (5)	5121	3568*	507	459	145	3002**	1523**	LASV
HF3 (1)	16963	3278*	163	342	87.5	2708**	595	LASV
HF4 (7)	167	681	4573*	1096	187.5	643	419	MARV
HF5 (3)	125	168	3558*	754	130	157	838	MARV
HF6 (5)	167	186	3369*	670	47.5	107	141	MARV
HF7 (25)	139	471	3696*	1629	446	329	2203**	MARV
HF8 (7)	11517	478	124	227	6461*	676	1896*	EBOV
HF9 (2)	631	193	235	130	1147*	305	324*	EBOV
***HF10(6)	3636	11023	15412	21477	3381	6842	9413	VHF

*MFI above cut-off, ** Possible cross-reactivity or co-infection, *** Suspected infecting virus cannot be determined.

MFI cut-off based on mean +3SD: MSP-1, 192; SUDV-GP, 5,532; LASV GP, 2,427; MARV-GP, 2,838; EBOV-NP, 1,212; EBOV-VP40, 651; EBOV-GP, 210

Discussion

The unprecedented scope of the recent outbreak of EVD in West Africa has underscored the need for rapid and accurate diagnosis of HFV. Interrupting the chain of HFV transmission relies heavily on laboratory support. Here, we report the development of a Luminex-based multiplex MIA incorporating recombinant proteins for the simultaneous detection of HFV-IgG specific antibodies. Luminex technology has several advantages over traditional ELISA and IFA including high assay throughput, minimal sample volume and multiplexing several analytes. The MIA described in this study required only 3 μL of plasma sample to test for four HFV pathogens and further includes testing for antibodies against MSP-1 and HSV-1.

Recombinant proteins are valuable alternatives to conventional antigens

The requirement of using high level biocontainment facilities for conventional antigen production and the associated safety concerns to use HFV, makes recombinant antigens a valuable alternative. Using S2 cells, we have successfully expressed and purified recombinant EBOV (GP, NP and VP40), MARV GP, SUDV GP and LASV GPC, which we used as antigens to develop in-house MIA for detection of HFV. The glycoproteins of Ebola, Sudan, Marburg and Lassa viruses mediate viral attachment and entry into the host cell and are antigens targeted by the host immune system (12-14). Our laboratory recently showed that recombinant EBOV-GP, VP 24 and VP 40 are highly immunogenic, stimulating both cellular and humoral responses (15). Moreover, EBOV NP shows high expression level in infected cells, making it a good target for the immune system (16). Also, antibodies have been shown to be directed against at LASV NP, GP1, GP2, and matrix protein (17, 18). Therefore, it is appropriate to use these proteins as detection antigens for our MIA.

Performance of MIA

Due to the logistical and safety issues in obtaining samples from confirmed VHF patients, we used human- or humanized -monoclonal antibodies to pre-validate our assay. The LASV-GP1 mAb was isolated from a patient in West Africa who had survived Lassa fever and who had developed sustained antibody titers (19). It was further shown to neutralize LASV Josiah strain. EBOV GP-specific mAbs have previously been used to purify EBOV-GP (15). Using these monoclonal antibodies, we have been able to show that our MIA is highly sensitive, specific, repeatable and reproducible. Our assay was sensitive enough to detect as low as 100 ng/mL LASV mAb. However, at this same mAB concentration, lower MFI were observed for EBOV-GP, MARV-GP and SUDV-GP conjugated microspheres. This may be due differences in the type of

monoclonal antibody used. LASV monoclonal antibody is fully human while EBOV, SUDV and MARV monoclonal antibodies are humanized. Humanization, which is the replacement of murine constant regions with that of human may alter the properties of the antibody and distort the interaction between the primary and secondary antibodies (20). This may probably be responsible for the low MFI of the SUDV mAb. The day-to-day variation of our MIA was within the general acceptable range of less than 15%. Therefore, the performance of the MIA is expected to be similar between operators.

Evaluation of cross-reactivity is very important for any serological test. With the use of the various HFV monoclonal antibodies, there was no apparent cross-reactivity between the different conjugated microspheres. When DENV-IgG positive control samples were used, there was also no apparent cross-reactivity with the other antigens. This shows that the MIA is capable of distinguishing various HFV. The MIA was capable of detecting individual antibodies from a cocktail of three different HFV antibodies. This means the assay is capable of detecting antibodies to multiple pathogens in the same individual. This is particularly important because once a single pathogen has been identified, healthcare workers may not readily suspect co-infections.

HFV may be silently circulating in Cameroon

Our results suggest that some Cameroonians have been exposed to HFV. We observed MFI greater than the cut-off for LASV, MARV and EBOV IgG in 2.2% of the samples. However, there were some samples where the suspected infecting virus could not be determined most likely due to cross-reactivity. In a large community-based survey in Gabon, 15.3% of the participants were seropositive for EBOV-IgG antibodies (21). However, in the Gabon study, the authors considered samples with optical density (OD) in the antigen-coated well twice as high as the OD control well to be positive. We used a more stringent cutoff of three standard deviations above negative controls as previously described. Meanwhile in Mali, the annual incidence of LHF was 6.3% with seroconversion frequently seen in pre-teenagers (22). Most of the presumed VHF-IgG positive individuals in our study were greater than 5 years old and had high MFI to at least one or both of our internal controls, indicating that they were able to mount an immune response to known endemic pathogens (malaria parasite and/or HSV-1). Overall, 76% of the Cameroonian samples had MFI greater than the cut-off for MSP-1. The four samples with MFI lower than the cut-off for MSP-1 in Table 1, were all negative for malaria by PCR. Meanwhile, for the US controls, the highest MFI for MSP-1 was only 187. This shows that our internal control is working; the samples are of good quality and the study participants were capable of

producing antibodies. However, in some of our patient samples, we observed high MFIs to more than one of the HFV antigens. In the absence of a confirmatory serological test, it is difficult to elucidate if this represents multiple exposure to HFV or cross-reactivity. Mohan and colleagues recently showed that human survivors of Ebola or MARV exhibit cross-reactive antibodies with NP being the most cross-reactive, while GP was most specific (23). Furthermore, antibodies from survivors infected by SUDV exhibited the highest cross-reactivity to EBOV (NP and VP40) and MARV (GP and NP), Reston Ebola virus (GP, NP and VP40), as well as against Tai Forest Ebola virus (NP and VP40) while MARV was least cross-reactive to the other filoviral antigens.

Conclusions

This study reports for the first time the development of an MIA incorporating recombinant proteins for the diagnosis of VHF. This is the first, high-throughput multiplex laboratory assay for serological detection of antibodies against LASV, MARV, SUDV and EBOV. When validated, this assay holds great potential to be used for large-scale epidemiology surveys, outbreak investigations and rapid diagnosis of VHF. Our results suggest that HFV may be silently circulating in Cameroon. We plan to validate this assay with human samples and subsequently conduct a community-based epidemiological survey of VHF in Cameroon.

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CHAPTER 6

Discussion

Discussion

At health facilities in tropical countries, fever is the most common presenting symptom, or chief complaint, among individuals seeking medical care. Fever invariably signifies systemic inflammation, often due to infectious etiologies (bacteria, parasite, fungi and viruses). A survey conducted in 2007 in 42 countries in Sub-Saharan Africa estimated 655.6 million fever episodes in children under five years of age (1). The incidence of fever varies from one country to another with an estimated two to nine febrile episodes per year in children younger than five years. Diagnosing and managing febrile illnesses in the tropics is very challenging, especially in the absence of diagnostic tools and focal signs/symptoms. But, if local epidemiology on the causes of fever is well understood by healthcare workers, the patients' age and geographic location can help in deciding appropriate diagnostic and treatment options. Nevertheless, there remains a large gap in our understanding of pathogen profiling in most malaria-endemic countries. The paucity of reliable data is in part due to the lack of diagnostic tools, active surveillance systems and the misplaced emphasis on malaria as the sole cause of fever. The overall objective of this dissertation was to identify infectious causes of febrile illnesses in Cameroon. This is particularly important because knowing the causes of fever is the first step in deciding appropriate treatment, thereby improving health outcomes.

To achieve our overall objective, we embarked on three aims. In Aim 1, we determined the diagnostic accuracy of various malaria testing methods, including clinical diagnosis, thick-film microscopy (TFM) and rapid diagnostic test (RDT) using PCR as the reference standard. Aim 2 assessed the prevalence of infectious causes of febrile illnesses in Cameroon. Lastly, in Aim 3, we have developed a novel microsphere immunoassay incorporating recombinant proteins as antigens and used it to determine the prevalence of infections with pathogens causing viral hemorrhagic fevers (VHF) in Cameroon. Our long-term goal was to develop rapid, sensitive and specific diagnostic tools for tropical febrile illnesses. The rationale was that improved accuracy in diagnostic testing will result in improved health outcomes and guard against the indiscriminate use of antimalarial drugs.

Accuracy of clinical diagnosis, TFM, RDT and PCR in the diagnosis of malaria

Before 2010, the WHO guidelines on the treatment of malaria recommended empiric treatment with oral antimalarial drugs for fever in children less than five years living in malaria-endemic regions (2). However, due to control efforts, such as the distribution of insecticide-treated nets (ITN), the burden of malaria and the proportion of febrile illnesses attributed to malaria have

decreased. Regions with previously high malaria transmission are reporting decline in incidence (3). Moreover, the availability of RDTs and rise in antimicrobial resistance, challenges the purpose of presumptive treatment of febrile illnesses. The WHO revised its fever treatment guidelines in 2010 to recommend antimalarial treatment only for confirmed cases by RDT or microscopy (4). To begin with, we investigated the accuracy of the various malaria diagnostic test methods in Cameroon. Our results showed that clinical diagnosis was the least accurate method of diagnosing malaria with a very low specificity, while RDT was the most accurate compared to TFM and clinical diagnosis. However, PCR detected many malaria infections that were missed by TFM and RDT, making PCR a valuable diagnostic tool especially for the purpose of malaria elimination. Because the symptoms of malaria overlap with that of other febrile illnesses, accurate diagnosis of malaria will ensure that patients receive appropriate treatment. It has been a common practice for healthcare workers in malaria-endemic countries to presume that all fevers are due to malaria. In fact, 98% of our study participants were presumed to have malaria because of fever, but less than 50% of them were actually infected by the malaria parasite. Surprisingly, most of the malaria-negative patients were treated with quinine, which is meant to treat severe or complicated malaria. Apart from the impact on the patient, improper use of antimalarial drugs leads to wasted resources and can contribute to the spread of drug resistance. Moreover, improper treatment of non-malarial febrile illnesses with antimalarial have been shown to lead to higher mortality (5). Therefore, diagnosis of all suspected cases of malaria using a parasite-based test, followed by appropriate treatment will decrease morbidity and mortality associated with febrile illnesses leading to reduction in the development of drug resistance, diagnosis and treatment of malaria negative patients for causes of fever other than malaria, and overall cost-effective healthcare services.

Despite ready availability of malaria RDT and microscopy technology in our study, treatment decisions by healthcare workers were mostly based on clinical signs and symptoms rather than on the parasite-based guidelines recommended by WHO. This further demonstrates that the availability of diagnostic tests on its own may not be sufficient to improve fever case management. Diagnostic tests results are meant to guide clinical decision-making, hence testing should not be separated from treatment decision. Therefore, there is a need for additional support, such as well-designed competency-based refresher courses for healthcare workers that can have a sustainable impact on treatment practices. Such training courses, as evident from a study by Mbacham and colleagues, are supposed to last for at least three days, in order to promote the behavioral changes required for healthcare workers to adhere to the

WHO treatment guidelines for malaria (6). Mbacham and colleagues report that basic training (one day) is insufficient to promote the behavioral change required for healthcare workers to adhere to malaria negative results. In reality, changing habitual clinical behavior is difficult. As Oxan and colleagues put it, there is no “magic bullet” in improving professional practice (7). Several reasons can be associated with poor adherence to malaria test results including, i) lack of confidence in laboratory results, ii) patient pressure for treatment with antimalarial drugs, iii) the tradition of treatment based on signs and symptoms, iv) lack of diagnostic tests for non-malarial febrile illnesses, v) limited data on the epidemiology of febrile disease in malaria-endemic areas, and vi) no clear algorithms for the management of non-malarial fevers.

Infectious causes of febrile illnesses in Cameroon

In order to improve our understanding of the epidemiology of febrile illnesses in Cameroon, we conducted a cross-sectional study to profile infectious etiologies in three different geographical regions. Our results showed that malaria was the leading cause of febrile illnesses in Cameroon. Our study also confirmed that the yearly outbreak of febrile illnesses in the Far North region of Cameroon was due to seasonal malaria transmission. Importantly, we identified other etiologies of febrile illnesses, including: dengue virus, *Toxoplasma gondii*, *Entamoeba histolytica*, *Shigella sp*, *Salmonella sp*, West Nile virus, respiratory viruses, chikungunya virus and sepsis, all of which should be considered by healthcare workers in the differential diagnosis of febrile illnesses in Cameroon. Previous studies from some tropical countries demonstrated a predominance of acute respiratory infections in febrile patients with high proportion caused by viral pathogens (8, 9). We demonstrated that some of the infectious etiologies differ based on age and geographic region. Acute respiratory tract infections were the most common cause of non-malaria febrile illnesses in young children while in older children and adults, typhoid and dengue fever were more common. In two of our study sites (Nkolbisson and Maroua), malaria was the main cause of febrile illnesses, while in Bamenda, respiratory viral pathogens were responsible for most of the febrile illnesses. Therefore, in a place like Bamenda, giving antimalarial treatment to most of these patients with viral infections will be of no clinical benefit. Instead, treating other complications that may arise because of viral infections, like pneumonia and sepsis (due to secondary bacterial infection), with antibiotics will benefit the patient. As evident in our results, the possibility of malaria co-infections should be kept in mind by healthcare workers especially if the patient is not responding to antimalarial treatment. By using a combination of white blood cell count, body temperature and procalcitonin levels, we were able to identify participants at risk of developing sepsis more rapidly as compared to blood

culture. This is particularly important because blood culture is known to lack sensitivity, require good laboratory infrastructure, and may take several days for the results to be available. A delay in diagnosing sepsis and administering antibiotics increases severity of the condition. Thus, if PCT is made available as some POCT in tropical countries, it could greatly improve sepsis management.

Because of the growing concerns in malaria-endemic regions of non-compliance to malaria negative results by healthcare workers, clear algorithms on managing non-malarial febrile illnesses are urgently needed. The WHO has published two documents, integrated management of childhood illness (IMCI)) and community level integrated community case management, to guide healthcare workers to identify and treat children with fever and other common childhood illnesses at healthcare facilities (10). The IMCI strategy by WHO uses a series of algorithms and flow charts to systematically assess danger signs that can trigger immediate hospitalization or referral. It also provides guidelines to classify illnesses like diarrhea, pneumonia, measles, fever, otitis media and malnutrition according to severity. Even though the IMCI has the potential to increase quality of care, its major drawback is low specificity due to overlapping signs and symptoms of some infectious diseases. In fact, our results show that very few signs and symptoms can be used to predict the cause of fever in febrile patients. As demonstrated by our study and others, algorithms to manage febrile illnesses need to take into consideration the heterogeneous distribution of infectious etiologies of febrile illnesses and the integration of relevant laboratory tests.

Microsphere immunoassay for detection of viral hemorrhagic fevers (VHF) in Cameroon

The value of diagnostic tools in the management and control of infectious diseases cannot be overemphasized. This dissertation reports the development of a novel multiplex MIA for HFV, which can be used for surveillance and also as a readout for vaccine and other immunological studies. This represents an important step toward a more effective screening platform for the simultaneous detection of multiple pathogens and allows for high sample throughput, fulfilling the objectives of a reliable, accurate, and cost-effective process of generating surveillance information. Using human/humanized monoclonal antibodies, we demonstrated that MIA is sensitive, specific and precise. Furthermore, using a cocktail of monoclonal antibodies we demonstrated that MIA can detect and distinguish antibodies directed against multiple HFV. Our results suggest that HFV, including EBOV, MARV and LASV, may be silently circulating in Cameroon.

Because several pathogens can cause febrile illnesses in the tropics, the availability of multiplex tests, which can quickly identify a pathogen from a group of pathogens that cause similar symptoms is of paramount importance so that appropriate therapy can be initiated and appropriate public health measures taken to curb the spread of disease. Furthermore, health care providers are faced with the daunting challenge to select the appropriate tests despite the fact that febrile illnesses are caused by many pathogens and are clinically indistinguishable.

Lessons learned and limitations

In the process of conducting these studies, several lessons have been learned that can be used to improve future study design. If we were to conduct this study again with enough financial resources, we would design the study to include other important pathogens that can cause febrile illnesses that were not included in the present study. Also, we would collect urine samples, CSF, nasopharyngeal and throat swabs for the diagnosis of urinary tract infections, meningitis and acute respiratory tract infections. We would also include chest X-ray to confirm diagnosis of pneumonia and blood culture to diagnose septicemia. Other pathogens would include HIV and *Rickettsia spp*. We would include a control group of healthy individuals and collect convalescent blood samples for serological diagnosis.

Furthermore, we would include children younger than 6 months, as little research data is available on etiologies of fever in young infants. Moreover, febrile illnesses in young infants can rapidly progress to severe disease. We would also expand the study sites to include all 10 regions of Cameroon and collaborate with countries in West and Central Africa for a multicenter study (West and Central Africa Febrile Illness Surveillance Network (WCFSN). This will enable us to have enough power to conduct statistical analysis including a better prediction model of pathogens causing febrile illnesses by using a combination of signs and symptoms, age and geographical location. It will be important to also extend the study duration to at least one year. This will enable us to have a better understanding of the seasonal variations of the various infectious etiologies of febrile illnesses.

In addition, we would devise a means to safely store electronic records of each patient's hospital book. This can be done by using mobile apps such as CamScanner by INTSIG Information Co. Ltd, which can be used to scan pages in the patient's hospital book into clear and sharp images or PDF, to email, print or save to a computer. The use of this app does not require an internet connection and can therefore be applicable under field conditions. This

would enable us to have comprehensive health information of each participant with regards to diagnosis and treatment. However, additional measures would have to be taken to ensure the safety, security and patient confidentiality of such information.

Conclusions

Collectively, our data provide the first evidence of several important pathogens that cause febrile illnesses in Cameroon. These results will be disseminated to the Ministry of Public Health and local medical community in Cameroon, with the potential of improving the health outcomes of patients with febrile illnesses. Moreover, our results and others may provide data for the development of algorithm for the management of febrile illnesses. This study reports for the first time the development of multiplex immunoassay incorporating recombinant proteins for the diagnosis of VHF. This assay has the potential to be used for surveillance of VHF in both endemic and non-endemic regions without the requirement of a high containment laboratory. In our future research, we plan to validate our MIA and will extend the spectrum of pathogens to include other causes of fever such as: Zika virus, chikungunya virus, Japanese encephalitis virus (JEV), Crimean-Congo hemorrhagic fever (CCHF) virus, and *Salmonella typhi/paratyphi*. We hope to stratify our multiplex diagnostic platform into febrile illness panels such as a systemic fever panel, an enteric fever panel and a hemorrhagic fever panel. We have already expressed Zika virus envelope protein and are in the process of expressing chikungunya virus envelope protein. We hope to express recombinant proteins for other pathogens including Rift Valley Fever virus, CCHF virus, *Salmonella typhi* and JEV.

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