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MICROBIAL ETIOLOGY OF ACUTE FEBRILE ILLNESS IN CHILDREN PRESENTING TO HOSPITALS IN GHANA

BY

NICHOLAS AMOAKO

(10513065)

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DECLARATION

I, Nicholas Amoako hereby declare that with the exception of cited references to the work done by people which have been duly acknowledged, this thesis is the result of my own research conducted under supervision. It has never been presented elsewhere either in part or whole to any examining body for another degree.

..... Date: 19th May 2020

Nicholas Amoako

Student

..... Date: 26 May 2020

Prof. Gordon A. Awandare

Principal Supervisor: Department of Biochemistry, Cell and Molecular Biology

..... Date: 25th May 2020

Dr. Samuel Duodu

Co-Supervisor: Department of Biochemistry, Cell and Molecular Biology

..... Date: 26 MAY 2020

Dr. Kwaku Poku Asante

Co-Supervisor: Kintampo Health Research Centre, Kintampo

..... Date: 20th May 2020

Prof. Justin Stoler

Co-Supervisor: University of Miami, United States of America

DEDICATION

This work is dedicated to Almighty God and to my wife, Ernestina and children, Rexford, Samuel, Henry, Veronica and Yvonne. I also dedicate this work to my mother Madam Afia Adowaa. I again dedicate this work to my sisters Emelia, Didaa, Monica and Gloria for their prayers and support.

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LIST OF ABBREVIATIONS AND ACRONYMS

AFI	Acute febrile illness
API	Analytical profile index
ARTI	Acute respiratory tract infection
AUC	Area under the curve
CI	Confidence interval
CNS	Coagulase negative staphylococcus
CSS	Cascading style sheets
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
EDTA	Ethylenediaminetetraacitic acid
GRA	Granulocyte
GE	Gastroenteritis
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCT	Hematocrit
HHV	Human herpesvirus
HGB	Hemoglobin
HIV	Human immunodeficiency virus
HTML	Hypertext markup language
IFN-γ	Interferon gamma
IG	Immunoglobulin (IgG, IgA, IgE, etc)

IL	Interleukin (IL2, IL4, IL5, IL10, IL13, IL17, IL21, IL23)
IMCI	Integrated Management of childhood Illness initiative
KHRC	Kintampo Health Research Centre
LRTI	Lower respiratory tract infection
LEKMA	Ledzokuku Krowor Municipal Assembly Hospital
LYM	Lymphocyte
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MCH	Mean cell hemoglobin
MCHC	Mean cell hemoglobin concentration
MCV	Mean cell volume
Q fever	Query fever
MLST	Multi-locus sequence typing
MLVA	Multi-locus variable number tandem repeat
MSF	Mediterranean spotted fever
MST	Multi-spacer typing
MS2	Bacteriophage MS2 is icosahedral virus that infect bacteria
NB	Naïve Bayes
NLR	Neutrophil-lymphocyte ratio
NMIMR	Noguchi Memorial Institute for Medical Research
NTS	Non-typhoidal *Salmonella*
OPD	Out-patient department
OR	Odds ratio
OROV	Oropouche fever

PCR	Polymerase chain reaction
PCT	Procalcitonin
PFGE	Pulsed-field gel electrophoresis
PGE2	Prostaglandin E_2
Pf	*Plasmodium falciparum*
Pf HRP2	*Plasmodium falciparum* Histidine-rich protein
PhHV	Phocine herpesvirus
RBC	Red blood cells
ROC	Receiver operating characteristic curve
RDT	Rapid diagnostic test
SFG	Spotted fever group
SLEV	Saint Louis encephalitis virus
SNP	Single nucleotide polymorphisms
TAC-PCR	TaqMan array card-polymerase chain reaction
TNF-α	Tumor necrosis factor alpha
RDW	Red cell distribution width
RF	Random forest
UTI	Urinary tract infection
VEEV	Venezuelan equine encephalitis
WACCBIP	West African Centre for Cell Biology of Infectious Pathogens
WBC	White blood cells
WGS	Whole genome sequencing
WHO	World Health Organization

ABSTRACT

Background

Acute febrile illness (AFI) is responsible for a significant number of childhood mortality and morbidity and remains a common clinical presentation at most hospitals. Lack of appropriate screening techniques present a challenge in identifying potential pathogens associated with AFI. This study investigated the microbial etiology of AFI among febrile children, evaluated the potential use of inflammatory mediators as biomarkers of fever, and developed a web-based model to predict the infection status of febrile children.

Methods

858 children aged 1-15 years with acute uncomplicated fever were clinically screened using point-of-care and advanced diagnostic methods. A panel of laboratory tests comprising malaria microscopy, malaria Rapid Diagnostic Test (RDT), complete blood count, blood and urine cultures and polymerase chain reaction (PCR) were employed to screen samples for parasitic, bacterial, and viral pathogens. Concentrations of serum cytokines and hematological parameters were respectively measured using a Luminex-based magnetic bead assay and a fully automated hematology analyzer. The test of sensitivity and specificity, as well as the area under the curve (AUC) of the receiver operating characteristic (ROC) of cytokines and hematological parameters, were used as measures of diagnostic accuracy to predict fever and in the selection of the suitable data mining technique to model malaria and bacterial infection status of febrile children.

Results

Etiologies of fever were identified in 43.7% (374/858) of children studied. From blood samples analyzed, 38.6% (331/858) tested positive for the *Plasmodium* parasite which was

the most frequent pathogen detected. From 140 blood and 137 urine cultures performed, 59 organisms were identified. The most common organisms isolated were *Staphylococcus aureus* (4.7%), *Escherichia coli* (3.2%), Group D *Streptococcus* (2.5%), *Pseudomonas aeruginosa* (1.8%), Non-typhoidal *salmonellae* (1.4%), Coagulase negative staphylococci (1.4%), *Citrobacter freundii* (1.1%), *Enterobacter clocoae* (1.1%), *Salmonella* Typhi (0.9%), *Streptococcus pneumonia* (0.7%) and *Klebsiella pneumonia* (0.4%). Pathogens detected using TaqMan-based PCR from 166 blood samples included: Dengue virus (1.2%), *Coxiella burnetti* (0.6%), *Rickettsia* (3.0%), HIV (0.6%) and *Plasmodium falciparum* (37.9%). Of the enrolled children, 3.2% had *Plasmodium*-bacteria co-infections: *Plasmodium-Staphylococcus aureus* (0.9%), *Plasmodium*-dengue (0.3%), and *Plasmodium-Rickettsia* (0.6%). From the cytokine analysis, tumor necrosis factor (TNF-α) with sensitivity of 84.4% (95% CI: 75.5-91.0) and specificity of 72.2% (95% CI: 46.5-90.3) was the best predictor of fever, having AUC for the ROC curve to be 0.7. Lymphocyte (LYM-%) was the best hematological predictor of fever, with sensitivity of 65.2%, specificity of 67.3% and a ROC of 0.78. Naïve Bayes model, which incorporated only clinical symptoms, proved useful for the development of the interactive tool to predict infection status of children with AFI.

Conclusion

Malaria remains a major contributor of AFI in the study area, despite additional diagnoses of bacterial and viral origin. Dengue virus, *Rickettsia felis* and *Coxiella burnetti* were detected among the children but not clinically diagnosed. Febrile illnesses due to co-morbid infection are common and call for differential diagnosis of AFI to ensure judicious use of drugs and to avoid evolution of drug resistance. In addition, routine hematological

parameters including lymphocyte, and circulating cytokines such as TNF-α are useful and independent prognostic factors for fever. A web-based clinical decision tool has been developed to predict infection status of febrile patients.

CHAPTER ONE

1 INTRODUCTION

1.1 Background

Fever, also known as pyrexia, is an indicator of localized or systemic infection and a common clinical sign encountered in clinical pathology (Turkulov et al., 2011). It is a pathological process characterized by an elevation of core body temperature that exceeds the normal daily variation (> 0.5°C) in which pyrogen cause upward resetting of hypothalamic thermostatic set point. Fever is often not considered as a disease but as a sign that the body's immune system is against an unwanted agent (Karakitsos & Karabinis, 2008). A wide range of diseases can manifest as fever either single or in combination, resulting in a number of symptom(s). Fever can also be an important symptom in non-infectious conditions associated with malignancies and immunologic diseases (Turkulov et al., 2011; Zenone, 2006).

Acute febrile illness (AFI) is defined as a rapid onset of fever occurring for less than 7 days and typically associated with many clinical presentations (Ittyachen & Ramachandran, 2015; Kulkarni et al., 2010). In most developing countries, there is a high burden of AFI which sometimes accounts for more than 50% of all illnesses presented at the out-patient departments (Capeding et al., 2013; Kyabayinze et al., 2010; Lee et al., 2007). Globally, AFIs are common causes of childhood hospitalization contributing significantly to morbidity and mortality (Liu et al., 2012). In Africa, more than six million children die annually as a result of treatable or preventable illnesses many of which are febrile in nature (Elfving et al., 2016). There is generally scarcity of information about specific etiologies of febrile illnesses which impedes the ability to make accurate diagnoses and to provide effective treatment or implement prudent public health interventions. Consequently, most cases of AFIs are usually diagnosed

and treated presumptively as malaria due to overlapping clinical presentations (D'Acremont et al., 2014). The prevalence of malaria is still high in sub-Saharan Africa especially in West Africa, although there are reports of malaria decline in Eastern Africa sub-region (D'Acremont et al., 2014; WHO, 2018). Microscopy and malaria rapid diagnostic test are currently the only routine diagnostic tools used to guide febrile patient management. However, in situations where a sick person presents with fever and tests negative for malaria, clinical symptoms are often used to define provisional diagnoses and determine appropriate treatment regimens. In some instances, when parasites are not detected, patients are still treated with malaria drugs, often in combination with some antibiotics for fear of possible bacterial co-infections (WHO, 2018). Data regarding the cause of AFIs are limited or may be lacking in malaria endemic regions. Limited access to laboratory and diagnostic facilities as well as lack of the qualified personnel make identification of the real cause of AFIs problematic (Asante et al., 2016; Kallander et al., 2004).

Recent studies on etiologies of AFIs in Africa have highlighted a broad spectrum of potential causative pathogens that require different diagnostic approaches (Hogan et al., 2018; Prasad et al., 2015). Even though there is evidence of a gradual shifting in the epidemiology of childhood febrile illnesses in most parts of the world (Hogan et al., 2018b), malaria continues to play a leading role in childhood morbidity and mortality, accounting for over 61% (266,000) of under five deaths worldwide (Dalrymple et al., 2017; WHO, 2018). The contribution of non-malarial infections to infant mortality cannot be underestimated (Bryce et al., 2004). Particular interest are the recent outbreaks in West Africa of Dengue and Lassa fever virus infections, which also present symptoms similar to those of malaria (Akhuemokhan et al., 2017; Oyero & Ayukekbong, 2014; Ridde et al., 2016). These occurrences emphasize urgent need for

increased surveillance of non-malaria febrile diseases. Many of the studies that have assessed the etiology of AFIs in Africa have focused on hospitalized patients, particularly in regions with substantial declines in malaria transmission (Gething et al., 2007). On the contrary, the spectrum of causative pathogens responsible for fever among outpatients is poorly investigated. Few studies reporting the etiology of AFI have either targeted bacterial (Mahende et al., 2014) or viral co-infections (Church & Maitland, 2014) but not focused on the whole spectrum of fever-causing pathogens. Therefore, there is urgent need to improve on knowledge surrounding the etiology of outpatient febrile illness to optimize care and improve clinical outcomes.

Early identification of infection remains a challenge in the management of febrile patients. Differential expression of hematological and biochemical indices such as platelet count, white cell counts and serum cytokines have been suggested as useful biomarkers for monitoring early sign of infection (Kapasi et al., 2016; Lubell et al., 2015). Although positive correlation between these biomarkers and infection have been demonstrated, such studies are mostly conducted on admitted new-born or infants without emphasis on febrile patients.

Data mining technology such as Machine learning (ML) has been developed as new strategic approach for the early diagnosis and treatment of diseases. A range of ML techniques such as, Naïve Bayes (NB) classifiers, Decision Tree (DT) , Random Forrest (RF) and Lasso regression have been widely employed to model disease outcome (Kourou et al., 2015). This study employed a combination of laboratory methods to characterize the pathogens responsible for AFI and explored the usefulness of inflammatory mediators and hematological indices, as predictors of fever. It also compared different ML models and selected the best model to create a web-based tool to predict infectious status of febrile patients.

1.2 Problem statement and justification

Acute febrile illness is a widely known clinical characteristic in both children and adults seeking medical care in Ghana. Although malaria is known to be endemic in Ghana for a long time and considered a major cause to febrile diseases, the contribution of malaria to real disease state and death rate in recent times has been a subject of debate due to reported decline in its prevalence in several parts of Africa (O'Meara et al., 2010; Saran & Cohen, 2017). There is a growing concern about a high proportion of febrile illnesses which has been attributed to malaria morbidity without justification (O'Meara et al., 2010). It is believed that other diseases with overlapping symptoms to malaria may contribute to the high morbidity and mortality rates reported that are not recognized due to diagnostic challenges (Altaras et al., 2016; Breman, 2001; D'Acremont et al., 2009). This misrepresentation of malaria prevalence creates fear and places substantial burden on both individual caregivers and Government or funding agencies that either bear the cost of treatment or subsidize the cost of medications to affordable levels.

In most Ghanaian health facilities, febrile patients presenting with respiratory symptoms are almost always presumptively treated with both antibiotic and antimalarial drugs. Very little effort is often made to determine the underlying cause of the symptoms; many of which have been shown to be due to bacterial and viral infections (Feikin et al., 2013; Medici et al., 2004; Nokes et al., 2004). Existing information on bacterial and viral causes of AFIs is very scanty in Africa and Ghana in particular, but the situation is more daunting for viral infections. Reports from few studies conducted on bacterial causes of respiratory diseases in children from Ghana have led to the inclusion of vaccines against pneumococcal and *Hemophilus influenza B*

disease as part of the childhood immunization schedule in Ghana (Asuman et al., 2018).

Compared to malaria, tuberculosis and HIV, the burden of other tropical diseases has not been adequately addressed in many African countries including Ghana. Some studies in Africa recently confirmed the clinical overestimations of malaria burden, whilst invasive bacterial and viral infections are underestimated (Abokyi et al., 2015; Crump & Kirk, 2015; Kwarteng et al., 2015). Viruses, including Lassa, dengue and chikungunya have been reported to account for a significant proportion of hemorrhagic fevers among febrile patients in Nigeria and Burkina Faso (Sow et al., 2016). At the moment, contribution of bacterial zoonoses and arboviral infections to AFIs in Ghana remains virtually unknown. Therefore, investigation into the major etiological agents of AFIs needs to be intensified to ensure accurate diagnosis and management, especially among children. Generally, AFIs in Ghana are being handled through case management with drugs and vaccines but the risk factors associated with such diseases are often neglected and not properly documented.

Another worrying factor complicating the management effort is self-medication which impedes diagnosis and increases the occurrence and spread of antimicrobial resistance (Ilic et al., 2012). Moreover, inadequate microbiological facilities to diagnose bacteremia or viremia make presumptive diagnosis the only realistic option in some areas (Petti et al., 2006). This means, improved diagnostics is essential in the treatment of all fever related diseases and this requires better knowledge of the spectrum of circulating pathogens that commonly infect the population to cause fever.

This study investigated the etiology of AFIs in children by assessing a range of pathogens that may be associated with childhood febrile illness. It further investigated

the diagnostic potentials of host inflammatory mediators for predicting fever and developing a simple point of care diagnostic model that will help clinicians select the optimum test type to perform to aid with the diagnosis of febrile illnesses. The model is expected to inform medical practitioners on what to suspect or think about when malaria test is negative, and to ensure that management of AFI does not focus on the presumptive administration of antimalarials and antibiotics.

1.3 Study hypothesis and objectives

1.3.1 Hypothesis

In this study, we hypothesized that different inflammatory or hematological mediators and clinical presentations are induced by different pathogens of AFI and that the likelihood of predicting infection status of a patient for appropriate treatment can be developed using infectious disease models incorporating clinical, demographic, biochemical and hematological variables.

1.3.2 Main Objective

The primary objective of this study was to investigate the microbial etiology of AFI by using a combination of laboratory-based screening methods and clinical data to accurately detect and characterize the pathogens involved.

1.3.3 Specific Objectives

1. To identify and characterize bacteria and parasitic etiologies of AFIs using standard phenotypic methods recommended by WHO based on microscopy, rapid diagnostic test (RDTs) and microbiological cultures.

2. To identify and characterize viral, bacteria and parasitic etiologies of AFI using molecular-based detection techniques including real time PCR (TaqMan array cards) and sequencing approaches.

3. To evaluate the predictive values of serum cytokines and hematological parameters as biomarkers of AFI.

4. To develop a predictive model to inform clinicians of the probability of infections to decide if a febrile child should be subjected to the infection diagnostic tests or not.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Fever and acute febrile illness (AFI)

Fever is defined as regulated increase in internal body temperature where pyrogens cause upward resetting of hypothalamic set point of 36.8±0.7°C or 98.2±1.3°F (Mackowiak, 2005). Normal body temperature may vary from one person to another depending on age, sex, environmental factors, time of day and activity level (Lim et al., 2008). Fever is one of the cardinal signs of inflammation, but it may also herald an onset of severe or potentially fatal illness. Fever can arbitrarily be classified into acute, sub-acute and chronic fever depending on duration (Dinarello, 2004; Lim et al., 2008). Acute fever usually occurs less than one week (between 2-5days) in duration and may be characterized by infectious diseases such as viral or bacterial related respiratory tract infections and malaria. Sub-acute fever are those occurring not more than 14 days in duration without apparent cause despite inpatient evaluation as may be seen with intra-abdominal abscess (Dinarello, 2004). Chronic or persistent fever occur usually more than 14 days in duration and are characteristic of persistent bacterial infections such as tuberculosis or viral infections, typical of HIV, and several chronic infections such as the cancers (Dinarello, 2004). In many cases, acute fever may progress and become chronic if not managed or treated well.

Depending on the magnitude of the body temperature, fever can further be categorized into low grade fever (38.1°C -39°C), moderate grade fever (39.1°C - 40°C), high grade fever (40.1°C-41.1°C) and hyperpyrexia (41.1°C and above) (Ogoina, 2011). The magnitude of body temperature may have some prognostic and diagnostic indications. The level of fever may sometime correlate with severity of disease as demonstrated in an experimental dengue and shigellosis virus infections (Mackowiak et al., 1994) and

in *Plasmodium* infections where the presence of high fever (hyperpyrexia) may denote severe disease with poor prognosis (Mackowiak, 2005; WHO, 2000). However, the general clinical state of the individual provides a more useful predictor of severe illness than the magnitude of fever (Graneto, 2010).

Acute febrile illness is a rapid onset of fever, occurring for less than 7 days and usually self-limiting and presumed to originate from an infectious source (Chrispal et al., 2010). In many tropical countries, dengue, malaria, *Rickettsia*, *Coxiella*, *Leptospira*, *Bartonella*, and Chikungunya virus, typhoid fever and influenza like illnesses have been identified as common causes of AFIs (Chappuis et al., 2013; Cifuentes et al., 2013; Acestor et al., 2012). Other agents might be important causes of AFIs but are rarely considered by clinicians and often under-diagnosed. AFIs often have similar non-specific clinical presentations early in the clinical course when most patients seek medical care. In addition, rapid point-of-care diagnostics are often not readily available. Surveillance for AFIs is mostly passive and focuses on clinical identification of active cases and voluntary case reporting. As a result, burden of disease for the etiologic agents of AFI are likely underestimated (Dayan et al., 2015). A better understanding of the major causes of AFI is needed to guide clinical management, develop diagnostics, inform public health policy, and direct prevention efforts (Chappuis et al., 2013).

2.2 Pathogenesis of fever

The febrile response is thought to be influenced by mediators generally called pyrogens (Romanovsky, 2007). Depending on where they are produced, pyrogens can be classified either as “exogenous” (those produced outside the host) or “endogenous” (those produced within the host). Generally, exogenous pyrogens can be part or whole microorganism or their products, such as toxins, that trigger febrile response. The

Gram-negative cell wall component of bacteria, known as lipopolysaccharide (LPS) is a typical example of exogenous pyrogen. Endogenous pyrogens on the other hand are those produced by immune cells such as lymphocytes, neutrophils or macrophages in response to exposure to exogenous pyrogens and are responsible for induction of inflammatory cytokines including interleukins such as IL- 1, IL-6, interferon gamma and tumor necrosis factor (TNF), among others (Romanovsky, 2007).

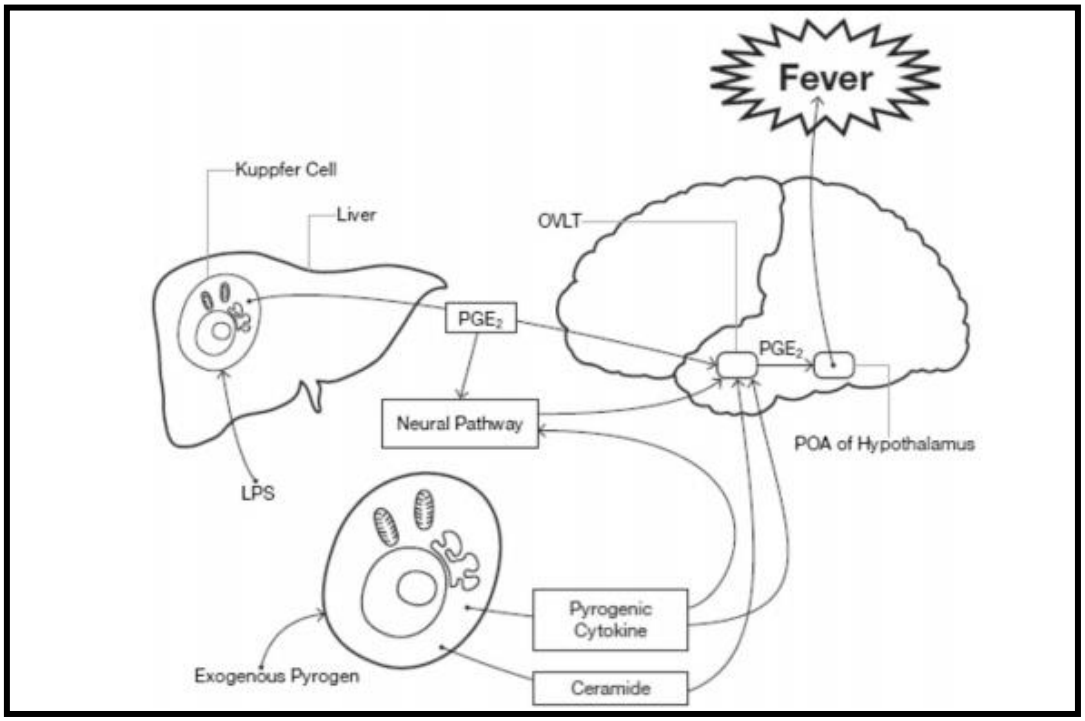
The initiation and regulation of the febrile response are dependent on the pyrogenic and cryogenic (anti-pyretic) properties of the infecting organisms or substances (Walter et al., 2016). While pyrogens can directly or indirectly initiate fever, cryogens on the other hand act to prevent excessive temperature elevation and thereby preventing abnormally high fever. It is the harmony between interactions of pyrogens and cyrogens that determine the level and duration of the febrile response to any immune challenge (Walter et al., 2016).

The interaction of endogenous pyrogens with a section of the brain called the organum vasculosum of the lamina terminalis (OVLT) results in the production of special proteins implicated in the induction of fever (Walter et al., 2016). OVLT forms part of anterior hypothalamus within the lamina terminalis of the brain, charged with many reflex functions.

Several exogenous pyrogens act by stimulating cytokine production either directly or indirectly by acting on the OVLT. The stimulation of OVLT primarily leads to increasing production of prostanoids, particularly prostaglandin E2 (PGE2), which acts in the pre-optic nerve of the hypothalamus and accelerate the metabolic functions of various warm sensitive neurones whose activities lead to increase body temperature (Walter et al., 2016). Alternatively, a bioactive lipid compound known as ceramide,

performs a proapoptotic and a cell signaling role, by acting as a intrinsic messenger independent of PGE_2, to generate febrile response (Walter et al., 2016). In the case of bacteria entering the human body (bacteremia), the lipopolysaccharides component of the bacteria cell wall can also trigger minor production of PGE_2 from hepatic Kupffer cells to energize warm sensitive neurones. The production of PGE_2 causes elevation of the hypothalamic set point through the autonomic nervous system which leads to heat production and conservation. This mechanism is achieved through processes such as muscle contraction, shivering, release of hormones (epinephrine) or vasoconstriction. Generation of fever is also thought to occur by signaling via the Toll-like receptor flow, which may not be dependent of the NF-kappa B cytokine inter-connections.

There are other neural pathways that may account for cytokine productions involved in the maintenance of homeostasis rather than the induction of fever (Romanovsky, 2007).



Source: (Walter et al., 2016)

Figure 2-1: Mechanism proposed to generate fever

Sentinel cells are stimulated by exogenous pyrogens to produce endogenous pyrogens which activate production of fever in the pre-optic area (POA) of the hypothalamus by the ceramide and prostaglandin E2 (PGE_2). Kupffer cells in the liver also produce PGE_2 in reaction to lipopolysaccharide, which can again stimulate the POA through the nerve of the organum vasculosum.

There have been several arguments about the usefulness or otherwise of fever. Some studies have indicated that fever is beneficial in disease healing processes by enhancing the mobilization and activation of leukocytes and phagocytes to fight disease (Carol et al., 1997; Schaffner, 2006). Fever is known to decrease the endotoxin effect of bacterial LPS after infection, and this leads to increased proliferation of T cells, which play a crucial role in the healing process (Schaffner, 2006). The negative aspect of fever stems from the fact that whenever there is 1^{o}C rise in temperature, there is also a 13% rise in the basal metabolic rate, oxygen consumption and increased energy requirements, which can be detrimental to the well-being of the affected individuals (Farthing et al., 1994).

Fever is a consequence of pathogenesis of most illnesses or a manifestation of illness and continuous to be a fundamentally important characteristic of both new and emerging diseases, whether from infectious or non-infectious sources. It is very crucial for clinicians and research scientists alike to continue to expand and harness their knowledge in the understanding of the febrile response to infections in order to improve on prevention, management and diagnosis of all diseases characterized by fever.

2.3 Diseases associated with acute febrile illness

2.3.1 Urinary tract infection

Urinary Tract Infection (UTI) refers to the microbial invasion of the urinary tract, which is supposed to be naturally sterile (Zorc, Kiddoo, & Shaw, 2005). UTIs are among the most common human diseases among adults and children, both at the hospital and community levels. The etiological agents of UTI generally infect the cells of the urinary tract through attachment and subsequent spread from the urethra to the

bladder or kidney and eventually through the bloodstream to cause bacteremia and other renal complications (Kaper et al., 2004).

Although fungi, viruses and other organisms can infect the urinary tract, UTIs are caused predominantly by bacteria (Zorc et al., 2005). Gram-negative bacteria are the most common type of bacteria implicated and these include *Escherichia coli (E. coli), Klebsiella, Proteus* and *Pseudomonas species.* Some other aerobic Gram-negative bacteria, belonging to the family *Enterobacteriaceae*, such as *Citrobacter and Salmonella* are reported to cause UTI. However *E. coli* are the predominant bacteria, accounting for 70 to 90 % of all UTI cases in both children and adults (Riccabona, 2003; Schalger, 2001).

Despite that *E. coli* is a major cause of UTI, other Gram-positive bacteria with hemolytic properties such as *Streptococcus saprophyticus* and *Staphylococcus aureus* are gaining significance (Lutters & Vogt, 2000; Ugbogu et al., 2010). Acute pyelonephritis, which is an advanced form of urinary tract infection due to bacteria colonization of the urethra and the kidney, has generally associated been with fever (Bass et al., 2003). Pyelonephritis occurs in the upper urinary tract and that includes the urethra, collecting system and the renal parenchyma. UTI due to fungal infection is rarely reported. Only few cases have been diagnosed in immune-compromised patients and among those who use invasive devices such as intra venous (IVs) devices and catheter for longer period of time (Vasudevan, 2014).

The prevalence of UTIs may vary depending on sex, age, race, nutritional and circumcision status. Females are known to be more predisposed to UTIs generally as compared to males, except in early childhood (Akinkugbe et al., 1973). This is primarily due to physiological factors such as the shorter length of the females' urethra

and its proximity to the excreta passage, where infecting pathogens can easily infect and colonize the urinary tract. Sexual intercourse, pregnancy and childbirth are other factors that contribute to the increased cases of UTI in females (Geerlings et al., 2000). It is estimated that more than 50% of women in their reproductive age are most likely to experience a UTI in their lifetime (Foxman et al., 2000; Geerlings et al., 2000).

It is reported that boys in their first three months of life have a higher UTI prevalence (3%) as compared to girls (1%) of the same age bracket (Riccabona, 2003). However, women at their puberty stage stand a higher risk of contracting UTI (5%) but the prevalence remains the same among males (3%) of similar age group (Hooton et al., 2000). Another research has indicated between 10-12-fold increase in risk of UTI among uncircumcised men, which could be as a results of bacteria colonization of the mucosal surface of the foreskin (Wiswell, 2000). Higher prevalence of UTI among white children particularly among white girls has been reported than blacks of the same age (Shaw et al., 1998). This racial difference can be explained on the basis of genetic dissimilarities in production of carbohydrates containing compound such as mannose that prevent the adherence of bacteria to the walls of urinary tract; most white women have been shown to lack the genes needed to produce such carbohydrates (Jantausch et al., 1994).

Malnourished people are more prone to UTI showing higher prevalence of between 8-35% compared to well-fed children (Bagga et al., 2003). The seriousness of malnourishment has a relationship with the level of bacteriuria (presence of bacteria in urine) and this is belief to be due to a weakened immunity, usually overwhelmed by infectious agents (Bagga et al., 2003). In Ghana, close to 8% of pregnant women who

attend antenatal clinics are reported to have bacteremia with *E. coli* being the most dominant bacterial isolated (Turpin et al., 2007).

To diagnose UTI, a couple of clinical signs and the presence of uropathogens in urine are considered. A test is considered positive for UTI when there are more than 100,000 bacteria colony forming units per milliliter of urine culture (Mariani et al., 1989; Medina-Bombar et al., 2003). Accurate diagnosis and treatment of UTIs are important to reduce its associated pain and to prevent prolonged and unnecessary use of antibiotics (Akram, Shahid, & Khan, 2007). Treatment of UTI is best guided by the results of culture and antimicrobial susceptibility tests. The management of UTI has been based on the commonly used antibiotics including cefuroxime, amoxicillin/clavulanic acid, trimethoprim/sulphurmethoxazole and fluoroquinolones (Franco, 2005). In recent times however, treatment of UTI has become difficult due to the emergence of resistant bacterial strains such as *E.coli* to the commonly used antibacterial drugs (Newman et al., 2011). Resistance to antimicrobial agents has significant public health implications and contributes to rising cost of UTI treatment in most countries in sub-Saharan Africa.

2.3.2 Gastroenteritis

Gastroenteritis (GE) refers to inflammation of the stomach and intestinal mucosa. It is mostly characterized by the onset of acute diarrhea with or without fever, vomiting, abdominal cramps and dehydration (Johnston et al., 2010). Acute diarrhea resulting from GE is an important disease in terms of mortality. The mortality is mostly common among children less than 5 years of age, particularly those below age of two years whose immature immune systems make them susceptible (Perkins et al., 2011; WHO, 2018). Gastroenteritis can be caused by parasites, viruses or bacteria. Parasitic causes

of GE account for about 1% to 8% of all cases of diarrhea among pediatric patients (Navaneethan & Giannella, 2008). Parasites such as *Giardia*, *Cryptosporidium* and *Entamoeba histolytica* are well known etiological agents of GE (Navaneethan & Giannella, 2008). The most frequently identified viral pathogens implicated in GE in Ghana include norovirus, sapovirus, rotavirus, adenoviruses and astrovirus (Tam et al., 2012). Viral GE is very common, and it is transmitted by the fecal-oral route or through ingestion of contaminated drinking water or food. Close to 50% of patients with GE presenting with fever and headaches may be due to rotavirus infections (Jones & Rubin, 2009). Besides viruses, bacteria are the second most common cause of GE in pediatric patients in both developing and developed countries, accounting for 2% to 10% of reported cases (Dennehy, 2005). The most common bacteria include *Salmonella*, *Shigella*, and *Campylobacter*. Other notable bacteria include enterohemorrhagic *E. coli* (EHEC) and *Clostridium difficile*, although these bacteria tend to be uncommon in the pediatric population (Dennehy, 2005). With proper management, the prognosis for bacterial gastroenteritis tends to be very good, especially in developed countries. Nevertheless, viral GE may be more common than is currently recognized since healthy carriers have been shown to excrete 105 to 1011 virus particles per gram of stool (Manatsathit et al., 2002).

2.3.3 Meningitis

Meningitis is a highly contagious and fatal illness characterized by inflammation of the meninges which are protective membranes that covers the brain and spinal cord. It manifests as an acute form of illness, giving rise to complex symptoms including severe headache, sudden onset of fever, stiff neck, tenderness of the back, permanent damage to the brain and can result in mobility disorders (Ministry of Health, 2010a; Welch & Nadel, 2003). The severity of the disease is dependent on the causative

pathogen. Bacteria, viruses, parasites, amoeba and fungi are known causative agents of meningitis (CDC, 2012).

Although viral meningitis has been reported, meningitis due to bacterial infections is the most severe and responsible for significant morbidity and lifelong disabilities worldwide (CDC, 2012; Scheld et al., 2002). The highest incidence of the disease occurs in sub Saharan Africa where the disease is frequently reported with outbreaks in crowded populations (Brooks et al., 2006; Harrison, 2000; Scheld et al., 2002). Most of the cases in Africa are restricted to the "Meningitis belt" which comprises 22 countries from Senegal to West of Ethiopia and covers the northern part of Ghana. The 3 most common etiologic agent of bacterial meningitis are *Streptococcus pneumoniae* , *Hemophilus influenzae* type b (Hib) and *Neisseria meningitidis*, which account for over 90% of reported cases of all bacterial meningitis cases among young children (CDC, 2012; Peltola, 2000). Among these bacteria, *S. pneumonia* has been most implicated in the cases, with the disease burden being high in children aged 4–18 months and causing a fatality rate of about 25% (Trotter et al., 2005). Reduction in cases of pneumococcal infection in recent times could be as a result of introduction of the pneumococcal vaccines. Several pneumococcal conjugate vaccines are available for the prevention of the disease, but choice of the conjugate vaccine depends on the type of *S. pneumoniae* strains circulating in a geographical area (Nigrovic et al., 2008).

Treatment is mostly by use of antibiotics usually orally or parenterally, depending on the disease severity. Penicillin is often used together with second and third generation cephalosporin and levofloxacine (Twum-Danso et al., 2003; Welch & Nadel, 2003). A study in Saudi Arabia revealed that 43% percent of the isolates from patients with invasive pneumococcal infections were resistant to penicillin although 76% were

sensitive to ceftriaxone (Twum-Danso et al., 2003). Cerebro-spinal meningitis (CSM) caused by *N. meningitides* is the second most common form of bacterial meningitis with a reported fatality rate between 3-30% (Feigin & Pearlman, 2004). The epidemiologic landscape of meningococcal disease is always changing due to increasing cross-border migrations and international travels. While serogroup A is common in Africa and Asia, and serogroups B is predominantly found in North America and Europe, the serotype C is global in distribution (Anderson, Glode, & Smith, 2004; Jelfs & Munro, 2003). In many countries, the proportion of the meningitis caused by serogroup Y has increased tremendously over the past decade, where it now accounts for about one third of meningococcal reported cases (Brooks et al., 2006; Rosenstein et al., 1999). The serogroup W-135 is common in the Middle East and parts of Africa where large epidemics have been reported (Taha et al., 2000). The coming into being of serogroup W-135 meningococcal disease among Hajj pilgrimage has resulted in vaccine recommendations for pilgrims being changed to the quadrivalent meningococcal polysaccharide vaccine (A, C, W-135, and Y) since 2002 (Al-Mazrou et al., 2004). This type of vaccine is said to be effective against all meningococcal diseases (Ministry of Health, Ghana 2010c; Trotter et al., 2005).

The *Hemophilus influenza* type b (Hib) strain is also a major cause of meningitis in children and infants (Resman et al., 2011). All diseases resulting from *Hemophilus* type b infections are spread by exhaled droplets, containing the bacteria which invade the bloodstream or may spread to the meninges to cause meningitis. Although the introduction of Hib conjugate pentavalent vaccines has resulted in decreasing incidence of Hib infections amongst children, some cases of *Hemophilus* infections are still being reported. A study in Ghana by Nielsen et al (2012) indicated that infections caused by *H. influenza* was about 0.4% which is consistent with a Nigerian study where the

investigators recorded 0.5% prevalence rate for *H. influenzae* as the cause of meningitis in children (Kumanda, 1983; Nielsen et al., 2012). High frequency of 19% *H. influenza* was associated with meningitis in Gambian children (Howie et al., 2013). Viruses implicated in meningitis infections include *mumps*, which can spread through mosquitoes and bites of certain insects. Fungal meningitis caused by *Cryptococcus* and *Candida* is rare but has been reported in humans with predisposing factors such as HIV/AIDS infections, leukemia, or people suffering from immunosuppression.

2.3.4 Malaria

Human malaria is a disease caused by a protozoan parasite of the genus *Plasmodium* which spreads through the bite of the female anopheles mosquitoes. Several species of the *Plasmodium* parasite cause the disease, but *Plasmodium falciparum* (Pf), *Plasmodium malariae* (Pm), *Plasmodium vivax* (Pv), *Plasmodium ovale* (Po) and recently *Plasmodium knowlesi* (Pk) are known to infect humans (Cox-Singh et al., 2008). The parasite lives and develops in human hepatocytes and erythrocytes as obligate parasites. *P. vivax*, *P. falciparum* and *P. ovale* invade reticulocytes (immature erythrocytes) preferentially, but *P. falciparum* can invade erythrocytes of all ages. Nearly 80% of the global cases of malaria-associated death occur in sub-Saharan Africa and are mostly caused by *P. falciparum* with pregnant women and children under-5 years of age being the most affected (WHO, 2016).

As a leading cause of morbidity and mortality in Ghana, malaria is responsible for over a third of all outpatient visits each year (Ministry of Health, 2010b). Low socio-economic status, poor housing and not sleeping under insecticide-treated mosquito nets are some of the risk factors for malaria transmission (Bisoffi Z, 2013; Kenneson et al., 2017). The clinical manifestation of malaria is not very specific and varies greatly such that it

can easily be misdiagnosed with other febrile diseases. Nevertheless, most patients presenting with fever to health facilities in Africa are still presumptively diagnosed as malaria. The initial symptoms for uncomplicated malaria are usually nonspecific and include fever, headaches, chills, joint pains, nausea and vomiting. Severe or complicated malaria may present as cerebral malaria which manifest as coma, severe anemia, hypoglycemia, acute renal failure, acute pulmonary edema or metabolic acidosis (WHO, 2000). It is estimated that close to 2% of patients who recover from cerebral malaria may experience epilepsy as a result of brain damage (WHO/UNICEF, 2003).

In endemic countries, the case management of malaria is often based on clinical judgment otherwise called presumptive diagnosis (Chandler et al., 2008). This results in a high probability of false positive diagnosis leading to the over-diagnosis and over-treatment of malaria in the management of febrile illness (Chandramohan et al., 2002; Nankabirwa et al., 2009). The current treatment for malaria as defined by the national policy guidelines in most endemic countries is the artemisinin combination therapy (ACT). The drugs combination routinely used include artemether lumefantrine (AL) and artesunate-amodiaquine (ASAQ) (Webster, 2014). Laboratory confirmation of malaria is normally through a demonstration of malaria parasites in blood films using light microscopy, but RDT or PCR are also used where available (Hamer et al., 2007). Despite the change of malaria guidelines encouraging parasitological diagnosis, studies have shown that overall malaria testing rates in health facilities are still low in many African countries (Chandler et al., 2008; Zurovac et al., 2006).

2.3.5 Acute respiratory tract infection

Acute respiratory tract infections (ARTI) are those infections that affect the respiratory tract and are classified into lower and upper respiratory tract infections. ARTIs are usually caused by a number of bacterial and viral agents, but in a few instances by fungi, mostly in immunocompromised individuals (Low & Rotstein, 2011). Nearly 45%-60% of the lower respiratory tract infections are caused by viruses (Yun et al., 1995). The upper respiratory tract diseases include otitis media, common cold, pharyngitis and sinusitis and lower respiratory tract infections include diseases such as bronchiolitis, laryngitis, tracheitis, and pneumonia. Bronchiolitis and pneumonia are major causes of childhood ARTI deaths through sustained progressive respiratory failure as a result of inflammation of the lungs (Walker et al., 2013). Common viral etiologies of ARTIs include influenza virus (serotypes A and B), respiratory syncytial virus, parainfluenza virus, rhinovirus, coronaviruses, adenovirus, and human bocavirus (García et al., 2010).

The commonest bacteria responsible for ARTIs are *Hemophilus influenza, Streptococcus pneumoniae and Mycoplasma pneumoniae* but *S. pneumoniae* is known to be a major cause of lower respiratory tract infection in infants less than 5 years in the developing countries (Klugman et al., 2003). ARTIs case-management is incorporated into integrated management of infant and childhood illness (IMCI) strategic plans and success of treatment depends on the prompt referral to hospitals and correct management with oxygen or antibiotics. The current recommendation for treating ARTI is the use of co-trimoxazole and intramuscular penicillin or chloramphenicol, depending on age. Management includes administering oxygen, especially those with signs and symptoms of severe pneumonia. Vaccination with a nine-valent pneumococcal conjugate vaccine has been demonstrated to reduce the incidence of

virus-associated pneumonia responsible for most childhood hospitalization (Klugman et al., 2003).

2.3.6 Q fever

Q fever is a systemic infection caused by a bacterium known as *Coxiella burnetti*. It is usually transmitted from infected animals to humans through direct contact or inhalation of a spore-like form of the bacteria. The disease has a rapid onset of fever following an incubation period of between 1 to 60 days. Q fever infections are usually self-limiting within a few days after infection, but chronic forms of disease may persist from months to years (Brouqui et al., 2007; Sow et al., 2016).

Laboratory diagnosis is essentially to establish the presence of the pathogen by culture in blood, but serology and PCR tests have only recently been included in routine diagnosis of the disease. Q fever is managed by treatment with doxycycline (200 mg/day) for two weeks but chronic form of Q fever usually requires treatment with hydroxychloroquine and doxycycline for at least 18 months period (Dijkstra et al., 2012; Million et al., 2010). Prevention of this zoonotic disease relies on occupational advice and veterinary measures including animal vaccination in conjunction with vaccination or chemoprophylaxis in humans (Ruiz & Wolfe, 2014).

2.3.7 Rickettsiosis

Rickettsiosis refers to a group of illnesses caused by a variety of bacteria including those responsible for typhus fever caused by *Rickettsia prowazekii*, *Rickettsia* Typhi and scrub typhus caused by deadly *Orientia tsutsugamushi* (Paris et al., 2012). One of the well-known causes of Rickettsia disease *is Rickettsia felis* which is increasingly recognized as an emerging cause of febrile illness in Africa. *Rickettsia felis* is a Gram-negative bacterium that belongs to the spotted fever group (SFG) of *Rickettsia species*

(Botelho-Nevers & Raoult, 2007). In humans, these intracellular bacteria infect endothelial cells and cause acute onset of fever with skin manifestations such as rashes at the site of the arthropod bite.

Rickettsia conorii belongs to the SFG of Rickettsia that causes Mediterranean Spotted Fever (MSF) in sub-Saharan Africa and Mediterranean countries. *R. conorii* spreads through the bite of brown dog tick, *Rhipicephalus sanguineus* which is the main reservoir for the bacteria as evidenced by transtadial and transovarial transmission co feeding behavior (Brouqui et al., 2007; Parola, Paddock, & Raoult, 2005). However, the role of domesticated dogs in maintaining this zoonotic transmission remains unknown even though dogs are the natural hosts of the vector (Levin et al., 2014).

Clinical diagnosis is clearly made in the early phases of the disease, especially when skin manifestations are prominent. Laboratory diagnosis is usually restricted to serology tests that are only available in specialist laboratories but suffer from cross-reactivity problems (Brown & Macaluso, 2016). There are no well-established rapid diagnostic tests currently available and the use of the Weil-Felix serology test is no longer recommended due to poor sensitivity and specificity (Kularatne et al., 2005). Rickettsial infections are usually treated with doxycycline given for 7-10 days depending on the species involved. Prevention relies on avoidance of insect bite, vector control and chemoprophylaxis with doxycycline.

2.3.8 Leptospirosis

Leptospirosis is a systemic infection caused by *Leptospira spirochaetes* which are spiral shaped and obligate bacteria that share common features of both Gram-negative and Gram-positive bacteria. The bacterium is highly motile and transmitted from infected animals through direct or indirect contact of contaminated urine or fresh water

or wet soil, with the skin or mucous membranes. Leptospirosis is said to be a widespread zoonotic disease and is more common in developing countries but associated with certain high-risk activities such as farming and water sports (Nally et al., 2001). It is known that nearly all mammals can become infected, and rodents mainly act as carriers due to chronic infection of their proximal renal tubules.

Leptospirosis has many manifestations which mimic the clinical presentations of many other diseases. The disease manifest after an incubation period ranging from 2 to 26 days with abrupt onset of fever and non-specific clinical features such as headache, myalgia and muscle tenderness in the lower trunk and legs. A substantial number of people who are infected with *Leptospira* may have very mild or subclinical symptoms and may not seek medical attention immediately (Ashford et al., 2000; Charon et al., 2002).

An investigation into the outbreak of Leptospirosis in Nicaragua reported 29·4% (25 out of 85) seropositive individuals diagnosed with febrile illness (Ashford et al., 2000). In a community survey in the Seychelles, 9% of the participants tested positive as evidence of recent infection and 37% showed evidence of previous infection, but no participant reported of current symptoms of Leptospirosis (Ashford et al., 2000).

The diagnosis of Leptospirosis can occasionally be made by dark-field microscopy (DFM) or special cultures of urine or blood (Thaipadungpanit et al., 2011). Serology is the main test used, but these are complicated due to the large number of different serovars and are usually negative at initial presentation (Limmathurotsakul et al., 2012). Diagnostic PCR tests have been developed, but are likely to be unavailable in many developing countries (Thaipadungpanit et al., 2011).

2.3.9 Dengue

Dengue is a systemic viral disease caused by dengue virus and commonly characterized by acute fever. The virus belongs to the family *Flaviviridae* and genus Flavivirus and is transmitted by bites of *Aedes* mosquitoes that usually breed in urban areas (van den Hurk et al., 2012). There are four serotypes of dengue virus (DENV-1, DENV-2, DENV-3 and DENV-4) and infection with a particular serotype may not provide lifelong immunity to the other infecting serotypes. The disease is said to be the most common arbovirus infection worldwide and accounts for 3%-11% of febrile illnesses in most endemic countries (Poloni et al., 2010). The disease remains under-recognized and under-reported and found in most tropical countries with the potential to cause outbreaks and spread quickly where *Aedes* mosquitoes are found (Amarasinghe et al., 2011). The past few decades have seen about 30-fold increase in incidence of dengue infections, expanding from country to country and from urban to rural setting (Jentes et al., 2010). The worst affected continents are South and North America, Asia and parts of Africa. Dengue infections annually are estimated at 50 million people with about 2.5 billion people estimated to be living in dengue transmitting countries (Samir Bhatt et al., 2013; Rackoff et al., 2013). Dengue is often transmitted extensively when there is a spread of the vector to new geographical locations coupled with increased international travel, unplanned urbanization and rapid population growth (Stoler et al., 2014). In Ghana, the vector which transmits the disease has been around for over a century (Patterson, 1979) and has persisted in many mosquito surveys (Appawu et al., 2006; Opoku et al., 2007). The first dengue case to be reported in Ghana was the isolation of DENV 2 in Finnish travelers who had visited Ghana in 2000-2005 (Huhtamo et al., 2008b). Several seroprevalence studies in west Africa (Amarasinghe et al., 2011;

Collenberg et al., 2006; Jentes et al., 2010) and other parts of Africa (Vairo et al., 2012) have all confirmed the transmission of the disease.

The dengue virus mostly infects mononuclear phagocytes and results in sub-clinical illness, after an incubation period of 3-14 days. The disease may present with an acute onset of classic dengue fever (DF) which is defined as the presence of fever and other symptoms such as headache, arthralgia, myalgia, skin rash, mild bleeding and reduced white cell counts. The disease may also present as Dengue hemorrhagic fever (DHF) which is characterized by fever, thrombocytopenia, and bleeding or plasma leakage. One may experience dengue shock syndrome (DSS), which may comprise of all DHF symptoms in addition to renal and circulatory failure. All the Dengue types usually present with an undifferentiated febrile illness with symptoms of nausea, headache, severe body pain, myalgia, and vomiting. Both DHF and DSS mostly occur in patients who have previously been infected by another dengue virus serotype and are thought to be associated with a process of "antibody-dependent immune enhancement" that leads to more serious illnesses (Halstead & Deen, 2002).

The standard treatment for patients with suspected dengue is supportive care consisting of oral-rehydration therapy, bed rest, paracetamol and avoidance of aspirin. The rapid spread of dengue virus has therefore renewed global interest in the development of vaccines and therapeutic drugs. At the moment, there is no vaccine to prevent dengue infection. The only means of dengue prevention is through the control of its vector which is said to be both expensive and ineffective (Halstead & Deen, 2002).

A laboratory confirmation of dengue virus infection can be made by isolation of the virus in cell culture, viral nucleic acid detection using PCR, identification of antigen

(NS1) using Enzyme-Linked Immunosorbent Assay (ELISA), and serologic assay for the detection of virus specific antibody.

2.3.10 Bacteremia

Bacteremia is the invasion and rapid circulation of bacteria through the vascular or circulatory system. It is characterized by fever, chills and general malaise (Berkley et al., 2005). A wide range of microorganisms are known to be responsible for bacteremic infections. These organisms however differ from one locality to the other, depending on antimicrobial susceptibility patterns (Meremikwu et al., 2005). Significant differences exist in the causative organisms of bacteremia in different age groups, with Gram-positive organisms being commonly isolated bacteria in neonates. Group B *Streptococcus* is one of the commonest causes of neonatal bacteremia (Were, Davenport, Hittner, et al., 2011). In adults and children however, non-typhoidal *Salmonellae* and *S. aureus* are the predominant etiological agents of bacteremia

(Nielsen et al., 2012).

The infection may be defined clinically and confirmed with positive blood cultures. Culturing of cerebrospinal fluid and urine may also be used when localized infections like meningitis and UTIs are suspected. Bacteremia account for nearly 12 million of childhood deaths in developing countries and most children lose their lives before their fifth birthday (Brent et al., 2006). Although, *S. aureus* may be a normal commensal that colonizes the mouth, nose, skin and other parts of the body of healthy individuals (Del Rio et al., 2009), it is able to enter the bloodstream or the adjourning tissues through a breach of the skin or mucosal barriers to cause bacteremia (Hakeem et al., 2013). The virulence factors of these bacteria, together with the host defense mechanisms, play essential roles in determining whether the infection will spread or be contained. *S.*

aureus is a major cause of bacteremia and it is associated with high morbidity and mortality because the bacteria are difficult to treat (Naber, 2009). In Uganda, *S. aureus* accounted for 60% of the commonly isolated bloodstream pathogens in febrile children (Kizito et al., 2007). This agreed with studies conducted by Meremikwu (2005) and Awoniyi et al. (2009) in Nigeria that also reported *S. aureus* as a major organism isolated from blood representing 48.7% and 28% respectively of the bacteria isolated (Awoniyi et al., 2009; Meremikwu et al., 2005).

A study in Ghana also revealed *S. aureus* as one of the major etiologic agents of bacteremia among children (Evans et al., 2004). In Mozambique, *S. aureus* was prevalent among neonates diagnosed with bacteremia (Sigauque et al., 2009; Were et al., 2011). Nevertheless, in contrast to other findings across Africa, Reddy et al (2010) argued that, bacteremia caused by S. aureus had been overestimated over the years by some laboratories that had often misidentified Coagulase negative *staphylococci* as *S. aureus* (Reddy et al., 2010).

Non-typhoidal Salmonellae (NTS) are known to cause severe bacteremia especially in children with fatal consequences (Kariuki et al., 2006). In a cross survey, nearly 60 % of bacteremic cases diagnosed among children were caused by invasive NTS (Reddy et al., 2010). Human salmonellosis including typhoid fever due to serovars Typhi and paratyphi A, B, and C are reported to be more common in developing countries where sanitary conditions are very poor (Evans et al., 2004). Typhoid fever as the cause of bacteremia affects people of all age groups, but children are most prone to the disease probably because they possess immature gut lymphoid tissues with reduced gastric acidity (Evans et al., 2004). In a study conducted in Ghana, as high as 40.7% of

Salmonella isolates from febrile patients were due to *Salmonella* Typhi (Groß et al., 2011).

Despite the availability of new antimicrobials, many healthcare practitioners are faced with challenges in treating bacteremia. Multi-drug resistance to most first line drugs have been observed in Ghana (Groß et al., 2011; Ministry of Health, 2010b). In recent times, fluoroquinolones and third generation cephalosporins are the only options for the treatment of bacteremia due to enteric bacteria.

2.4 Epidemiology of AFI

Acute febrile illness (AFI) remains a common syndrome among children seeking treatment in resource-limited settings (Feikin et al., 2011; Lubell, 2011). Although there has been a significant decline in the mortality rate of malaria among children in the past two decades, other febrile illnesses especially bacteremia and respiratory infections are responsible for over 1.6 million deaths every year and account for about half of total global childhood disease burden (Liu et al., 2012). Given the limited access to diagnostic tools in resource-limited settings, the current estimates of specific etiologies of AFIs may not correctly describe overall trends and patterns. The etiologies of infectious diseases are fast changing due to globalization, increased human travel and revamped animal industries (Stoler et al., 2015). For instance, dengue virus has spread rapidly in recent times throughout the whole world with nearly half the world's population being at high risk of getting dengue virus infection (Poloni et al., 2010). Recent reports of dengue outbreaks in Angola, Somali, Burkina Faso and Kenya all provide evidence of serious local transmission of the virus in sub-Saharan Africa (Caron, 2013; Tarnagda et al., 2014).

In Bangladesh, AFIs was more closely associated with typhoid fever in preschool children than older persons (Naheed et al., 2010). In southern Asia, typhoid and paratyphoid fever as well as dengue were the most commonly pathogens detected with the prevalence range of 23% to 25% among febrile patients (Owais et al., 2010; Thompson et al., 2015). The causes of fever in rural Southern Laos in patients showed that 52% had evidence of more than one diagnosis of a specific infection (Mayxay et al., 2013) where dengue was the most common detected pathogen (30.1%), with leptospiral (7.0%), Japanese encephalitis virus (3.5%), scrub typhus (2.6%), spotted fever group *rickettsia* (0.9%), murine typhus fever (0.4%) and unspecified flavivirus infection (0.9%). The study confirmed the presence of rickettsial and leptospiral infections as important diseases and treatable causes of AFIs for the first time in rural Laos and recommended intensification of surveillance.

Tomashek *et al* carried out a cohort study of AFI in Puerto Rico using diverse methods and detected chikungunya virus, influenza A or B virus, dengue and other respiratory viruses (Tomashek et al., 2017). Unlike the AFI study in Puerto Rico, there was no evidence of *Rickettsia* and *Coxiella* among the study population. Another AFI study in Cambodia (Chheng et al.,2013; Chheng et al., 2009) identified dengue virus (16.2%), Japanese encephalitis virus (5.8%) and scrub typhus (7.8%) in a survey of febrile patients. Six percent (6.0%) of febrile episodes had culture-proven bloodstream infection, including *Salmonella enterica serovar* Typhi, *S. pneumoniae*, *E. coli*, *H. influenzae*, *S. aureus* and *Burkholderia pseudomallei*. A similar study conducted by Kasper (2012) in south-central Cambodia found 1% of febrile patients were co-infected with two or more pathogens including dengue and influenza virus with malaria (Kasper et al., 2012). This phenomenon of co-incidence of both invasive bacterial especially

Salmonella enterica Typhi, *Plasmodium* and dengue virus indicates the complex nature of febrile illness etiology in various populations.

2.5 Febrile illness in the Caribbean and Latin America Region

Several infectious pathogens have been implicated in the etiology of febrile illness in the Latin America and the Caribbean region. Dengue and leptospirosis are considered the most predominant fever associated diseases in most health facilities, although it is very challenging to distinguish between the two diseases (Forshey et al., 2010). On many occasions, post-mortem results from patients who died from suspected dengue-related fatality revealed that most of them normally had Leptospirosis infection as well (Bruce et al., 2005). In addition, Venezuelan equine encephalitis virus (VEEV) is less frequently reported but it is difficult to differentiate it from dengue, although co-infection occurs in most cases. This was evidenced in a prospective study in Venezuela, where 55.2% of the samples were serologically positive for VEEV and dengue (Valero et al., 2001), suggesting that the disease might have been caused by both pathogens.

In the Amazon region of Ecuador, *Rickettsia, Leptospira, Coxiella,* and *Brucella* spp were the major pathogens detected among undifferentiated febrile patients using a combination of diagnostic tests (Manock et al., 2009). In the state of São Paulo, Terzian et al., used RT-PCR to identify arboviruses among febrile illness patients and found 71% to be dengue-infected with 1.5% of the patients also positive for Saint Louis encephalitis virus (SLEV) which was least expected (Terzian et al., 2011). Vasconcelos et al. (2009) investigated Oropouche fever (OROV) in the Brazilian Amazon and found that 64% of the patients' samples were positive for the OROV. Oropouche fever infection is a common arboviral infection in Brazil affecting about half a million people every year during the rainy season with sporadic occurrences in dry seasons

(Vasconcelos et al., 2009). Among febrile patients diagnosed with meningo-encephalitis in the state of Amazonas, Bastos *et al*. (2012) detected the presence of OROV in 3% of cerebrospinal fluid samples evaluated, showing evidence of co-infections (Bastos et al., 2012).

2.6 Febrile illness in sub-Sahara Africa/WHO African Region

Historically malaria has been a major contributor to overall AFI in the African region. The use of serology, microbial cultures and rapid diagnostics for malaria in recent times, have led to the recognition of non-malarial causes of fever. Studies in Africa that used a combination of microbiologic and molecular methods have identified different etiological agents in varied ranges as follows: malaria (1.3% to 64.4%), *Coxiella* (0.1% to 2.6 %,) and *Brucella* spp (0.5 to 2.0%), adenovirus (1.4% to 28.8%), human herpesvirus 6 (1.2%-7.9%), parvovirus B19 (0.1 to 1.3%) (Crump et al., 2013; D'Acremont et al., 2014). The outpatient pediatric study conducted in Tanzania detected a variety of respiratory viruses with adenovirus contributing up to 30% of pathogens identified, and this accounted for more than 50% of the fever recorded (D'Acremont et al., 2014). Fungal etiologies of bloodstream infections among febrile adults in a prospective study in northern Tanzania found *Cryptococcus neoformans* (9%) and *Histoplasma capsulatum* (1.0%) among the febrile patients (Crump et al., 2011).

In many areas of West Africa, people are at risk of acquiring tick-borne relapsing fever through the bite of fast-feeding ticks, spirochete, *Borrelia crocidurae* (Nordstrand et al., 2007). There is lack of information regarding the prevalence of tick-borne relapsing fever among humans but spirochetes are known to circulate in small mammals such as rodents and ticks that live among people. The potential for people to

acquire relapsing fever is not known even though a few human cases have been reported. Some studies have provided clinical and field evidence of spirochetes being detected in the blood of acutely ill patients (Million et al., 2009; Nordstrand et al., 2007). However, one study in Mali identified spirochetes in 11.3% of 726 rodents and 2.2% of 278 ticks, giving an evidence of widespread occurrence of relapsing fever spirochetes as potential human public health problem(Schwan et al., 2012).

Lassa virus (LASV) has long been reported in West Africa including Mali, Côte d'Ivoire and in Ghana recently. LASV is maintained in nature in rodent hosts, specifically, the multimammate rat known as *Mastomys natalensis* which is said to be common several communities in West Africa.

It is estimated that 300,000 LASV infections occur in West Africa, resulting in about 5,000 deaths each year (Ogbu, Ajuluchukwu, & Uneke, 2007). Human acquire the infection through contact with infectious rodents or ingestion or inhalation of virus-laden particles. Person-to-person transmission is also well documented and can result in outbreaks, especially in nosocomial environment, leading to mortality rates in >50% during infections (Fisher-Hoch et al., 1995). In a prospective case-control study in Nigeria, Lassa virus was identified as an important cause of fever, although malaria parasitemia was found to be relatively high in the entire cohort (Akhuemokhan et al., 2017). In Egypt, a hospital-based surveillance for acute febrile illness with emphasis on community-acquired bloodstream infection, reported positive cultures for *Salmonella enterica* serogroup Typhi (5%), and *Brucella* (3%) with an additional 2% accounting for other cultivable bacteria (Afifi et al., 2005). Recently, Hogan and colleagues (2018) in a case-control study determined the burden of malaria co-infections and their association with parasite densities. The overall results showed that 51% of febrile

Ghanaian children with malaria parasitemia were co-infected with one or more other pathogens (Hogan et al., 2018a).

In a town of Kedougou in south-eastern Senegal, Sow et al (2016) investigated the concurrent infection of malaria with arbovirus in patients presenting with AFI (Sow et al., 2016b). The results also showed different infections including malaria, yellow fever, and Zika, chikungunya and dengue virus. Among the virus-infected individuals, 48.7 % were co-infected with malaria parasites. Zika virus was the most common arbovirus in the co-infections with malaria (88.9 %) and elevated body temperature (above 40 °C) was significantly associated with those showing dual infection of malaria parasites and arbovirus compared to those patients with mono-infection. A study in western Kenya, found prevalence of bacteremia with *P. falciparum* to be 11.7%, with non-typhoidal *Salmonella* spp. as the most common isolates (Were et al., 2011). The other pathogens isolated were *Salmonella enterica* serovar *Typhimurium, Salmonella typhimurium*, *Salmonella enteritidis* and *Salmonella arizonae*. These results further underscore the importance of differential diagnosis of AFI in Africa.

2.7 Management of AFI

Management of febrile illness varies due to the diverse nature of febrile diseases and their context-specific characteristics. This has led to the adaptation of different management strategies and treatment regimens. A few policy documents guidance for the management of acute fever has been formulated (WHO/UNICEF, 2003). These include the Integrated Management of Childhood Illness (IMCI) initiative, instituted by the WHO with the primary objective of reducing childhood mortality, especially for children less than 5 years of age.

The IMCI guidelines rely on management of disease, based on simplified clinical algorithms, with or without laboratory test confirmation, and endorse empirical treatment of fever. Most countries including Ghana have adopted the IMCI approach to manage diseases associated with childhood febrile illness, including malaria, ARTI, urinary tract infection and sepsis. Depending on health challenges and specific needs of a particular country, several adaptations and modifications have been made to the generic IMCI guidelines issued by the United Nations Children's Fund (UNICEF) and WHO. This includes guidelines for management of human immunodeficiency virus (HIV), diarrhea, malaria, pneumonia and other febrile illnesses. The principles of the IMCI guidelines are incorporated into pre-service curricula and training programs and the approach is implemented in all healthcare facilities in IMCI implementing countries.

IMCI initially recommended that all fever presenting at a healthcare facility be treated as malaria in malaria-endemic regions especially in Africa because of relatively weak health systems and limited diagnostic tools. IMCI guidelines are to ensure that severe febrile illnesses are identified through an evaluation of danger signs and managed accordingly. However, the IMCI clinical assessment algorithms have not been well implemented in many countries due to diagnostic challenges and poor referral systems (Bryce et al., 2004). Although increased access to rapid diagnostic tests has significantly improved, the diagnosis of malaria in endemic areas and the differential diagnoses of other childhood febrile diseases have not made much progress. The management of non-malaria febrile illness greatly depends on robust clear assessment and interpretation of clinical signs and symptoms and therefore relies on the opinion and skills of the attending clinician.

2.8 Diagnosis of AFI

Reliable laboratory techniques are often not available at the time of care for most febrile illnesses across the globe. Hence many clinicians make diagnosis based on presenting clinical symptoms and travel or medical history of patient (Berkley et al., 2005). Due to overlap between symptoms of febrile illness, algorithms have been developed for workers at the lower levels of healthcare where laboratory diagnosis is not available. This algorithm is said to increase the probability of diagnosing febrile illness particularly for malaria, which has been shown to be 88% sensitive as compared to presumptive diagnosis by pediatricians (Okebe et al., 2010). Laboratory confirmation of *Plasmodium* is carried out in blood films using light microscopy (Bates et al., 2004). The use of RDTs to detect the presence of antigens in the patient blood has become routine. Serological tests are also commonly employed to detect specific antibodies in the patient specimen. Antigen-based ELISA assay is also used to identify the causative organism in the diagnosis of febrile illness. Moreover, standard microbiological cultures of blood, feces, urine or bone marrow remain the gold standard for the identification of organisms in majority of AFIs cases.

2.9 Advanced diagnosis of AFI

In the past few decades, molecular typing techniques have greatly superseded phenotypic methods for laboratory diagnosis in terms of efficiency, sensitivity and specificity. The traditional phenotypic methods have not shown sufficient discriminatory power and ability to establish genetic relationships between isolates. Most frontline diagnostic laboratories across the world can now undertake standard identification and antimicrobial susceptibility testing for bacteria; coupled with a varying level of molecular-based detection of organisms in biological specimen which had lead to improved diagnosis of AFI associated pathogens.

2.9.1 Nucleic acids identification techniques

The use of Polymerase Chain Reaction (PCR) technique has radically improved the detection of microorganisms in biological specimens (Liesenfeld et al., 2014). PCR has enabled identification of fastidious microorganisms that would not ordinarily grow in the normal culture conditions (Fournier et al., 2014). Additionally, several multiplex PCR assays have been utilized to detect multiple pathogens simultaneously (Bouricha et al., 2014). The use of real-time-quantitative PCR assays has provided a further boost to molecular based pathogen identification in clinical samples.

Recently, the TaqMan Array Card (TAC) platform, developed by Life Technologies, has offered a spatial multiplexing of up to 384 pathogen targets in a single analysis. The United States Center for Disease Control (CDC) initiated the use of TAC for detection of 21 respiratory pathogens. The AFI-TAC array card is specially designed and uses a quantitative reverse transcription-PCR (qRT-PCR) platform for parallel detection of protozoa, bacteria, viruses and well suited for surveillance and clinical studies (Kodani et al., 2011; Liu et al., 2016).

2.9.2 Culture based-matrix-assisted laser desorption/ionization-time of flight mass spectrometry

Within the past few years, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has been introduced and approved for the rapid identification of varieties of bacteria, viruses and even fungi for both clinical and research purposes. MALDI-TOF MS can identify bacteria to species level within few minutes. This new technology is being introduced in the field of virology to identify and study viral genome mutations from clinical samples. Multiplex MALDI-TOF MS has been successfully utilized to detect human herpesviruses (HHVs) from a wide variety of clinical specimens (Sjöholm etal., 2008). Peng et al. used this technique to

diagnose mouth, hand and foot disease that is caused by enteroviruses including echovirus, poliovirus and coxsackievirus (Peng et al., 2009). The potential use of this new approach for the identification of novel host biomarkers for accurate and early diagnosis of bacteria septicemia was recently demonstrated in study of infants with septicemia and enterocolitis (Ng et al., 2010).

The major advantage of this technique is the identification of a number of pathogenic strains at the same time, which has opened up new possibilities to diagnosis and discover the epidemiological characteristics of many organisms (Oeth et al., 2009). Future applications of MALDI-TOF MS in clinical laboratories is expected to be established soon to provide more accurate identification of pathogens on clinical specimens, including the detection of resistance biomarkers and specific virulence of organisms.

2.9.3 Target gene DNA sequencing

Due to non-culturability of large proportion of microorganisms and for discovery of novel ones, many fragment and sequence-based techniques have been developed. DNA fragment-based typing using methods including pulsed-field gel electrophoresis (PFGE) and multi-locus variable number tandem repeat analysis (MLVA) are being employed to analyze specific etiologies from samples referred to specialized laboratories. PFGE and MLVA have long been considered as 'gold standard' non-sequence based genotyping methods for pathogen detections.

MLVA is currently the reference genotyping method for most fastidious bacteria. Paranthaman and colleagues (2013) used MLVA to accurately identify the source of a multidrug-resistant *Salmonella enteric serovar* Typhi outbreak in 2011 in England (Paranthaman et al., 2013). MLVA has a high discriminatory power and it's fast, easy-

to-perform, and less expensive but it is less efficient to detect certain species of organisms which lacks tandem repeats such as *Mycoplasma hyopneumoniae*, (Minion et al., 2004).

However, sequence-based typing techniques have become the new reference standard for the detection of organisms rather than the known reference non-sequence based typing tools (i.e. PFGE, MLVA) because they offer much greater discriminatory power. Typical example is multilocus sequence typing (MLST). MLST is based on the combination of genotypes from many genes, including housekeeping genes and has become one of the frequently used sequence-based genotyping methods (Maiden et al., 2013). Different types of MLST assays have been applied to different specimen for the identification of pathogens with highly variable genes among strains, such as *S. aureus, E. coli, or N. meningitides* (Fournier et al., 2007; Kang et al., 2013; Zhang et al., 2013).

A recently developed ribosomal-MLST technique, based on a combined ribosomal protein subunits was employed to discriminate strains within *N. meningitidis* with great success (Jolley et al., 2012). Additionally, whole genome MLST, with the corporation of several loci, was employed to identify bacteria to the strain level with high identification scores (Jolley et al., 2012; Maiden et al., 2013).

Contrary to the MLST, multi-spacer typing (MST) sequencing technique has been introduced, which relies on combined signatures sequences from variable intergenic spacers between aligned genomes of different organisms instead of relying on the actual genes for the identification of pathogens (Drancourt et al., 2004). Although MST was first developed for *Yersinia pestis,* its application has been extended for typing various bacteria, including *Coxiella burnetti* (Glazunova et al., 2005; Zhu et al., 2005). Single nucleotide polymorphism (SNP) analysis is another sequence typing approach

for pathogen identification. Huijsmans et al (2014) used SNP typing to identify 5 genotypes of *Coxiella burnetti* that were involved in the two separate outbreak of Q fever in the Netherlands (Huijsmans et al., 2011). DNA microarrays are another diagnostic tools based on probes specifically designed for individual or all genes present in the genome (Bryant et al., 2004). Geue and colleagues (2014) used this method to differentiate pathogenic between Shiga-toxin producing *E. coli* from other non-pathogenic strains (Geue et al., 2014). The method can be fully automated, sensitive, fast and above all, high throughput.

2.9.4 Whole genome sequencing

The development of next-generation genomic sequencing (NGS) bench-top sequencers (MiSeq-Illumina and Ion Torrent Personals sequencers) has made genomic sequencing highly compatible with the routine clinical microbiological workflow in many advanced laboratories (Didelot et al., 2012; Zhang et al., 2006). Whole-genome sequencing (WGS) has given access to the entire genetic content of pathogens and it is considered the greatest in sequence based-genotyping method. It allows for culture-free identification of a range of organisms in complex samples, including environmental, clinical and food samples in a fair and impartial manner and therefore enabling the identification of difficult-to-culture pathogens such as *Chlamydia trachomatis* (Andersson et al., 2013; Seth-Smith et al., 2013). WGS permit unbiased calling of SNPs and core-genome MLST types, and this also unable extraction of MLST data to lineage-specific markers to identity pathogens.

WGS may be used to reveal the presence of antimicrobial resistance genes or genotypes that may be associated with virulence and pathogenicity of organisms or to discover genetic patterns that may be important to study the clinical manifestation of a disease.

Furthermore, WGS was used to investigate different strains of methicillin-resistant *Staphylococcus aureus* (MRSA) from different countries that lead to the reconstruction of inter-continental transmission patterns for four decades, which revealed the transmission characteristics of those bacteria within hospital environment (Harris et al., 2000). Even though WGS assays are rapid and reliable; they are associated with higher costs and require the service of highly skilled personnel to performance. These factors limit the routine use of these diagnostic molecular methods in most hospitals or reference laboratories.

Recently, whole genome 16S rRNA metagenomics are being applied in many laboratories to identify and describe new bacteria species. 16S rRNA genes are present in all bacteria and contain both variable and conserved sequences that undergo mutations at a slower but constant rate over a long time and therefore can serve as molecular clocks (Rajendhran, J Gunasekaran, 2011). The variable regions of the 16S rRNA sequence provide unique signatures to any particular bacterium and provide vital information about their evolutionary relationships. Various studies have applied 16S metagenomics to identify variety of bacteria species causing human infections from different specimen (Faria et al., 2015; Frickmann et al., 2015; Gyarmati et al., 2015).

2.9.5 Biomarker diagnostics in clinical management

The identification of pathogen-specific biomarkers may be one approach to overcome the present diagnostic uncertainty in clinical management of febrile illnesses. In the event of dwindling antimicrobial agents, prognostic biomarkers are increasingly becoming important for guiding treatment and predicting severity of diseases (El Haddad et al., 2019). An attempt to reduce the indiscriminate use of antibiotics in critically ill individuals, several acute phase proteins including proadrenomedullin

(proADM), procalcitonin (PCT), and C-reactive protein (CRP) are being employed by clinicians to decide whether febrile illness is due to infection or malignancy (Debiane et al., 2014).

CRP has been useful in differentiating bacterial and viral infections, particularly among patients diagnosed with pneumonia and other respiratory infections (Diez-Padrisa et al., 2010; Lubell et al., 2015). Among septic patients, the course of CRP concentrations was highly associated with onset of pneumonia and changes in its levels could be used to indicate its resolution (Povoa et al., 2011). Oguz et al. (2011) investigated CRP levels in fungal and bacterial infections among young children and showed that CRP concentrations were lower in patients with bacterial infections than in fungal patients(Oguz et al., 2011). In rural Laos, CRP was rated superior to pathogen specific rapid tests for dengue and scrub typhus in their ability to inform antibiotic treatment for patients (Lubell et al., 2015).

The potential use of procalcitonin (PCT) as a biomarker of bacterial sepsis has been described. PCT is the prohormone of calcitonin but its levels during infection is regulated differently from the hormonal activities of the mature hormone. In a study to evaluate the diagnostic potential of PCT in patients with different types of meningitis, the investigators reported of significantly higher PCT concentrations in patients with bacterial meningitis compared to those with non-bacterial meningitis (Ibrahim et al., 2011). Traditionally, the management and treatment of meningitis, has relied on the microbial analysis of lumbar puncture and cerebrospinal fluid. In recent times however, PCT has emerged as an important, less-invasive diagnostic alternative, and has removed the pain and suffering of patients during lumber puncture (Reinhart & Meisner, 2011). El Haddad and colleagues demonstrated the potential of both proADM

and PCT to distinguish tumor fever from infectious fever in cancerous patients (El Haddad et al., 2018).

One member of the immunoglobulin super family, known as Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1) is said to be up regulated during fungi or bacterial infections. A study by Gibot et al. (2012) reported that the value of serum sTREM-1 levels used as an indicator of sepsis was better than those of PCT when used as biomarker of disease (Gibot et al., 2012). In addition, increasing levels of CD64 expression on the cell surface of polymorphonuclear and monocytes cells has been implicated as critical step in the immune response to bacterial infections and could serve early indication of sepsis in children (Icardi et al., 2009).

Differential expression of inflammatory cytokines has been linked to certain disease conditions (Heper et al., 2006; Panacek et al., 2006). Among critically ill neutropenic patients, levels of IL-6 and IL-8 were very much different between those with microbiologically confirmed infections as compared to those with undiagnosed febrile illness (Engels et al., 1998). In neonates, increased plasma levels of IL-6 and IL-8 could predict an early onset of sepsis with high sensitivity and specificity (Ng et al., 2010). A combined cytokine score has also been associated with severe disease outcome for patients with sepsis, and predicted to be more superior to those of CRP and PCT (Andaluz-Ojeda et al., 2012). Some pioneering works have been done to assess the potential role of host inflammatory mediators as biomarkers to diseases. Elevated levels of proinflammatory cytokines have been associated with disease progression or severity in humans (Lyke et al., 2004). Decreased levels of proinflammatory cytokines, such as IL-12 and IFN-γ, may be associated with enhanced malaria pathogenesis in humans (Lyke et al., 2004).

Anti-inflammatory cytokines play a vital role in malarial pathogenesis through their ability to modulate the proinflammatory response. In the case of malarial anemia, IL-10 concentration increases steadily with rising severity of disease and parasite density (Ong'echa et al., 2008; Reinhart et al., 2012). One study evaluated the diagnostic performance of IL-4 to identify bacterial infection with 90% sensitivity and 76.5% specificity (Haran et al., 2013). Despite their importance in host-pathogen interactions, the role of cytokines as biomarkers of fever remains to be established.

The role of blood cell components as biomarkers has been evaluated in various disease states. Some of the blood indices studied include leukocyte counts, neutrophil counts, white cell counts (WBC), erythrocyte sedimentation rate, red cell counts, lymphocyte counts. WBC and neutrophil counts are the most frequently studied biomarkers of infections. High sensitivities (88.0%) and specificities (92.5%) to detect bacterial infections have been reported in a study that compared WBC counts in cerebrospinal fluid of meningitis patients compared to individuals with no meningitis infections (Linder et al., 2011). The neutrophil-to-lymphocyte Ratio (NLR) is considered as another useful parameter to distinguish bacterial from viral infections (Bekdas et al., 2014). Fan et al. (2018) evaluated the potential use of routine hematological parameters as disease markers and concluded that NLR, eosinophil, and RDW were useful and independent prognostic factors for development of ischemic stroke (Fan et al., 2018). The N/L ratios have been evaluated as a biomarker of recovery in patients suffering from severe dental infections (Dogruel et al., 2017; Fang et al., 2013).

Some limitations associated with the use of biomarkers for disease prediction have been reported. CRP is said to discriminate between patients better only during early course of infection (Monneret, 1997; Sakr et al., 2008). Plasma levels of CRP could remain

high for many days even when infection is treated or the level may remain high during inflammatory states of non-infectious causes, as seen in cases of autoimmune or rheumatic disorders and in malignant tumors (Meisner et al., 1999). Non-specific elevation of PCT levels in the absence of specific infection have been reported in persons under stress, after surgery or during cardiac shock (Schuetz, 2010). High levels of PCT have been documented in non-infectious systemic inflammation during the administration of different types of immunotherapy, such as granulocyte transfusions, cytokines or related antibodies therapies (Reinhart et al., 2011).

Combinations of infection biomarkers that reflect on various components of the host inflammatory response have been suggested to overcome the limitations of using just a single biomolecule as a biomarker. Such combination of biomarkers may be advantageous in the diagnosis of fever as they indicate a useful parameter to evaluate the prognosis of individuals presenting with fever.

2.10 Challenges in the management and diagnosis of AFIs

2.10.1 Epidemiologic challenges

Having good knowledge of the epidemiology of febrile illness in an area is necessary for informing public health interventions and strategies. In most cases, there are huge challenges in the compilation of such knowledge, and the main handicap is the non-existence of accurate data in many countries especially in sub-Sahara African. Most febrile patients may not commonly attend healthcare facility for treatment during febrile episodes, and so quite a substantial proportion of cases are missed, resulting in underestimation of the true burden of the AFIs. Another limiting factor is that seasonal variation of disease dynamic are not accounted for as most febrile illness studies are

conducted over short periods and do not cover the entire calendar year to record events various time points (Bell et al., 2001; Mayxay et al., 2013).

2.10.2 Diagnostic challenges

2.10.2.1 Challenge of rapid diagnostic test (RDT)

RDT uses immunochromatographic lateral flow for antigen detection and rely on the capture of dye-labeled antibodies to give a visible band on nitro-cellulose strip. The performance of the rapid test can be influenced by varieties of factors such as the type of pathogen and the level of infection; the target antigen and the capture antibody; the expression levels of the targeted antigens and the presence of certain isomers. Moreover, the experience and level of training of the person performing the test could influence the interpretation of RDT results. In this study, we used malaria Pf (HRP II) and (PAN pLDH) antigen detection test device (AZOG Inc.) for rapid diagnosis of malaria which has been approved by WHO for malaria testing. For malaria RDTs, cross reactivity with self-induced antibodies such as rheumatoid factor can result in false positive tests. The target antigen, PfHRP2 only detects *P. falciparum* infection and thus likely to miss other *Plasmodium* species (*P. malariae, P. vivax,* and *P. ovale*) in areas where non-*falciparum* malaria is co-endemic. Cross reactions of PfHRP2 with non-*falciparum* malaria parasites could give false positives for *P. falciparum* infections. Some RDTs are reported to give false negative results at higher levels of parasitemia with fetal consequences.

2.10.2.2 Challenge of clinical diagnosis

In many resource-limited settings, clinical records and physical examinations are the mainstays of diagnosis for febrile illness. The use of clinical algorithms promotes presumptive treatment of febrile disease which has been shown to have a high

sensitivity but low specificity (Chandramohan et al., 2002). This low specificity is attributed to the overlapping signs and symptoms shared by many febrile related illnesses, culminating in over-diagnosis of the disease in question (Chandramohan et al., 2002). Once malaria is ruled out, a trial and error method is used to sequentially resolve the disease condition, one after the other to get the most probable diagnosis for patients. While this may be slow, the patient's condition may also get deteriorated leading to fatal clinical consequences.

2.10.2.3 Challenges of microbiological cultures

Microbial cultures continue be the gold standard for diagnosing bloodstream infections, but this approach is limited by being relatively slow and resource intensive. Cultures vary in sensitivities according to the type of sample or fluid used, volume of samples collected and host factors including the patients' age bracket, co-morbidities, previous antibacterial agent used and the disease stage (Rosenblum et al., 2013). For instance, blood cultures are known to be 40% to 80% sensitive for the detection of invasive bacterial pathogens such as *Salmonella* species and can even be more sensitive during the first week of illness when the bacterial load is high compared with subsequent weeks. But the sensitivity for the detection of the same *Salmonella* spp in bone marrow cultures can be as high 90%, which is unaffected by prior antibiotics usage. In general, stool, urine and rectal swabs cultures have <40% sensitivity (Pang & Peeling, 2007). Some pathogens, such as *Rickettsia*, *Leptospira* and Dengue usually required cell-based cultivation systems with enriched culture media for their growth. The complicated procedures involved in culturing may be hazardous to the laboratory staff, which limits their routine application in most clinical laboratories.

2.10.2.4 Serologic test challenge

Serology can be used to detect a wide range of pathogens, but this method is limited by the need for both acute and convalescent phase samples to establish seroconversion levels. Although, one-time sampling can be ideal for presumptive diagnosis, the cutoff values may vary in different locations, which limit their usage for diagnostic confirmation. This makes it hard to confirm if a real positive test reflects acute disease or not. The low sensitivity and specificity levels of latex agglutination test have also been observed in other serologic assays, including the widely used Widal test, especially at initial stages of disease in many settings (Dutta et al., 2006). The detection of immunoglobulin as biomarkers may lack sensitivity and accuracy, especially if samples are taken in the early stages of the illness. In addition, serologic assays may exhibit cross-reactivity with other related species. Serologic assays for most flaviviruses may be influenced by extensive cross-reactivity between the species (Cleton et al., 2005), which may present big challenge for clinical diagnostics. Due to cross-reactivity issues, many patients may have positive serologic test results but negative bacteriological test and this makes interpretation very challenging. Although, ELISA has been used to study the normal antibody response during infection, the results may be limited by lack of specificity.

2.10.2.5 Molecular diagnosis challenge

Molecular-based diagnostics has led to increased detection of a range of pathogens in bloodstream, respiratory tract, gastrointestinal tract and central nervous system. However, rate of detection are pathogen and tissue specific. This was evident for the molecular detection of *Rickettsia* by PCR which had a sensitivity of 90% when skin biopsy samples were used but the results fell to 50% when blood sample was used instead (Andrews & Ryan, 2015). On a few occasions, PCR has been reported to have

low sensitivity for the detection of invasive pathogens because of their low titer in the blood (Andrews & Ryan, 2015). Malaria diagnosis by PCR is said to be sensitive but is generally expensive to employ routinely and often used for research purposes in most countries.

High cost of running is seen as a limitation of implementation of real-time PCR in smaller laboratories with limited resources. The cost of reagents for performing real-time PCR excluding DNA extraction can be more than three times higher than the cost of conventional PCR for the same number of samples.

2.11 Empirical clinical data and machine learning techniques

Accurate diagnosis of disease remains a challenge in medical field. In recent time, however, new strategies for the early diagnosis and treatment outcome have been developed through data mining technology (Kourou et al., 2015). Large data from patient records are being made available to the medical research community for proposes of generating predictive models for diagnostic purposes. Using Machine Learning techniques, predictions on future events can be achieved based on current information (Obermeyer et al., 2016).

Machine learning process involves an estimation of unknown dependencies in a given set of data and the use of such estimated dependencies to predict new outputs of the same data set (Kourou et al., 2015). Two types of ML methods available are the Supervised Learning (SL) and Unsupervised Machine Learning (UL) (Niknejad & Petrovic, 2013). SL typically models a response based on a set of available predictors while in UL, one infers patterns within a dataset with no specific set of responses. Thus, inferences are drawn from dataset consisting of input data without labelled response. The most important task in ML process is data classification. ML functions to

classify data items into one of many predefined classes. Whenever data classification model is developed by means of ML techniques, training and generalization commands are generated, edited and finally applied to make predictions or estimations.

Besides medicine, the use of ML for predictions purposes can be applied in different kind of domains including health communications, risk management and decision support systems (Cruz & Wishart, 2006). Risk management play a vital role in medical health insurance organizations where insurers classify their client into standard or substandard applicants based on their policy rate and the premiums. By having information on client medical status including laboratory test results, insurers can make evidence-based decisions to optimize, validate and refine the regulations that govern their premiums. Zhang et al. (2009) conducted classification on breast cancer metastasis by using available published gene expression profiles (Zhang et al., 2009). By comparing multiple methods such as logistic regression, Support Vector Machine, LogitBoost and Random Forest, they were able to classify patients into high and low risk breast cancer patients. Mantzaris et al (2008) used different input variables such as age, sex, height and weight as classifiers and made predictions on osteoporosis using Probabilistic Neural Network (PNN) and Multi-Layer Perception (MLP) and reported on the prognosis rate for the disease (Mantzaris et al., 2008).

In this study, the performance of three supervised machine learning algorithms namely Lasso regression, Naive Bayes, and Random Forest were compared and the suitable one used to develop a predictive model to inform clinicians of the probability of infections of patients reporting with fever.

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Study sites and settings

This study was conducted at the Kintampo Municipal Hospital, Kintampo and the Ledzokuku Krowor Municipal Assembly (LEKMA) Hospital in Teshie. The two hospitals serve different geographically and demographically heterogeneous populations with quite different rates of malaria transmissions, nutrition status and poverty levels.

Teshie is a peri-urban area in Greater Accra Region of Ghana with about 172,000 residents. LEKMA Hospital is 450 bed capacities Hospital and has all the units of a general hospital including laboratory, radiological facilities, maternity, a surgical theatre and a well-equipped pediatric ward with pediatricians. Malaria transmission in Teshie is low with annual entomological inoculation rate (EIR) of less than 50 infective mosquito bites per person per year (Klinkenberg et al., 2008). The area has two malaria transmission seasonal peaks that coincide with two main rainy seasons spanning from March to May, and October to November.

Kintampo is a semi-urban area in the forest-savanna middle belt of Ghana in the Brong Ahafo Region with a population of about 43,000. Malaria transmission is high with annual entomological inoculation rate of 269 infective bites per person for the year which peaks between April and October (Dery et al., 2010; Owusu-Agyei et al., 2009). Kintampo Municipal Hospital is a local hospital serving a semi-urban and relatively poor population who are predominantly subsistence farmers and traders. The hospital has all the units of a general hospital and serves as first point of referral for the surrounding peripheral health facilities. Health and Demographic Surveillance System

(HDSS) is being maintained at Kintampo by Kintampo Health Research Centre (KHRC) that records detailed demographics of all residents including, births, deaths and migrations at 4 monthly intervals. Every household in Kintampo has been digitized making the selection and tracing of individuals to their homes very easy.

3.2 Study design, population and eligibility

A cross sectional study design was used to seek consent and recruit children aged 1-15 years presenting at the outpatient departments of the two hospitals from October 2016 to August 2017. The study participants were children with acute fever defined as individuals with measured axillary temperature ≥37.5°C and occurring less than 7 days prior to hospital attendance. Children are considered the most high-risk group for contracting infections due to their immature immune system and their playing habits which expose them to various infectious agents. Afebrile children with temperatures

<37.5°C were also enrolled alongside and data used for the development of a predictive model and evaluation of biomarkers of fever. The inclusion criteria took into account the afebrile children as well.

3.2.1 Inclusion criteria

The inclusion criteria for enrolment into the study were;

1. Absence of chronic illness, as determined clinically by study clinician.
2. Parent or guardian's willingness to give written informed consent.
3. Willingness of older children (10-15 years) to provide assent.
4. Residency in the study area for the past two months was required for the purpose of collecting convalescence samples when needed or for a repeat of a laboratory test after the first visit.

5. Parent or guardian and children willing to allow blood draw and provide other samples needed were eligible and recruited as study participants.

3.2.2 Exclusion criteria

The exclusion criteria for the study were;

1. Refusal to give informed consent
2. Children showing signs of severe disease such as chronic kidney failure, dementia and others complicated diseases as determined by the study clinician.
3. Minors unaccompanied by parents/guardians were not enrolled either.

3.3 Study procedure

The study team comprising a field worker and a nurse identified children within the required aged group and measured their axillary temperature, height and weight as routinely done for patients at the OPD. The study information, procedures and all other activities were explained to every parent or guardian; and each individual were allowed ample time to decide whether or not to participate in the study. Our study team also obtained written informed consent from all parents or guardians and assent in the case of minors, from the two hospitals. The patients were then sent to the study clinician who physically examined and enrolled them into the study, using the study's inclusion and exclusion criteria outlined.

A detailed structured questionnaire was administered by the attending clinician or field staff to collect socio-demographic and clinical (presenting signs and symptoms) data. In addition, results of point-of-care laboratory tests were also recorded. The data collected also included information on prior usage of antibiotic and antimalarial drug treatment in the week preceding OPD attendance. After obtaining informed consent, each child was physically and clinically examined by the study clinician who requested for specific

laboratory investigations based on presenting signs and symptoms and clinical judgment. Any child suspected or confirmed to have any disease received appropriate treatment according to the Ghana Health Service treatment guidelines. Presented in Figure 3-1 is a flow chart of the study procedures and activities.

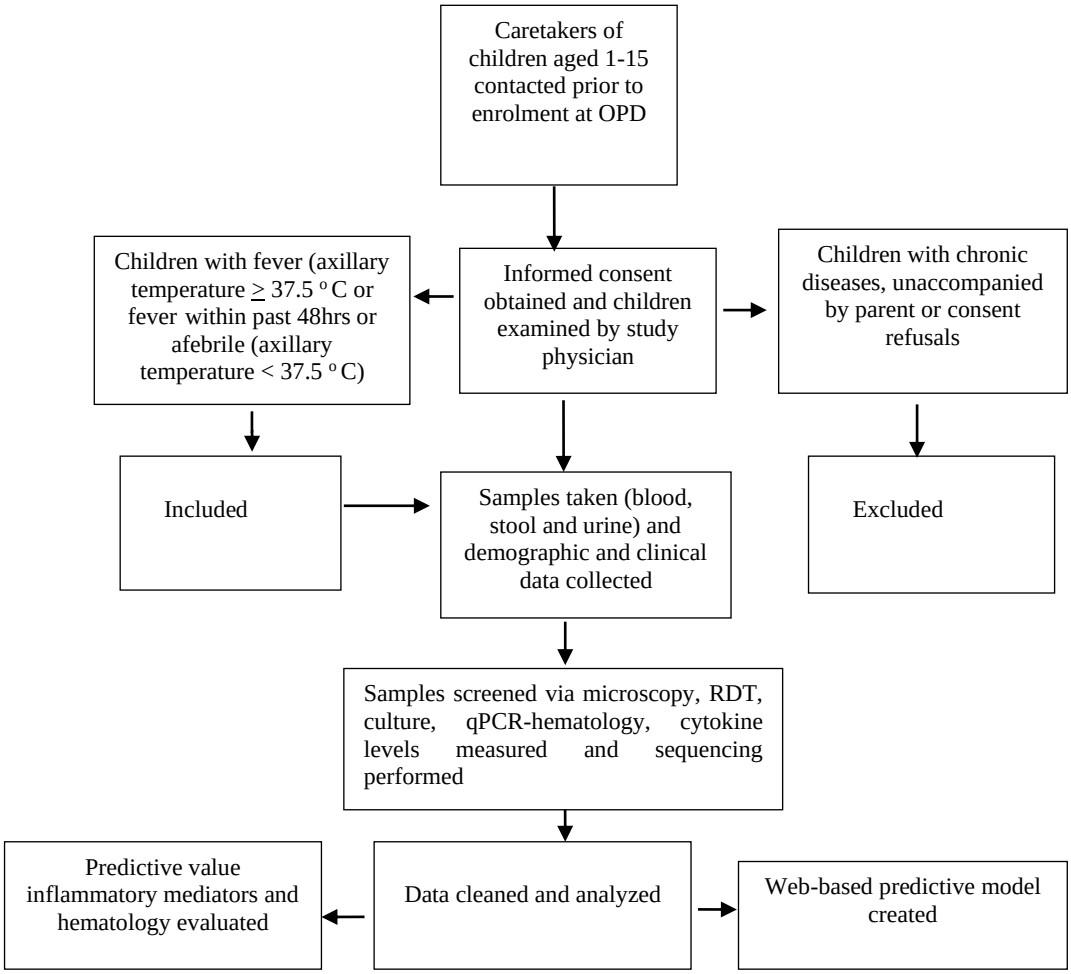


Figure 3-1: Flow chart of study recruitment procedure and activities

3.1 Sample size determination

Sample size was calculated, taking into account the low rate of detection of AFI associated pathogens in febrile patients (Crump et al., 2015). The sample size was determined using the Fisher's formula;

$$SZ = \frac{r+1}{r} \frac{SD^2 (Z_{\alpha/2} + Z_{\beta})^2}{d^2}$$

Where;

SZ= sample size required

SD= standard deviation

$Z_{\alpha/2}$ = standard normal variate for level of significance

Z_{β} =standard normal variate for power

d = effect size

r = odds ratio of case to control

Setting $Z_{\alpha/2}$ =1.96, d=0.842, α= 0.05, we assumed odds ratio of 2.5 and a power of 80% at significance level of 5% between febrile (defined as case) and afebrile (control) children. Making a provision of 10% for contingencies, a sample of 858 for febrile cohort and 655 for the afebrile children was calculated using the STATA's direct software version 12 (STATA Corp, College Park, 33 TX, USA). Hence a total of 1513 children were recruited for the whole study cohort.

3.2 Sample collection, transportation and storage

Depending on the number of laboratory investigations to be carried out at the request of the attending clinician, between 5-7 mL of venous blood was collected from each child into a EDTAK2 anticoagulant vacutainer tube, by a qualified phlebotomist after disinfection of the skin surface. Whenever blood culture was requested, a minimum of 2.5 mL of blood was inoculated into a BD BACTEC Peds Plus/F culture vials (BD, Maryland, USA, Lot no. 7108604) and transported within 24 hours of collection to the clinical laboratory at Kintampo Health Research Centre (KHRC) or Noguchi Memorial Institute for Medical Research (NMIMR), Legon, for children recruited in Kintampo study area and LEKMA Hospital, respectively. From the blood collected, 2.5 mL was aliquoted into EDTA vacutainer tubes and stored immediately -80 ^{o}C, and some of the

remaining blood was used to prepare thick and thin blood films for malaria parasite identification. The sample was also used to run patient's full blood count using ABX Micro 60+ automated analyzers (Horiba-ABX, Montpellier, France) available at both LEKMA Hospital and KHRC. The rest of the sample was centrifuged and serum separated and stored in Eppendorf tubes (Alpha laboratories, UK) at -20 ^{o}C for later use in other downstream analysis.

Nasopharyngeal samples were collected from study participants with signs of respiratory infection by trained medical personnel. In this case, a sterile swab with cotton ended applicator (Thermo Scientific, Waltham, MA) was inserted through the nose into the nasopharynx; making sure the tip reached a distance equivalent to that from the ear to the nose of the patient. It was rotated three times to ensure that it picked any microbes in there and thereafter removed and the swabbed specimen placed in a vial containing M4-RT universal transport medium (Copan Diagnostics Inc) and stored at -80 ^{o}C. For the study participants showing signs of urinary infections, about 10mL of urine was collected for bacteria culture and other routine urine analysis. Stool (about 10g) was taken from participants with diarrhea and subjected to wet mount microscopic analysis or bacteria culture. All laboratory results were immediately reported to the treating clinician without delay for the benefit of the patient.

3.3 Ethical considerations

Approval to conduct this research including all subsequent amendments was granted by the ethics committees of Kintampo Health Research Centre, Noguchi Memorial Institute for Medical Research, University of Ghana and Ghana Health Service. The study aims and procedures were explained to parents or guardians of potential study

participants and a written/signed informed consent was obtained before enrolment into the study.

Every participant was given a unique identification number and all data was entered into the recruitment form and research database using unique participants' identification numbers. The link between the unique subject number and the participant's identity was maintained securely. Computerized data were stored in a password-protected computer, and only study investigators had access. The results of the clinical assessments and laboratory results were made available to the treating clinician and the parents as soon as they were ready. The study participants did not incur any additional costs for their participation into the study. All participants received the standard care regardless of their enrollment status (febrile or afebrile participant) into the study.

3.4 Phenotypic characterisation of febrile pathogens (Laboratory analysis)

3.4.1 Malaria parasite identification and quantification

For each participant, thick and thin film blood smears were prepared on the same slide and stained with 3% Giemsa. The thick smears were examined microscopically with an oil immersion objective lens to quantify the parasitemia. The parasitemia was determined by counting the number of leucocytes per field in the thick blood film. In most cases, asexual parasites were counted against 200 leucocytes using a hand tally counter. For low parasitemia (parasites < 1000 parasites per 200 WBCs per field), parasite counts were made against 500 WBCs. However, for high density parasitemia (10,000 parasites/field), the count was made against 200 WBCs. The parasites were quantified (number of parasites/µL blood) for medium parasitemia based on the formula below:

$$\text{Parasite density (parasites/µL)} = \frac{\text{Parasites counted X absolute WBC counts*}}{\text{Number of leucocytes counted (200 or 500)}}$$

*absolute WBC = child's own WBC count

Slides were declared negative if about 200 fields were examined in the thick film without seeing any parasite. As quality control for proper staining and reading of blood slides, known positive and negative blood films were stained with each newly prepared batch of working Giemsa solution. The stained films were evaluated using the criteria from the Malaria Blood Film Giemsa Staining Quality Control Log Sheets (WHO, 2003). In both study sites, each slide was examined by two independent microscopists and if the results for the two readers were found to be discordant, a third microscopist was used to ascertain the true parasite density. The microscopic data was also compared with malaria RDT results, as approved by WHO for routine malaria testing.

3.5 Bacterial culture and identification

As a gold standard, blood cultures were routinely carried out for all study participants with suspected bacteremia by qualified laboratory personnel. Blood for culturing was collected using BACTEC culture vials. Briefly, 2.5−3mL of venous blood were inoculated into standard BD BACTEC Peds Plus/F culture vials containing enrichment broth and transported to the laboratories. The culture vials were incubated in a BD BACTEC™ 9050 automated blood culture machine for 5 days and declared negative if there was no signal of growth within the 5 days of incubation. Any blood that flagged positive was Gram-stained and sub-cultured onto appropriate media (7% sheep blood agar, chocolate agar and MacConkey) and incubated at 37 ^{o}C for 18-24 hours with 5% CO_2 to obtain bacterial colonies. All bacteria isolated were identified using their Gram stain reactions and confirmed by the pattern of biochemical reactions using standard procedures. Smears for Gram stain were prepared by adding a drop of sterile

physiological saline solution (0.85%) onto a clean labeled slide, and then desired colonies were picked and added to the saline solution to obtain uniform mixtures. The smears were then air-dried at room temperature and heat fixed. Gram staining was done using standard procedure (Appendix-1). The Gram stain results were immediately communicated to the attending clinician for patient management. Besides the daily performance of internal quality control assessment at bacteriology units of both Kintampo and Noguchi where cultures were performed, the laboratories also participate in external quality assessments for hematology and bacteriology with the College of American Pathologists (CAP) and the United Kingdom National External Quality Assessment Scheme (UK NEQAS), and also comply with the principles of Good Clinical Laboratory Practice.

Urine and stool samples were selectively cultured (at the request of attending clinician) on cysteine lactose electrolyte-deficient (CLED) agar and hektoen enteric agar (HEA) or deoxycholate citrate agar (DCA) respectively. Anaerobic cultures were established using the gas (CO_2) pack anaerobic system and incubated for 18-24 hours at 37 ^oC (Cheesbrough, 2006).

3.5.1 Biochemical tests

Standard microbiological culture procedures were followed. Gram negative rods and members of the *Enterobacteriaceae* were identified based on their biochemical reactions. Gram positive bacteria were identified using the coagulase test (Appendix 2) and catalase test (Appendix 3). Other basic tests including motility, production of indole and the use of cytochrome oxidase were also performed to aid bacterial identification. Catalase activities were detected with catalase reagent droppers (BD, Franklin Lakes, USA), according to the manufacturer's instructions. The alpha- and

beta-hemolytic *Streptococci* groups were differentiated using optochin and bacitracin and disc test respectively. *Streptococci* belonging to the Lancefield group A, B, C, D, F or G were identified using *Streptococcal* grouping kits (Oxoid, Basingstoke, England). *Staphylococcal* agglutination kits (Oxoid, Basingstoke, England) were employed to distinguish *Staphylococcus aureus* from *Staphylococcus intermedius* and Coagulase negative *Staphylococci*. All the tests were performed according to the manufacturer's instructions.

The Analytical Profile Index-API 20E (bioMérieux, Marcy l'Etoile, France) test was used to either identify the organism or as confirmatory test. The API 20E consists of plastic strips containing miniaturized compartmental wells with each well containing a special dehydrated substrate for the tests compiled to identify a group of closely related bacteria species especially the *Enterobacteriaceae* (Appendix 8).

3.5.2 Bacteria identification by matrix-assisted laser desorption ionization time-of-flight

Some bacteria were identified using the Bruker Biotyper matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) instrument (Bruker Daltonics, Coventry, UK) at the Bacteriology Department of the NMIMR, University of Ghana. By this method, positive culture broths were sub-cultured to obtain pure bacteria isolates. Individual pure samples were processed by adding a mixture of 5µL each of acetonitrile (AN) and 20% trifluoroacetic (TFA) acid to 1-3 bacterial colonies and incubated for 15 minutes at room temperature. This was spotted in duplicate onto a MALDI-TOF MTP target plate (Bruker Daltonics, Coventry, UK). The preparation was overlaid with 2 µL of matrix solution (α-HCCA in 50% AN and 20% TFA). The matrix-sample mixture was crystallized by air drying for few minutes at room temperature. Measurements of molecular weight of the high abundance proteins were

obtained with an Auto flex II mass spectrometer (Bruker Daltonik) fitted with a nitrogen laser. Data were acquired using AutoXecute acquisition control software and analyzed by standard pattern matching (using default settings) against the spectra of over 3500 species used as references in the Bruker database. In each spectrum, more than 20 peaks (mass range: 1000-10000) were considered and the generated peaks were compared with those in the Bruker's database. Valid identification scores as given by Bruker Daltonik MALDI Biotyper was 2.0 or above and this was enough for a reliable identification to the species level.

3.6 Biomarker identification

3.6.1 Measurement of blood cytokine levels

The MILLIPLEX® MAP 13-plex kits from Millipore (EMD Millipore, Billerica, MA, USA) were used to quantify serum concentrations of 13 cytokines. The quantification was performed on the XMAP technology platform (Luminex corporation, Austin, TX, USA), which simultaneously quantified all the analytes in the samples in a 96 well format. These kits with the same lot numbers were used to quantify 8 pro-inflammatory cytokines (GM-CSF, TNFα, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-12) and 5 anti-inflammatory cytokines (IL-5, IL-4, IL-7, IL-10, IL-13) in serum samples obtained from both febrile and afebrile children. These analytes were selected based on their relevance in immune modulation in response to infection.

The assays were performed following the manufacturer's protocol without any modification (Appendix 9). Readings from the background wells were prevented while the readings for the quality control and the standards wells were all within the correct reference ranges of the kits. Prior to running the assay, every sample was thawed and clarified by centrifugation (2000 rpm/10 minutes) and the samples were randomly

distributed across plates and assayed in duplicates. Whenever a sample recorded percentage co-efficient of variation of more than 15%, that record was not used in the data analysis.

3.6.2 Measurement of hematological parameters

Full blood counts (FBC) for all blood samples were determined using the ABX Micros 60+ (Horiba ABX, Montpellier, France) which is a 5-part differential auto-analyzer. The parameters considered were white blood cells (WBC), lymphocytes (%), lymphocytes (absolute), monocytes (%), monocytes (absolute), granulocyte (%), granulocyte (absolute), red blood cell (RBCs), hemoglobin (Hgb), hematocrit (Hct), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin corpuscle (MCHC), platelets (Plt), platelet distribution width (PDW), red cell distribution width (RDW), and mean platelet volume (MPV). Three levels of daily quality controls were routinely carried out to be certain of the analyser's performance in estimating low, normal and high parameters of blood counts. Each printed result was checked and signed by the operator and a senior supervisor according to standard operating procedures (SOPs) before being sent out for patient evaluation and data entry.

3.7 Evaluation of parameters as fever biomarkers

3.7.1 Hematological parameters as biomarkers of fever

To evaluate the diagnostic performance of full blood count or hematological parameters for predicting fever, age-specific reference values for hematological parameters for healthy children established in the Kintampo North Municipality and Kintampo South District, Ghana was used (David et al., 2014). This was done by defining population specific cut-off points for normal blood counts which were coded as zero, and abnormal blood count coded as one. Table 3.1 shows a summary of the reference values cut-off

points used in the analysis. Whenever differences existed between males and females, gender-specific reference values were used instead as indicated by asterisk (Table 3.1). The accuracy of the diagnostic tests was evaluated based on their sensitivity (SEN), specificity (SPE), positive predictive value (PPV), and negative predictive value (NPV) probabilities. The area under the receiver operating characteristic (ROC) curve generated in the STATA software was recorded.

The ROC curves were used to evaluate the sensitivity and specificity of the diagnostic parameters being tested. ROC curves were obtained by plotting sensitivity scores (true positive) against 1-Specificity (false positive). The area under ROC curves (AUC) were used as the overall measure of the test performance. AUC of 0.5 or below represents a correct diagnostic probability no better than chance while an AUC of 1 represents perfect diagnostic accuracy (Grzybowski et al., 1997; Schmitz et al., 2002). Cut-off scores were chosen to have seasonally high sensitivity and specificity values and a good test was defined as the one with high sensitivity and specificity.

Table 3-1 Hematological reference values for healthy children

Parameters	Age Category (years)				
	0.5-4.9	5-12	13-17	$13-17	
				M	F
WBC (x 10^9/L)	5.1-17.6	4.1-11.9	3.7-9.4		
***RBC (x10^{12}/L)**	3.2-5.6	3.5-5.3	-	3.79-5.69	3.4-5.4
***HGB (g/dL)**	8.0-12.7	9.1-13.5	-	10.4-14-8	9.4-14.2
***HCT (%)**	24.4-38.8	27.3-41.5	-	31.1-45.1	29.4-44.9
PLT (x10^{12}/L)	110.0-637.0	117.0-417.0	113.0-363.0	-	-
MCV(fl)	56.0-87.0	68.0-89.0	67.0-93.0	-	-
MCH(Pg)	16.9-29.7	21.4-30.3	21.2-32.0	-	-
MCHC(g/dL)	30.0-36.9	30.9-36.0	30.5-36.6	-	-
RDW (%)	12.5-21.6	11.5-17.9	11.6-16.1	-	-
***PDW (%)**	8.8-25.4	12.1-20.5	-	11.9-20.8	12.4-22.6
***LYM (%)**	34.9-75.6	29.6-62.5	-	26.5-56.7	25.7-60.2
MON (%)	4.9-13.6	5.9-13.3	4.9-14.4	-	-
***GRA (%)**	18.5-59.7	28.3-62.4	-	33.6-64.4	30.8-64.0
LYM (#)	2.3-11.9	1.6-5.8	1.4-4.0	-	-
MON (#)	0.2-1.0	0.2-1.1	0.2-0.9	-	-
***GRA (#)**	1.5-8.5	1.6-6.2	-	1.4-5.4	1.6-5.2

M=male, F=female, #=absolute number, %=percentage, $ Data used when gender parity or difference exist, *=parameters with gender parity, WBC= White blood cells, RBC=red blood cells, HGB=hemoglobin, HCT= hematocrit, PLT= Platelets, MCV= mean cell volume, MCHC= mean cell hemoglobin corpuscle, RDW= platelet distribution width, PDW= Platelet distribution width, LYM= Lymphocytes, MON= Monocytes, GRA= Granulocyte

3.7.2 Serum cytokines as biomarkers of fever

To evaluate the predictive performance of serum cytokines for the study population, an age-specific reference cytokine values for healthy children published in the literature was used (Kim et al., 2011; Kleiner et al., 2013). The individual cytokine reference cut-off points for normal and abnormal cytokine levels were appropriately coded using the age-specific study population. The values for healthy children have been summarized in Table 3-2.

Table 3-2: Reference cytokine levels for healthy children

Cytokine	Reference values
GM-CSF	0.5-72.8
IFN-γ	1.14-12.7
IL-10	1.0-19.8
IL-2	0.4-6.4
IL-13	0.9-36.5
IL-1β	0.2-14.0
IL-1	2.9-18.3
IL-4	0.3-90.1
IL-5	0.3-20.1
IL-6	0.2-37.7
IL-7	0.3-42.1
IL-8	4.2-13.6
TNF-α	1.9-26.8

GM-CSF= Granulocyte-macrophage colony-stimulating factor, IFN= Interferon gamma, TNF =Tumor necrosis factor, IL= interleukin

3.8 Molecular screening for AFI associated pathogens

Blood samples from one hundred and sixty-six (166) febrile children who had tested negative for malaria RDT and microscopy, blood culture and other point of care phenotypic tests were further screened with TaqMan qPCR. The samples were selected randomly from the pool of clinically and laboratory-declared negative individuals**.**

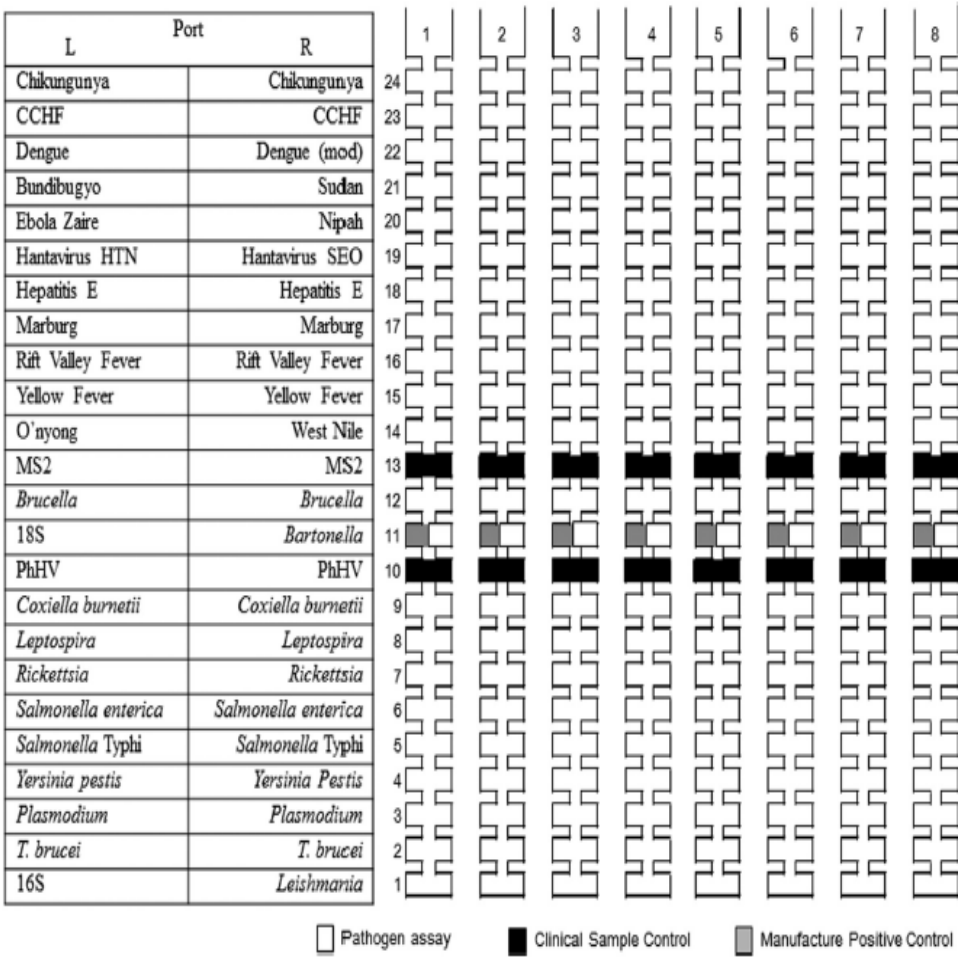
3.8.1 Extraction of total nucleic acid from whole blood for TaqMan PCR assay

Total nucleic acid (TNA) was extracted from whole EDTA blood using the high pure viral nucleic acid large volume kit (Roche) with slight modifications. Briefly, 2.5 mL EDTA blood was subjected to cell lyses in 2.5 mL extraction buffer containing proteinase K (Sigma, St. Louis, MO). The composition of the extraction buffer included poly (A) carrier RNA, RNA lysing-binding solution and two extrinsic controls. Addition of RNA carrier enhances binding of nucleic acids to high pure filtrate tube membrane and ensures the inactivation of RNases or DNases (reducing the chance of nucleic acid degradation).The sample was incubated at 70 ^{o}C for 15 minutes, after which 1.25mL of binding buffer was added and vortexed. The sample was then transferred into the upper reservoir of the high pure extender assembly and centrifuged at 4,000 x g for 5 minutes. The 50mL polypropylene tube was unscrewed and removed from the high pure extender assembly and both the flow-through and the 50 mL tube were discarded. The high pure extender or filter tube was placed in a new 2 mL collection tube and treated with 500 µL inhibitor removal solution and centrifuged at 8,000 x g for 1 minute. The filter tube was placed in a new collection tube and tube containing the filtrate discarded. The filter tube was washed twice with 450 µL wash buffer after centrifugation at 8,000 x g for 1 minute to get rid of contaminants such as salts and proteins. The filter tube was transferred into a labeled 1.5 mL microcentrifuge

tube and 150 µL pre-warmed elution buffer was added, incubated for 1 minute at room temperature and centrifuged at 8000 x g for 1 minute. The purified TNA was stored at -80°C until use. The two extrinsic controls, PhHV (10^6 copies) and MS2 (10^7 bacteriophage) (ATCC Manassas, VA; a gift from Professor Eric Robert Houpt, University of Virginia, Charlottesville, Virginia, USA) were added to the lysis buffer to serve as build-in controls to evaluate the success of the extraction and amplification efficiency of the RNA and DNA targets. One extraction blank was included in each batch of extraction to check laboratory contamination and if found positive for a given target, the results obtained for that target and the entire batch of extraction were not considered valid.

3.8.2 AFI TaqMan qPCR assay

The AFI TaqMan array card used in this study was the 384-well format (Life Technologies, Applied Biosystems; Foster City, CA, USA). The left and right ports of the card were coated with the pathogen-specific primer-probes oligonucleotide sequences as well as the manufacturer's positive internal controls derived from both human and pathogen sources. A total of 26 different pathogens were targeted in a single PCR assay but 6 samples with 2 control standards (a high and low nucleic acid extract of known concentrations) and no-template negative controls were run at a time (Figure 3-2).



Source: Liu et al., 2016

Figure 3-2: Configuration of TaqMan array card

Prior to usage, the card was removed from 4 ºC storage and equilibrated to room temperature in their original packaging. The nucleic acid samples extracted were thawed and clarified by brief centrifugation. In this assay, 75 µL of the TNA extract was mixed with 25 µL TaqMan fast virus one-step master mix (Life Technologies, Warrington, UK) in a 100 µL reaction mixture and pipetted into the inlet port of each channel on the array card as shown in Figure 3-3. The cards were briefly centrifuged

(1 min at 1200 rpm for two times), sealed and the inlet port together with the remaining excess PCR mix (99 µL) removed by following the manufacturer's instructions.

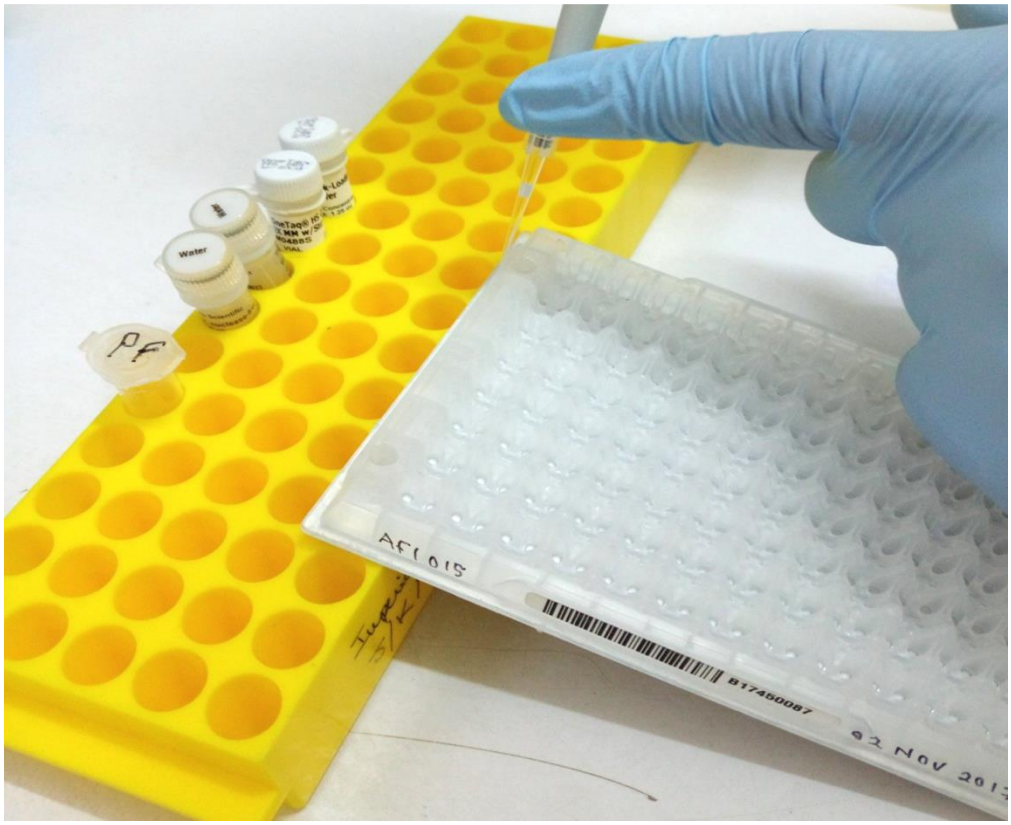


Figure 3-3: Loading of the PCR mix into TaqMan array card

The cards were then run on the ViiA 7 real-time PCR system in 1µL reaction volume (Life Technologies) using PCR cycling conditions as shown in Table 3.3.

Table 3-3: Cycling conditions for the TaqMan array card PCR

Steps	**Stage**	**Cycle**	**Temp/^{O}C**	**Time**
Reverse transcription	1	1	50	**10 min**
RT inactivation/initial denaturation	2	1	95	**5 min**
Amplification (PCR)	**3**	**45**	**95** **60**	**15 secs** **35 sec**

Temp= temperature, min-minutes, sec= seconds

3.8.3 Quality control for TaqMan array card (TAC) PCR

Two positive controls (synthetic plasmid DNA construct and transcript for RNA viruses) was run alongside and served as standards for the quantitative analysis. At the end of each TAC PCR run, the quality control (QC) summary was reviewed as outlined in Figure 3.4. The amplification curves were all carefully examined, and the baseline adjusted to ensure accurate calculation of the quantification threshold cycles and proper calling of positivity. A sample was considered positive when any of the duplicated reactions produced amplification or quantification threshold cycle of less than 35 (C_t < 35).

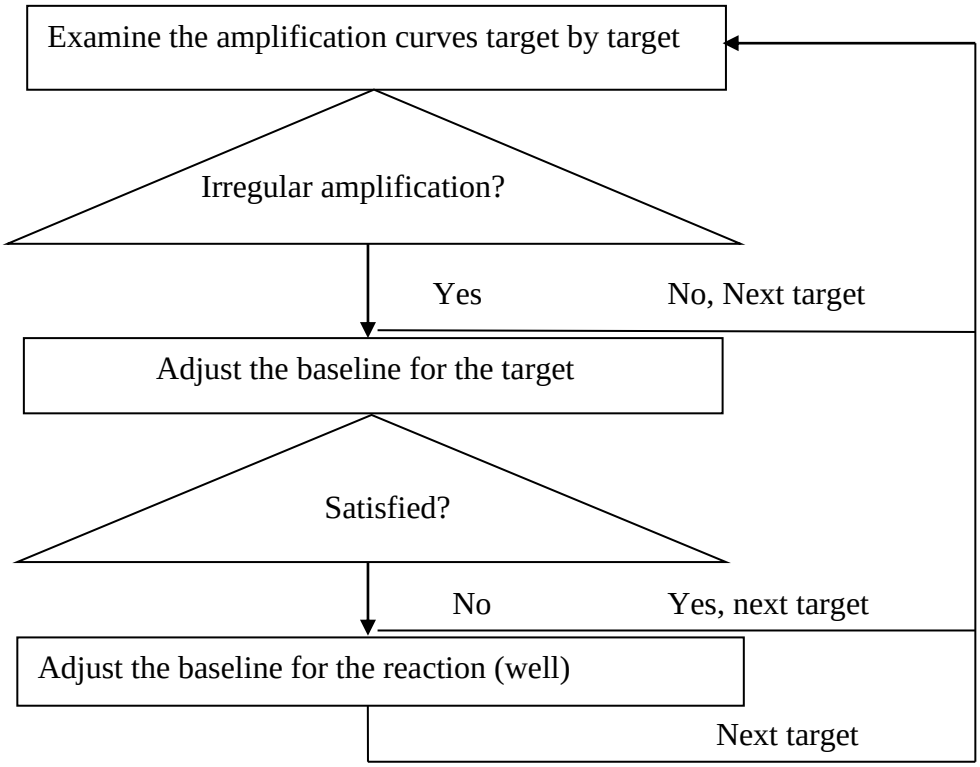


Figure 3-4: **Procedure for TaqMan array card data analysis**

3.8.4 Reverse transcriptase (RT)-PCR assay to determine viral serotype

3.8.4.1 RNA extraction

To confirm and ascertain the specific serotype of a dengue virus that was detected by the TaqMan array real time PCR, viral RNA was extracted from serum and purified using the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) following the manufacture's protocol. In this case, 140 µL of the serum sample was added to 560 µL of prepared Buffer AVL (AVL + RNA carrier), mixed by pulse-vortexing for 15 seconds and incubated at room temperature for 10 minutes. The RNA carrier was included to facilitate the binding of viral nucleic acids to QIAamp mini membrane and inactivate any RNase present (reducing the chance of viral RNA degradation). RNA was precipitated by adding absolute ethanol and the sample mixture was transferred into a spin column and centrifuged at 6000g (or 8000 rpm) for 1 minute. The filtrate in the collecting tube was discarded and the column placed in a fresh collection tube and washed twice with AW1 and AW2 buffers by centrifugation at 6000 g (or 8000 rpm) for 1 minute and 20000 g (14000 rpm) for 4 minutes, respectively. The purified viral RNA was eluted in 60 µL AVE buffer and stored at -20 °C until further use in the RT-PCR assay. The quantity and purity of the extracted RNA was checked using nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The ratio of RNA absorbance at 260 nm to 280 nm is generally used to assess the RNA purity and a ratio value of ~ 2.0 is considered as "pure" for RNA.

3.8.4.2 Conventional RT-PCR for dengue virus

Conventional RT-PCR was performed using the protocol described by Lanciotti et al, (1992) in Aeris G-96 well PCR system (Esco Micro Pte Ltd, Singapore) to target the viral envelope glycoprotein. The RNA extracted was amplified in a 25 µL reaction volume containing the following components: 10 mM Tris (pH 8.5), 50 mM KCl, 1.5

mM $MgCl_2$, 199 µM each of the four deoxynucleotide triphosphates, 5 mM dithiothreitol and 50 pmol for each primer.

DUC (5'-TCAATATGCTGAAACGCGCGAGAAACCG-3') and

DUS (5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'), 2.5 U of Amplitaq polymerase (Perkin Elmer, Norwalk, Conn) and 2.5 U of RAV-2RT. The PCR thermocycler programming included 1 hour incubation at 42 °C with 45 cycles of denaturation (94 °C, 30 seconds), primer annealing (55 °C, 1 minute), and primer extension (72 °C, 2 minutes). The PCR amplification products were electrophoresed on 2 % agarose gel (Peqlab Biotechnologie, Erlangen, Germany), stained with ethidium bromide, and viewed under UV light.

The PCR assay was conducted at the biosafety level-3 laboratory at the Department of Virology of the NMIMR, University of Ghana.

3.8.5 Dengue virus RNA sequencing

To confirm the identities of the circulating dengue virus serotypes detected by TaqMan PCR, the reaction mix for sequencing was set up using Big Dye Terminator (v3.1) cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA). Primer sequences for the amplification of the PCR fragments and for sequencing were; DUC (Forward primer); 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' and DUS (reverse primer); 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'. The reaction contained 1.5 µL for each primer (1 µM) in 10 µL final volume. The Dye-labeled PCR products were sequenced using an ABI 3130 sequencer (Applied Biosystems Carlsbad, CA, USA) following the manufacturer's protocol. The sequence chromatograms were manually edited using Sequencer 4.7 software (Gene Codes, USA). The edited sequence data obtained (forward and reverse) were used to create consensus (Contig) sequence designated as WACCBIP AFI/GH/227/2017 (Contig) and WACCBIP

AFI/GH/299/2017 which have been deposited in the GenBank with accession numbers MG937762 and MG937763, respectively.

3.8.6 Phylogenetic analysis of dengue viral sequences

Eighteen sequences, representative of the four known dengue virus genotypes (DENV-1, DENV-2, DENV-3, and DENV-4) were retrieved from GenBank and included in the data set used to establish the phylogeny of the two Ghanaian viral strains. The non-structural protein 5 (NS5) gene sequences were aligned using the Clustal W algorithm of MEGA version 7 software package (www.megasoftware.net). Following the determination of nucleotide substitution model, the genetic distance (nucleotide substitutions per site) was estimated using the Kimura-2 parameter model. Any non-uniform evolutionary rates among the sites were corrected using a discrete gamma distribution with 5 rate categories and basing on the assumption that some fraction of sites were evolutionarily not changing. All positions containing gaps and missing data were removed to ensure good alignment. Phylogeny was inferred by constructing a phylogenetic tree using the Maximum Likelihood method of MEGA (Kumar et al., 2016). Statistical support for tree branching was provided by 1000 bootstrap replicates and values ≥ 70 were considered significant.

3.9 Modelling infection status using symptoms and hematological parameters

3.9.1 Data types and pre-processing

Up to 30 clinical signs and symptoms obtained from the enrolled children (e.g. cough, headache, skin rashes etc.) and 15 hematological parameters (WBC, RBC, platelet etc) were used to model the infection status of patients. Malaria parasites were detected using microscopy and RDT tests. Other pathogens (bacteria viruses, parasites) were detected by TaqMan array qPCR (TAC) and microbial cultures.

Clinical symptoms, location, sex, age and hematological parameters were used to predict the malaria and bacterial infection status of AFI children using diagnostic test outcomes as proxy. Specifically, the bacterial infection status was predicted based on blood culture and urine culture. Malaria infection status was also predicted based on RDT, microscopy and TAC-PCR. The diagnostic tests outcomes were binary and coded as 1 if test is positive (if pathogens were detected) or 0 if the test was negative (no pathogen detected). Most of the covariates (e.g. clinical symptoms and location of hospital) were also binary and entered as such. Body temperature and full blood count parameters were entered into the models as either continuous or categorical variables. The variables were further categorized into three groups based on their 25th, 50th and 75th percentile. Patient's age was categorized into three groups: 1-2, 3-5 and 6-15 years old and entered into the model as a categorical variable.

3.9.2 Association of the selected predictors and data classification

Chi square test (α=0.05) was used to test the association of all identified variable that could serve as predictors for the model. Variables that were found to be associated with the test outcomes were duly classified and assigned as true associated variables. After determining the associated variables, the resulting data were split into 80% training and 20% test sets and used for the training and testing of the model as outlined in Figure 3.5.

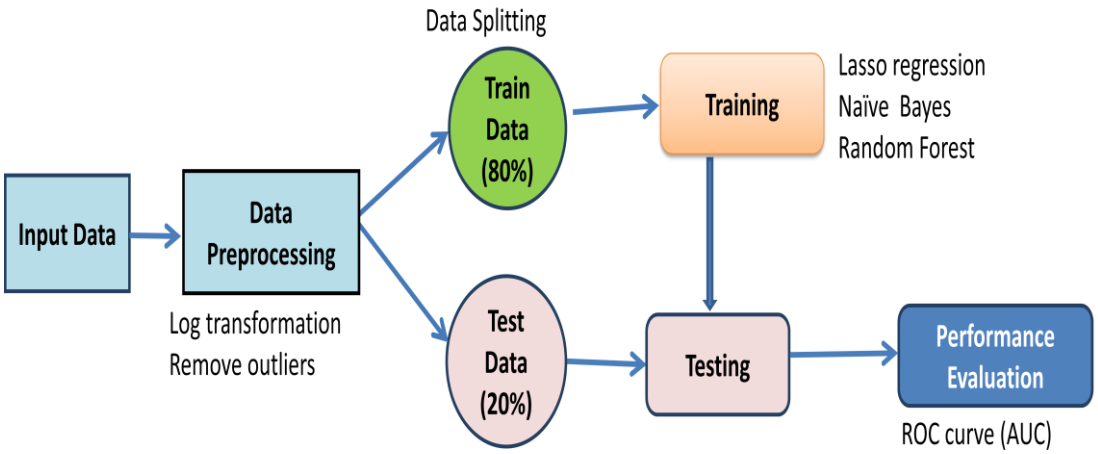


Figure 3-5: Workflow for data classification for model development

3.9.3 The model

Three machine learning modeling approaches were proposed to predict the infection status of patients (or test outcomes), namely (1) Lasso regression, (2) Random forest and (3) Naïve Bayes classifier. Briefly, Lasso regression uses regression coefficient shrinkage method to "select" variables. Given a suitable penalty term λ, Lasso regression reduces most regression coefficient to zeros, eliminating covariates that are not useful for prediction and thereby reducing over fitting to improve predictions. Since not all symptoms were recorded for all observations, some covariates were pre-screened (i.e. covariates with more than 10% missing values were removed before fitting the model). Hence all observations with missing covariates were omitted during the model fitting. We fitted Lasso regression using glmnet package in R and cross validation was used to determine the optimal penalty term λ using cv.glmnet function.

Random forest is a popular Machine Learning technique that is non-parametric and based on decision trees. It is very flexible and can account for complex interaction among covariates. By this method, several decision trees were fitted to bootstrap training samples. To decorrelate these trees, a random sample of m predictors were

chosen from p covariates as split candidates, i.e. whenever there is a split in a tree, only a small subset of p covariates could be used as splitting criteria. The decision trees were then aggregated to predict the response. Just like the Lasso regression, Random forest does not "tolerate" missing data and so we prescreened the variables before fitting. We fitted the random forest using Random Forest package, and cross validation was used (implemented using train function in caret package) to find the optimal parameter m.

Naïve Bayes classifier is a simple probability-driven classifier based on application of Bayes' theorem. Naïve Bayes endorses the use of Bayes classifier to accommodate multiple covariates by assuming that all features or predictors are independent of each other. Therefore, under this model, the symptoms and the location of hospital were assumed to be independent.

To simplify the calculation, we used categorized versions of body temperature and hematological parameters, in addition to the signs and symptoms in this model. The probability of positive test outcome (i.e. infection status), given a patient's set of symptoms or blood test results (hematological parameters) $S = (S_1, S_2, \dots)$ and location L is given as follow:

$$P(T = 1|S, L) = \frac{P(S, L|T = 1)P(T = 1)}{\sum_{k=0}^{1} P\,(S|T = k)P(T = k)} = \frac{[\prod_i P\,(S_i|T = 1)]P(L|T = 1)P(T = 1)}{\sum_{k=0}^{1}[\prod_i P\,(S_i|T = k)]\,P(L|T = k)P(T = k)}$$

Where $P(S_i|T = k)$, $P(L|T = k)$ and $P(T = k)$ are estimated from the data. Although the Naïve Bayes' strong assumption of independence of all variables is often unrealistic, it is able to deal with all features with missing values by dropping the probability term that corresponds to missing symptom S_j from$\prod_i P\,(S_i|T = 1)$. Naïve

Bayes classifier was fitted using customized codes. Since "blood test" results require more time and effort, we tested to see if it provided extra predictive power in combination with basic demographic information and clinical symptoms. All the three models were fitted using two sets of predictors. The first set contains only the symptoms, location and patient's age. The second set contained symptoms, location, age and the blood test readings (or hematological parameters). As a result, six models were compared in this study, thus 3 types of models multiplied by 2 sets of predictors.

3.9.4 Cross validation

We assessed the predictive performance of the models based on their out-of-sample predictive skill using ten-fold cross validation. The dataset was randomly divided into ten subsets. In the first fold of cross validation, the first two subset was the test set (held out from model fitting), and the remaining eight subsets were the training set. The training set was used to fit the model and predict the outcome of the test set (Figure 3-5).

To deal with the problem of missing data, Lasso regression and Random forest were refitted for each observation in the test set. Thus, all predictors with missing data (for the observations) were omitted from model fitting. In the case of Naïve Bayes classifier, the models were fitted by estimating the probabilities of using only the training set. Since Naïve Bayes can accommodate missing predictors, it was not refitted for each observation in the test set. Predicted probability of each observation in the test set was then obtained, and the procedure was repeated nine times by treating data from the second to the tenth subsets as test set. As a result, each observation was held out exactly once throughout the process and had an out-of-sample prediction. Using the out-of-sample predictions for individual observations, misclassification rate was

calculated (by classifying observation with probability > 0.5) and the area under the receiver operating characteristic (ROC) curve was determined. These metrics served as the out-of-samples prediction to measure the performance of the models. We also calculated the sensitivity and specificity of the models based on two rules: (1) by assuming the cut-off probability of 0.5 (equivalent to misclassification rate), and (2) by setting minimum sensitivity at 0.8. The latter can be done by examining the ROC curve. We then designed the interactive tool known as 'shiny' package in R, which allowed creation of a web application using R language and required little knowledge of HTML, CSS and JavaScript.

3.10 Data analysis

The data was analyzed using STATA version 12 special edition. All categorical variables were summarized as percentages and frequencies. Continuous variables following Gaussian distribution were summarized as means and standard deviations (SD), while the continuous variables that were skewed were summarized as median and inter quartile range (IQR). The sensitivity, specificity, positive predicted value, negative predicted value, odds ratios (OR) and the corresponding 95% confidence intervals were reported. Predictive values were obtained for test outcomes such as blood culture, urine culture, malaria microcopy and RDT and malaria TAC, or negative test. Naïve Bayer classifiers analysis was employed to validate the prediction equation. All explanatory variables were treated as continuous, except for sex which was dichotomized. Mean age differences and proportion were tested by the two-tailed t-test and the logistic regression analysis was used to examine the association of parental and environmental characteristics with respect to infection.

ROC curves were generated and the area under the curves, including the 95% CI, was calculated for each biomarker. The goodness-of-fit of the Machine Learning models was assessed by the Hosmer-Lemeshow logistic test. Purposeful selection was used to build the model, such that all predictors at $p \geqslant 0.50$ level of significant in univariate analysis were included in the final models. The Naïve Bayer classifier was applied in R software to make a "prediction equation" for the test outcome in the development of a web-based tool.

CHAPTER FOUR

4 RESULTS

4.1 Clinical, demographic and risk factors for infections of the study participants

1,513 participants aged from 1 to 15 years were recruited into the study. The participants were made up of 858 (57%) febrile and 655 (43%) afebrile children.

Table 4-1: Demographic and clinical parameters of study children

Characteristics	Study groups		
	Febrile (858)	**Afebrile (655)**	***p-value***
Age (years)			
Median (IQR)	3 (4.0)	3 (5.0)	0.011
Age categories (n, %)			
< 5	620 (72.3)	398 (60.8)	0.026
5-10	191 (22.6)	182 (27.8)	
>10	47 (5.1)	75 (11.4)	
Sex (n,%)			
Males	464 (54.1)	336 (51.3)	0.017
Females	394 (45.9)	319 (48.7)	
*Temperature (ºC)			
Median, (IQR)	38 (1.0)	36 (1.0)	0.036
Fever grade (n, %)			
Mild	594 (69.2)	0 (0.0)	
Moderate	219 (25.5)	0 (0.0)	
Severe	45 (5.3)	0 (0.0)	
Anthropometrics			
Weight (kg)			
Median (IQR)	13 (8.0)	14 (11.2)	0.450
Height (cm)			
Median (IQR)	89 (31.0)	90 (40.4)	0.023
Common clinical symptoms (n, %)			
Vomiting	239 (27.9)	207 (31.6)	0.016
Diarrhea	185 (21.6)	146 (22.3)	
Headache	489 (57)	356 (54.4)	
Cough	331 (38.6)	297 (45.3)	
Abdominal pain	183 (21.3)	108 (16.5)	
Lethargy	6 (0.7)	0 (0.0)	

*= Axillary temperature at enrolment (°C), mild fever = 37.5°C to < 39 °C), moderate fever (39 °C to < 40 °C), severe fever = ≥40 °C. IQR= inter quarter range, y= year, n= frequency and %= percentage of children

Among the febrile children, 363 and 495 were enrolled at Kintampo and LEKMA hospitals respectively. The median age of the febrile children from both study sites was 3 years (IQR= 4) and 54.1% of them were males (Table 4.1). With the median temperature of 38.4 °C, majority of the febrile children (69.2%) had mild body temperature (37.5 °C to < 39 °C) and 57% of them complained of headache. The median temperature of the afebrile children was 36 °C and 51% were males. There was no difference in the median age of two groups of children. The febrile children were matched with afebrile children as a control in the evaluation of cytokines and hematological parameters as potential markers of different pathogens. Most of the febrile children belong to the Akan ethnic group (25.4%); followed by the Ga-Adangbe ethnic group (23.2%) and less than 5% of the children did not know their ethnicity (Figure 4.1).

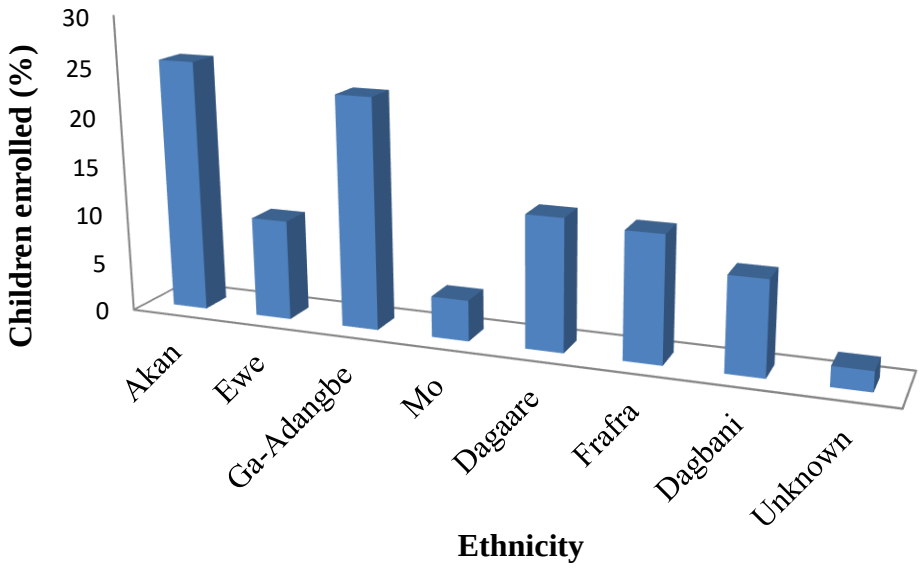


Figure 4-1: Percentage distribution of children enrolled in the study by ethnicity

To determine the risk factors that predispose children to zoonotic pathogens, the primary sources of drinking water and contact with domestic animals, prior to enrolment into the study were assessed. Drinking water for most of the febrile children was drawn from sources such as unprotected wells and rivers (Table 4.2). However, just over 20% had access to improved or potable drinking water (piped-borne, boreholes and sachet water). It was found that 15% (129/858) of febrile children have had contacted with domesticated animals, two weeks prior to seeking treatment for the current illness. After controlling for primary source of water including borehole, pipe-borne, sachet water, well and river water, it was found out that the risk of getting infected with pathogens in children using well water as a source of drinking water was increased by 120% relative to those who did not used well water (OR= 2.25, 95% CI; 0.91-0.97, p = 0.010). In other words, children who used well water were 2.3 times more likely to get infection compared to those who did not drink well water. The risk of the children who used sachet water, to get infected with pathogens, reduced by 45% relative to those who did not use sachet water (OR=0.55, 95% CI; 0.31-0.97, p= 0.038).

After controlling for animal contacts such as dogs, cats, cattle and others (Table 4.3), it was found that the risk of children who had contact with both cats and dogs to get infection increased by 314% relative to those who did not have contacts with cats and dogs (OR=4.14, 95% CI; 2.27-7.57, p= 0.031). However the risk of infection reduced by 35% among those who had contact with only cat compared to those who did not have contact with cat alone (OR=0.65, 95% CI; 0.37-1.16). However the association was not statistically significant (p = 0.189). The details of the association between animal contacts and risk of infection are summarized in Table 4.3.

Table 4-2: Binary logistic regression analysis of source of drinking water and risk of infection in febrile children

Characteristic	Infection n (%)	unadjusted analysis		*adjusted analysis	
		odds ratio (95% CI)	*p-value*	odds ratio (95% CI)	*p-value*
Source of drinking water					
Bore hole					
No	32 (3.7)	1		1	
Yes	10 (1.2)	0.32 (0.11-0.89)	0.001	0.27 (0.10-0.80)	0.002
Pipe borne					
No	84 (9.8)	1		1	
Yes	48 (5.6)	0.09 (0.61-5.28)	0.005	0.70 (0.63-0.87)	0.004
Sachet water					
No	371 (43.2)	1		1	
Yes	122 (14.2)	0.62 (0.41-0.99)	0.041	0.55 (0.31-0.97)	0.038
Well					
No	41 (4.8)	1		1	
Yes	61 (7.1)	3.01 (0.80-0.99)	0.008	2.25 (0.91-0.97)	0.010
River					
No	26 (3.1)	1		1	
Yes	63 (70.8)	2.93 (0.11-0.84)	0.001	3.83 (0.66-0.79)	0.001

n = number of children, Infection = presence of any disease causing pathogen, CI= confidence interval, No= no infection, Yes = presence of infection, *source of drinking water adjusted

Table 4-3 Binary logistic regression analysis of animal interaction and risk of infection in febrile children

Characteristic	Infection n (%)	unadjusted analysis		*adjusted analysis	
		odds ratio (95% CI)	*p-value*	odds ratio (95% CI)	*p-value*
Animal contact					
Cat					
No	59 (6.9)	1		1	
Yes	29 (3.4)	0.98 (0.65-1.30)	0.196	0.65 (0.37-1.16)	0.189
Dog					
No	48 (5.6)	1		1	
Yes	27 (3.1)	0.85 (0.82-1.66)	0.495	0.75 (0.42-1.32)	0.385
Both Cat and Dog					
No	9 (1.1)	1		1	
Yes	23 (2.7)	5.10 (3.11-9.23)	0.011	4.14 (2.27-7.57)	0.031
Fowls					
No	50 (5.8)	1		1	
Yes	17 (2.0)	1.98 (1.45-4.00)	0.041	1.81 (1.09-3.34)	0.043
Sheep/goat					
No	16 (1.9)	1		-	-
Yes	29 (3.4)	1.32 (0.92-1.99)	0.201	-	-
Cattle					
No	31 (3.6)	1		-	-
Yes	14 (1.6)	4.14 (2.27-7.57)	0.340	-	-
No animal contact					
No	377 (43.9)	1		-	-
Yes	129 (15.0)	1.07(0.97-1.18)	0.154	-	-

n = number of children. Animal contact = contacted with animals two weeks prior to enrolment, Infection = presence of any disease-causing pathogen, CI= confidence interval, No= no infection, Yes = presence of infection, *animal contact were adjusted

4.2 Correlates of caregivers characteristics and infection status of their children

A high proportion, 85.2% (751/858), of the caregivers of the children was mothers, 10.5% (90/858) were house helps and 1.98% (17/858) was fathers. Caregiver's educational attainment and occupation type were assessed to determine if they could serve as risk factors of their children getting infected with one pathogen or another (Table 4.4). There was an association between caregivers' level of education and infection. The risk of infection reduced with increasing education from basic to tertiary levels. Close to 13% of the febrile children who had infections have their caregivers having basic education whilst only 2% of febrile children with infections had caregivers with tertiary level of education. After controlling for educational level (basic, secondary, tertiary or no education) (Table 4.4), children whose caregivers had no formal education had increased risk of infection (261%) compared to those whose caregivers had formal education (OR= 3.61, 95% CI; 0.01 - 0.84, p = 0.012). Such children were 3.6 times more likely to infections compared to those whose caregivers had education.

Association between the caregiver's occupation and infections was observed (Table 4.4). Children whose caregivers were farmers were 2 times more likely to contract infections relative to those whose caregivers were not farmers (OR= 2.01, 95% CI; 1.65 - 7.10, p = 0.034). Children whose caregivers were professional had a reduced risk of getting infections (61%) when compared to non-professional caregivers (OR= 0.39, 95% CI; 0.31- 0.98, p = 0.046).

Table 4-4: Binary logistic regression analysis of socio-demographics of caregivers and infection status in febrile children

Characteristic	Infection n (%)	unadjusted analysis		*adjusted analysis	
		Odds ratio (95% CI)	*p-value*	Odds ratio (95% CI)	*p-value*
Education					
No Education					
No	80 (9.3)	1		1	
Yes	145 (16.9)	3.96 (0.66 - 0.98)	0.001	3.61 (0.01 - 0.84)	0.012
Basic					
No	159 (18.5)	1		-	-
Yes	111 (12.9)	1.00 (0.47 - 1.94)	0.902	-	-
Secondary					
No	154 (18.0	1		1	
Yes	89 (10.4)	0.92 (0.47 - 1.66)	0.398	0.72 (0.41 - 1.26)	0.314
Tertiary					
No	103 (12.0)	1		1	
Yes	17 (2.0)	0.15 (0.07 - 0.33)	0.001	0.18 (0.09 - 0.36)	0.001
Occupation					
Farmer					
No	38 (4.4)	1		1	
Yes	126 (14.7)	1.99 (0.51 - 0.79)	<0.001	2.01 (0.65 - 0.10)	0.034
Trader					
No	293 (34.1)	1		1	
Yes	154 (18.0)	0.80 (0.46 - 1.30)	0.252	0.71 (0.40 - 1.25)	0.247
Professional					
No	114 (13.3)	1		1	
Yes	44 (5.1)	0.30 (0.20 - 0.99)	0.012	0.39 (0.31- 0.98)	0.046
Unemployed					
No	20 (2.3)	1		1	
Yes	69 (8.1)	0.04 (0.01- 1.06)	0.432	0.99 (0.85- 1.14)	0.867

Infection=presence of any kind of pathogen, professionals= occupation as a paid job or worker in a paid job that requires some degree of training and skill, n= number of children, CI= confidence interval, %= percentage, *level of education and occupation types adjusted

4.3 Detection of AFI associated pathogens

4.3.1 Malaria infection

In all, malaria parasites were detected by PCR and microscopy in 38.5% (331/858) of febrile children. Malaria RDT was positive for 97.3% (322/331), giving a false positive rate of 2.7% (9/331) after confirmation by only PCR as gold standard. Of the PCR and microscopy positive malaria cases, 99.4% (329/331) were *P. falciparum* monoinfections and 0.6% (2/331) was *P. falciparum/ovale* mixed infections (Table 4.5). The number of participants who were RDT and microscopy positive for malaria from Kintampo and LEKMA were 63.6% (231/363) and 20.2% (100/495), respectively. Seven (7) out of the 9 discordant RDT-microscopy malaria results were confirmed as positive for *P. falciparum* by PCR with only 2 tested negatives by both PCR and microscopy. Parasite densities varied greatly in the two sites, with a median parasite count of 65,166/µL for Kintampo children and 13,380/µL for children in LEKMA. Thirteen (13) of the febrile children from both study sites were diagnosed to have severe malaria but none were classified as cerebral malaria. All the severe malaria cases were observed in children less than 5 years old.

4.3.2 Urinary tract infections

Urinary tract infections (UTIs) were diagnosed clinically in 15.9% (137/858) of the children, out of which only 24.1% (33/137) were culture positive (Table 4.5). Close to seventy-six percent, 75.9% (104/137) of urine from the suspected UTI children tested negative by culture. The most common pathogens isolated were *Escherichia coli and* Group D *streptococcus*, each constituting 21.2% (7/33); followed by *Enterobacter cloacae*, 15.1% (5/33) and *Pseudomonas aeruginosa*, 12.1% (4/33). The frequencies of other pathogens isolated are summarized in Table 4.5 and they include *Citrobacter freundii, Enterococcus spp,* non-typhoidal Salmonella, *Klebsiella pneumoniae and*

Staphylococcus aureus. The proportion of clinically diagnosed UTIs was highest among children more than 5 years old as compared to those less than 5 years. Moreover, UTI infections were more common with girls, 64% (88/137) than boys 35.8% (49/137) and this was statistically significant (p=0.045).

4.3.3 Bacteremia

Bloodstream infections due to bacteria were suspected in 16.3% (140/858) of febrile children recruited in the study by the study clinicians. In all, only 17.9% (25/140) of bacteremia diagnosed cases were confirmed through culturing by expert bacteriologist at NMIMR and KHRC, including 10.7% (15/140) in Kintampo and 7.1% (10/140) at LEKMA. No bacteria were identified in 82.1% (115/140) of the blood cultures requested by the attending clinicians. *Staphylococcus* spp. (10 *S. aureus* and 4 CNS) were the most commonly isolated organisms, accounting for 56.0% (14/25) of the isolates (Table 4.5). The second most common species identified was *Salmonella species* (3 NTS and 2 *S*. Typhi), constituting 20.0% (5/25). Other bacteria isolated were *Streptococcus pneumoniae*, 8.0% (2/25), *Pseudomonas aeruginosa* 6.7% (1/15), *Escherichia coli*, 8.0% (2/25) and *Klebsiella pneumoniae* 4.0% (1/25). Whilst 40% (10/25) of bacteremia cases were reported at LEKMA, that of Kintampo was 60% (15/25) and the difference was not significant statistically (p=0.051). The proportion of bacteremia increased with age and was highest in children aged more than 5 years, for whom the rate was 56% (14/25).

4.3.4 Gastrointestinal infections

Gastrointestinal infections were clinically diagnosed in 31.1% (267/858) of the study cohort. The most affected group was children aged less than 2 years, constituting 46.1% (123/267). Pathogens were detected in only 9.4% (25/267) of children screened for

gastrointestinal parasites, with intestinal flagellates being the predominant pathogen, being detected in 48.0% (12/25), followed by helminthes infection, 36.0% (9/25), and giardia infection, 16% (4/25) as shown in Table 4.5. No pathogen was detected in the stool samples of 90.6% (242/267) of the children tested. The proportion of gastrointestinal infections was non-significantly higher among children without malaria infection, 39.8% (210/527) than those with malaria infection, 17.2% (57/331).

Clinician diagnosed pneumonia and lower respiratory tract infections was 15.3% of the total children screened (Table 4.5). However, laboratory analyses of the nasopharyngeal samples collected have not been performed due to financial constraints. Hence incidence of respiratory tract pathogens among both febrile and afebrile children remained undetermined.

Table 4-5: Common clinically diagnosed diseases and pathogens detected using phenotypic-laboratory methods

Diseases	[#]Clinical diagnosis N (%)	Laboratory Confirmation n (%)	Species isolated	Frequency n (%)
Malaria	433 (50.1)	331/433 (76.4)	**P. falciparium*	329/331(99.4)
			**P. ovale*	2/231(0.9)
UTI	137 (29.5)	33/137 (24.1)	*E. coli*	7/33 (21.2)
			K. pneumoniae	1/33 (3.0)
			C. freundii	3/33 (9.1)
			E. cloacae	5/33 (15.2)
			E. spp	3/33 (9.1)
			GDS	6/33 (18.2)
			NTS	1/33 (3.0)
			P. aeruginosa	4/33 (12.1)
			S. aureus	3/33 (9.1)
Bacteremia	140 (16.3)	25/140 (17.9)	*K. pneumoniae*	1/25 (4.0)
			CNS	4/25 (16.0)
			E. coli	2/25 (8.0)
			NTS	3/25 (12.0)
			[$]*P. aeruginosa*	1/25 (4.0)
			S. Typhi	2/25 (8.0)
			S. aureus	10/25 (40)
			S. pneumoniae	2/25 (8.0)
Gastrointestinal infection	267 (31.1)	25/267(9.4)	*Giardia* spp	4/25 (16.0)
			Intestinal flagellates	12/25(48.0)
			Helminthes	9/25 (36.0)
Viremia	2 (0.2)	1/2 (50)	HIV-1	1/2 (50)
LRTI and pneumonia	131(15.3)	-	-	-
Non-microbial causes	20 (2.3)	-	-	-

K.pneumoniae =Klebsiella pneumoniae, C. freundii= Citrobacter freundii, E.cloacae= Enterobacter cloacae, Enterococcus spp= Enterococcus spp, GDS*= Group D streptococcus,* NTS= non typhoidal Salmonella, *P. aeruginosa= Pseudomonas aeruginosa, S. aureus=Staphylococcus aureus, CNS*= Coagulase negative staphylococcus, *E.coli = Escherichia coli, S.* Typhi = *Salmonella* Typhi, *S. pneumoniae= Streptococcus pneumoniae,* UTI= urinary tract infections, LRTI = lower respiratory tract infections, n= number, %=percentage, HIV= Human immunodeficiency virus, *P =*Plasmodium,* [$]*P=Pseudomonas,*[#]clinical diagnosis=diseases diagnosed by clinicians based on signs and symptoms only as obtained from patient's folder

4.4 Molecular screening and detection of AFI associated pathogens

Due to the limitations of the phenotypic-based pathogen detection methods, molecular based methods were employed to further investigate the etiology of the cases for which no pathogens had been identified. Using a multi-pathogen TaqMan real-time PCR–based assay, malaria parasites were detected in 37.9% (63/166) of the blood samples that tested negative by phenotypic-based laboratory methods. Zoonotic bacterial pathogens were detected in 3.6% (6/166) of the children screened. These included *Rickettsia felis*, 3.0% (5/166) and *Coxiella burnetti*, 0.6% (1/166). Viruses detected included HIV, 0.6% (1/166) and dengue virus, 1.2% (2/166) (Table 4.6).

Table 4-6: Pathogens detected among children diagnosed with acute febrile illness using customized AFI TaqMan array card

Pathogen detected	Frequency (%)
Plasmodium spp	61 (36.8)
Dengue virus	2 (1.2)
***Salmonella enterica* Typhi**	1 (0.6)
Rickettsia felis	5 (3.0)
Coxiella burnetti	1 (0.6)
HIV	1 (0.6)
No pathogen detection	95 (57.2)

HIV= human immunodeficiency virus, spp= species %= percentage

With the threshold cycle of less than 30, the amplification curves obtained by plotting amplification signals (ΔRn) against the number of cycles for the two samples which tested positive for dengue using the real time TaqMan array cards is presented in Figure 4.2. The amplification plots for other organisms which tested positive showed similar amplification curves but different quantification threshold (Ct) values which are not showed.

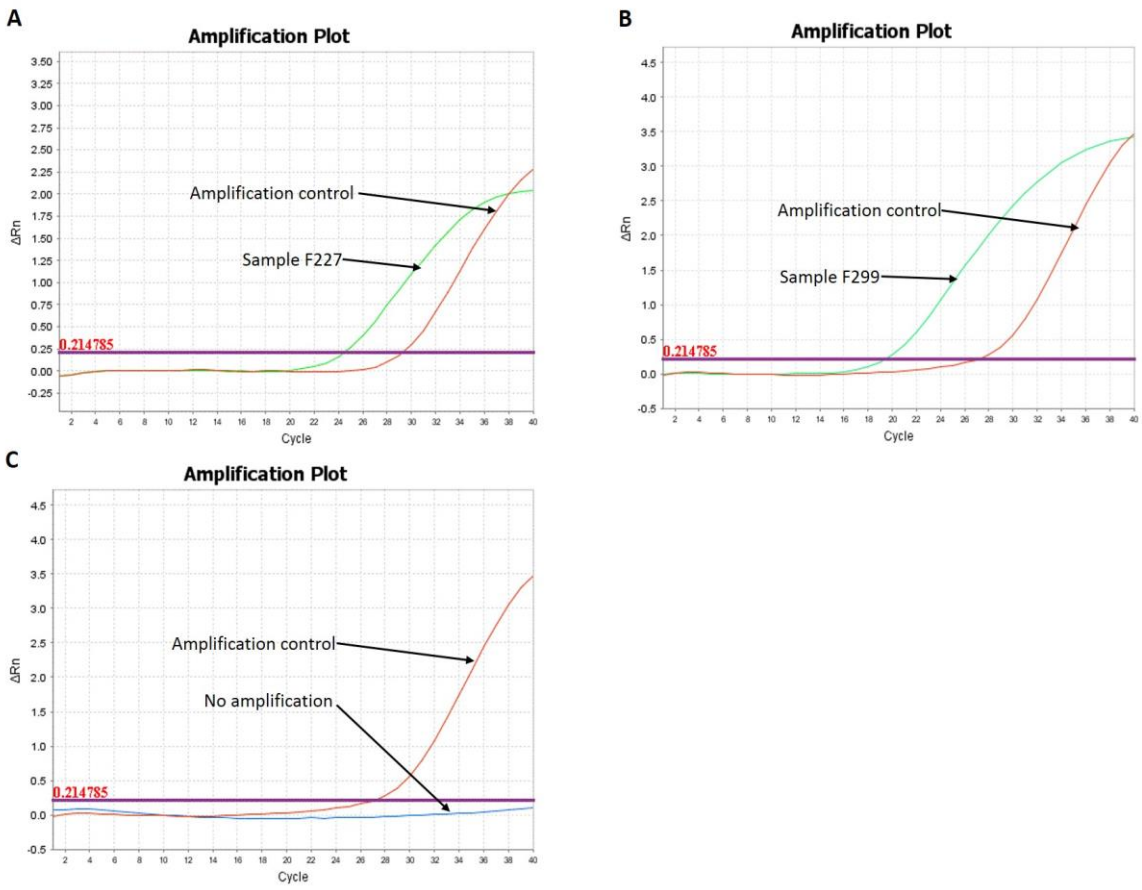


Figure 4-2 AFI-TaqMan array card amplification plots for two dengue positive samples and one negative sample

Between October 2016–July 2017, venous blood samples (2.5 mL of the 5.0 mL collected into EDTA vacutainer tubes) obtained from 166 febrile children who tested negative for laboratory methods used were screened for 26 AFI associated pathogens by using the real-time PCR customized TaqMan array cards. The cards have been designed in 384-well format, and each well contained 1 µL of reaction mixture (0.75 µL of the extracted total nucleic acid plus 0.25 µL of TaqMan Fast Virus 1-step Master Mix; Applied Biosystems, Foster City, CA, USA). The assays were run on Quant Studio 7 Flex real-time PCR system from Applied Biosystems, following the manufacturer's instructions without modifications. Two samples from the children tested positive for dengue virus: A) F227, which amplified with critical Ct of 24.40; and B) F299, which amplified with Ct of 19.35. C) a negative test showed by amplification signals (ΔRn) below the diagnostic threshold levels at Ct cutoff of 35. Nucleic acids of known concentration were included in the assay to serve as amplification controls (showed in plots). Two external controls (PhHV (10^6 copies) and MS2 (10^7 bacteriophage)) were spiked during the assay to monitor extraction and amplification efficiency, and one negative control was included for each batch of extraction to check laboratory contaminations. Ct represent cycle threshold.

Since the detection of dengue virus in the samples was unprecedented in Ghana, we sought to confirm these results using additional molecular methods. The presence of dengue virus in the samples was thus confirmed by conventional RT-PCR (Lanciotti et al., 1992) with the expected amplicon band size of 511bp (Figure 4.3).

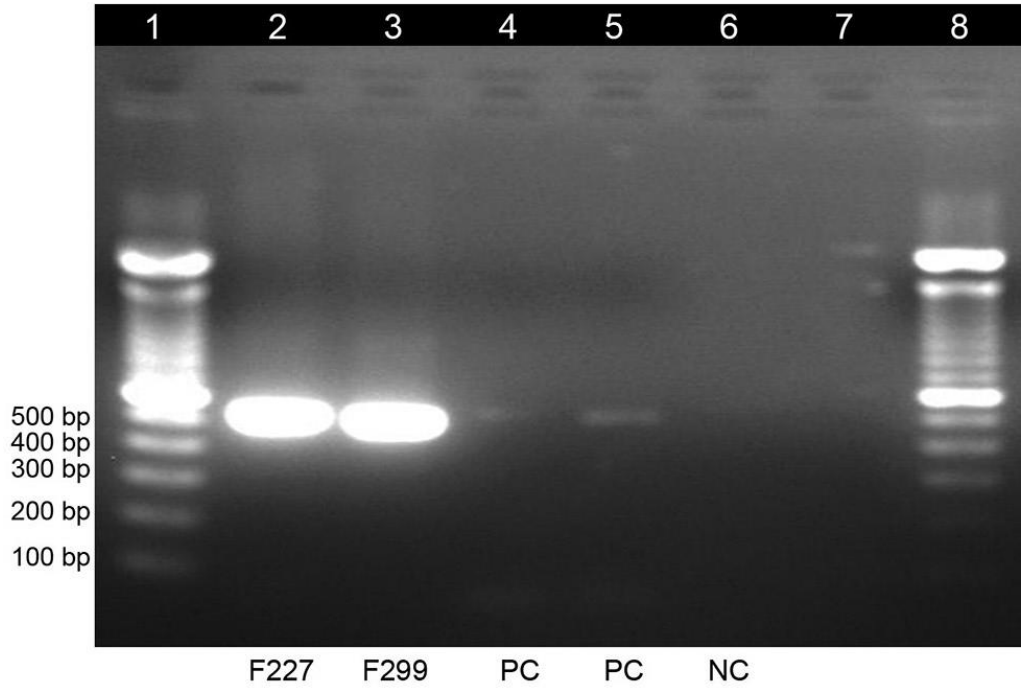


Figure 4-3: Agarose gel electrophoresis of dengue-specific RT-PCR product
The amplification products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and viewed under ultraviolet light. Lane 1 is a molecular weight marker; lanes 2 and 3 are test samples; lanes 4 and 5 are positive controls; lane 6 is negative control; lane 7 is empty and lane 8 is a molecular marker.

4.5 Serotyping by BLAST and neighbour-joining phylogenetic analysis of the Dengue virus

The nucleotide sequences of the detected virus strains have been deposited in GenBank under accession numbers MG937762 and MG937763. A BLAST search (https://blast.ncbi.nlm.nih.gov/blast.cgi) using a default settings of generated nucleotide sequences of the Ghanaian isolates showed that the two viruses were identical (100% homology) and closely related to the dengue virus serotype-2 strains isolated in epidemic outbreak in Burkina Faso in 2016 (GenBank accession number, KY627763.1). Phylogenetic analysis of the two isolates from Ghana when grouped with other African isolates showed a cosmopolitan genotype (Figure 4.4). Although the viruses showed a close resemblance to the Burkina strain, the phylogram demonstrated a unique lineage for the Ghanaian isolates.

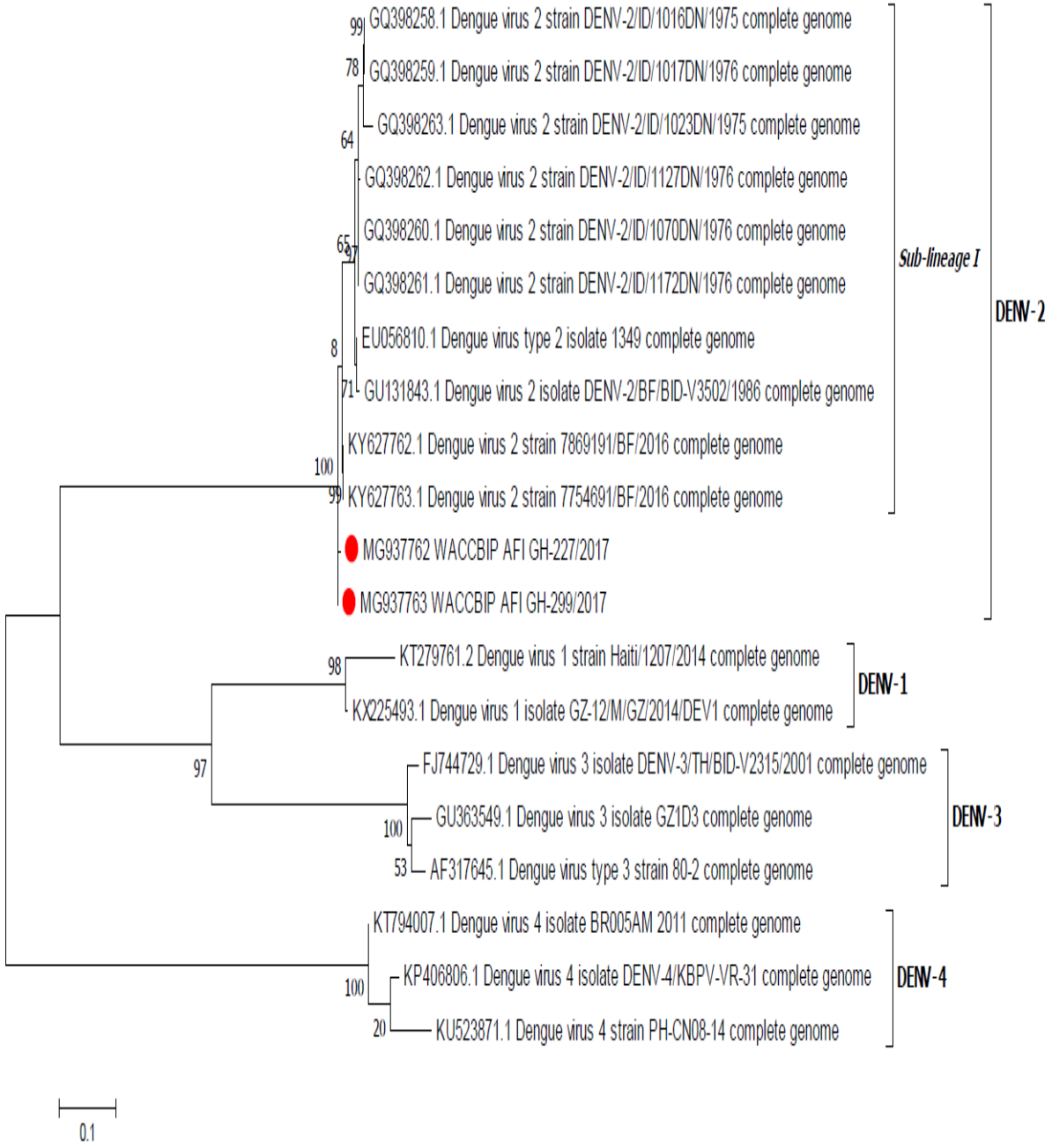


Figure 4-4: Phylogenetic analysis of dengue virus serotype-2 detected in Ghana
The phylogenetic tree was constructed using neighbor-joining method. Complete genomes obtained from 18 other dengue serotypes, DENV-1 to DENV-4 were included with DENV-2 sub-lineage. The red spot indicates strain sequence in this study. Scale bar indicates nucleotide substitutions per site.

4.6 *Plasmodium* co-infections among the febrile children

Among the febrile children screened with various methods, pathogens were detected in 43.7% (374/858) of them. Whilst 38.6% (331/858) of the children had *Plasmodium* infections, only 3.6% (12/331) of children with malaria had co-infection with other organisms. These include *Rickettsia felis-plasmodium, Klebsiella pneumoniae-plasmodium,* Dengue-*plasmodium* among others (Table 4.7).

Table 4-7: Occurrence of common pathogens co-infected with *Plasmodium falciparum* in study population

Pathogen	Prevalence n (%)	*Plasmodium* co-infection n (%)
Rickettsia felis	5.0 (1.3)	2 .0 (40.0)
Citrobacter freundii	3.0 (0.8)	2.0 (67.0)
Dengue virus	2.0 (0.5)	1.0 (50.0)
Streptococcus pneumoniae	2.0 (0.5)	1.0 (50.0)
Coxiella burnetti	1.0 (0.3)	1.0 (100.0)
Escherichia coli	9.0 (2.4)	1.0 (11.1)
Klebsiella pneumoniae	2.0 (0.5)	1.0 (50.0)
Pseudomonas aeruginosa	5.0 (1.3)	2 .0 (40.0)
Staphylococcus aureus	13.0 (3.5)	1.0 (7.7)

Note: Prevalence calculated as percentage of total number of children with infection (374), n=number of children, %= percentage

Plasmodium co-infections were observed more frequently in children with low parasitemia (<1000 parasites/µL) than those with high parasitemia (≥100,000 parasites/µL). The frequency of *Plasmodium*-bacteria co-infections was 3.2% (12/374) and out of which 66.7% (8/12) occurred among children with low parasitemia (1000/µL) as compared to 33.3% (4/12) for children with high parasitemia (100, 000/µL density). *Plasmodium*-bacteria co-infections was more common in Kintampo, 2.1% (8/374) compared to the children at LEKMA 1.1% (4/374). *Plasmodium*-viral infections were only 0.3% (1/374) among the febrile children.

4.7 Pathogen distribution among afebrile children

As controls, 655 afebrile children of the same age group and presenting to the same hospitals as the febrile children were recruited into the study (Table 4.8). No pathogen was detected in 79.5% (521/655) of the afebrile children by culture, RDT and microscopy. In 10.4% (68/655) of the afebrile children, malaria parasites, predominantly *P. falciparum* were detected, with median parasite count of 1895/µL (IQR, 2564 parasites/µL), although 12.5% (82/655) were clinically diagnosed to have malaria. The proportion of clinically diagnosed UTIs by study clinicians was 4.1%, (27/655) and that of bacteremia was 2.4% (16/655). However, pathogens were confirmed in only 7.4% (2/27) of the suspected UTIs and 6.3% (1/16) of bacteremic diagnosed afebrile children.

Table 4-8: Characteristics of afebrile non-hospitalized children

Characteristics	Frequency n (%)
Common clinical symptoms	
Vomiting	207.0 (31.6)
Diarrhea	146.0 (22.3)
Headache	356.0 (54.4)
Cough	297.0 (45.3)
Abdominal pain	108.0 (16.5)
Source of drinking water	
Bole hole	35.0 (5.3)
Pipe borne	71.0 (10.8)
Sachet water	298.0 (45.5)
Well	140.0 (21.4)
River	64.0 (9.8)
Animals contacted prior to enrolment	
Cat	67.0 (10.2)
Dog	54.0 (8.2)
Cattle	9.0 (1.4)
Fowls	16.0 (2.4)
Sheep/goat	28.0 (4.3)
Both Cat and dog	8.0 (1.2)
No animal contact	473.0 (72.2)
Infection	
***Plasmodium* spp parasitemia**	68.0 (10.4)
UTI	2.0 (0.31)
Bacteremia/Sepsis	1.0 (0.15)
Gastrointestinal infection	4.0 (0.61)
LRTI	3.0 (0.46)
Non-microbial causes of fever	521.0 (79.5)

UTI= urinary tract infections, LRTI= Lower respiratory tract infections and pneumonia. Data are presented as frequency n (%) unless otherwise indicated

4.8 Predictive performance of hematological parameters as predictor of fever

Data from a total of 1508 children, made up of 795 febrile and 713 afebrile children, were available for the analysis. Analysis was performed by defining population-specific cut off points for normal blood cell counts. Data were coded as normal using results from published reference normal healthy values (Table 3.1). The results of the diagnostic performance of parameters are presented under different sub-headings as red blood cell parameters, clotting parameters and white blood cell parameters. All the hematological parameters examined for their diagnostic ability to predict fever were selected based on their routine application to evaluate patient's health status by clinicians.

The probability of HGB to predict fever had sensitivity of 13.6 % (95% CI: 11.3-16.2) and a specificity of 89.3% (95% CI: 86.8- 91.5) at the cut-off level of 8.0 g/dL. As a result, the positive predictive value of HGB for fever was also low. The non-parametric AUC for the estimated sensitivity and specificity was 0.53 % and this does not make HGB a good predictor of fever. Similarly, sensitivity of RBC was 15.2% with a high specificity of 86.4% (95% CI: 79.0- 84.8) with a cut-off of 2.5 ($x10^{12}$/L) to predict fever presence, making it not a good predictor of fever. A good predictive parameter is expected to have sensitivity and specificity greater than 60 % with AUC greater than 0.5. The other red cell parameters examined namely, HCT, MCV, MCH and MCHC were also not good predictors of fever. Further details can be found in Table 4.9.

Table 4-9: Performance of red blood cell parameters as predictors of fever

Parameter	Febrile Children N=795		Afebrile Children N=713		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Positive predicted value (%) (95% CI)	Negative predicted value (%) (95% CI)	Odds ratio (95% CI)	Area under ROC (95% CI)
	Num. Pos. (TP)	Num Neg. (FN)	Num. Pos. (FP)	Num. Neg (TN)						
HGB (g/dL)	108	687	76	637	13.6 (11.3- 16.2)	89.3 (86.8-91.5)	58.7 (51.2-65.9)	48.1 (45.4-50.8)	1.32 (0.97-1.8)	0.53(0.50-0.57)
RBC ($x10^{12}$/L)	121	674	97	616	15.2 (12.8-17.9)	86.4 (83.7-88.8)	55.5(48.6-62.2)	47.8 (45.0-50.5)	1.10 (0.86-1.52)	0.52 (0.48-0.56)
HCT (%)	139	656	128	585	17.5 (14.9-20.3)	82.0 (79.0-84.8)	52.1(45.9-58.2)	47.1 (44.3-50.0)	0.97 (0.74-1.26)	0.50 (0.46-0.53)
MCV (fl)	45	750	58	655	5.7 (4.16- 7.5)	91.9 (89.6-93.8)	43.7(33.9- 53.8)	46.6 (44.0-49.3)	0.68 (0.45-1.01)	0.45 (0.40-0.50)
MCH (Pg)	32	763	39	674	4.03 (2.77-5.64)	94.5 (92.6-96.1)	45.1(33.2-57.3)	46.9 (44.3-49.5)	0.73 (0.45-1.17)	0.46 (0.40-0.52)
MCHC (g/dL)	176	619	221	492	22.1 (19.3- 25.2)	69.0 (65.5-72.4)	44.3(39.4-49.4)	44.3 (41.3-47.3)	0.63 (0.50-0.80)	0.44 (0.42-0.47)
RDW (%)	20	364	28	480	59.7 (15.0-72.3)	56.9 (49.9-63.7)	5.21(1.71-11.7)	94.5 (89-97.8)	0.94 (0.31-2.91)	0.55 (0.34-0.84)

Abbreviations: HGB= hemoglobin, RBC= red blood cell, HCT= hematocrit, MCV= mean cell volume, MCH= mean cell hemoglobin, MCHC= mean cell hemoglobin corpuscle and RDW= red cell distribution width, N, Num=number of children, TP=true positive, FN=false negative, FP=false positive, TN=True negative. Age-specific reference value for healthy children for red blood cell with specific cut-off point for normal and abnormal values were used. Data was duel coded and diagt command in STATA software was employed to calculate sensitivity, specificity, positive predicted value, negative predicted value odds ratios. Receiver operating characteristic curve (ROC) obtained by plotting true positive rate on y-axis and false negative rate on x-axis. Area under the was obtained by adding a set of vertical bars with width equals to the spaces between the points on the false positive rate axis and the heights equal to the step of height on the true positive axis. The area under the curve (AUC) was used as test significant to measure predictive performance

Meanwhile, the probability of RDW as a hematological predictor of fever when examined had the highest sensitivity of 59.7% among the red blood indices with the cut-off value of 13% (Table 4.9), although it cannot be described as a good predictor of fever, having a ROC area of 0.55.The probability of having fever for RDW decreases with a unit increase in RDW concentration (OR=0.94, (95% CI; 0.31-2.91) (Table 4.9). Absolute platelet (PLT) count and platelet distribution width (PDW) expressed as percentage were the two blood clotting factors evaluated. Both PLT and PDW were not good predictors of fever due to low sensitivity values recorded (Table 4.10).

The results of white cell parameters evaluated as fever predictors are summarized in Table 4.11. Among those parameters, lymphocyte (LYM) with a cutoff value of 46% had the highest sensitivity of 65.2% and specificity of 67.3%. ROC curve for LYM is showed in Figure 4.11 with AUC of 0.78 (95% CI; 0.64-0.89). This indicates that 65.5% of children with fever will be correctly identified by lymphocyte when used as a predictor of fever. In terms of absolute numbers, sensitivity of lymphocyte to predict fever dropped to 35% at a cut-off value of 6 (x 10^9/L), indicating a poor prognosis of fever. Granulocyte number (GRA) was found to be a good predictor of fever with sensitivity of 60.3, specificity of 70.2 and AUC of 0.65 using a cut-off of 26.0. The predictive performance of other white cell parameters, including monocyte (MON) and total white blood cells (WBC) are summarized in Table 4.11.

Table 4-10: Performance of blood clotting factors as predictors of fever

Parameter	Febrile Children N=795		Afebrile Children N=713		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Positive predicted value (%) (95% CI)	Negative predicted value (%) (95% CI)	Odds ratio (95% CI)	Area under ROC (95% CI)
	Num Pos. (TP)	Num. Neg. (FN)	Num Pos. (FP)	Num. Neg. (TN)						
PLT ($x10^{12}$/L)	179	616	77	636	22.5 (19.7-25.6)	89.2 (86.7-91.4)	69.9 (63.0-75.5)	50.8 (48.0-53.6)	2.4 (1.80-3.20)	0.60 (0.57-0.64)
PDW (%)	41	753	38	672	5.16 (3.73-6.94)	94.6 (92.7-96.2)	51.9 (40.4-63.3)	47.2 (44.5-49.8)	0.9 (0.61-1.51)	0.50 (0.44-0.55)

Abbreviations: PLT= platelets and PDW= platelet distribution width, N,Num=number of children, TP=true positive, FN=false negative, FP=false positive, TN=True negative, Pos=Positive, Neg=Negative Age-specific reference value for healthy children for platelets and platelet distribution width with specific cut-off point for normal and abnormal values were used. Data was duly coded and diagt command in STATA software was employed to calculate sensitivity, specificity, positive predicted value, negative predicted value odds ratios. Receiver operating characteristic curve (ROC) obtained by plotting true positive rate on y-axis and false negative rate on x-axis. Area under the was obtained by adding a set of vertical bars with width equals to the spaces between the points on the false positive rate axis and the heights equal to the step of height on the true positive axis. The area under the curve (AUC) was used as test significant to measure predictive performance

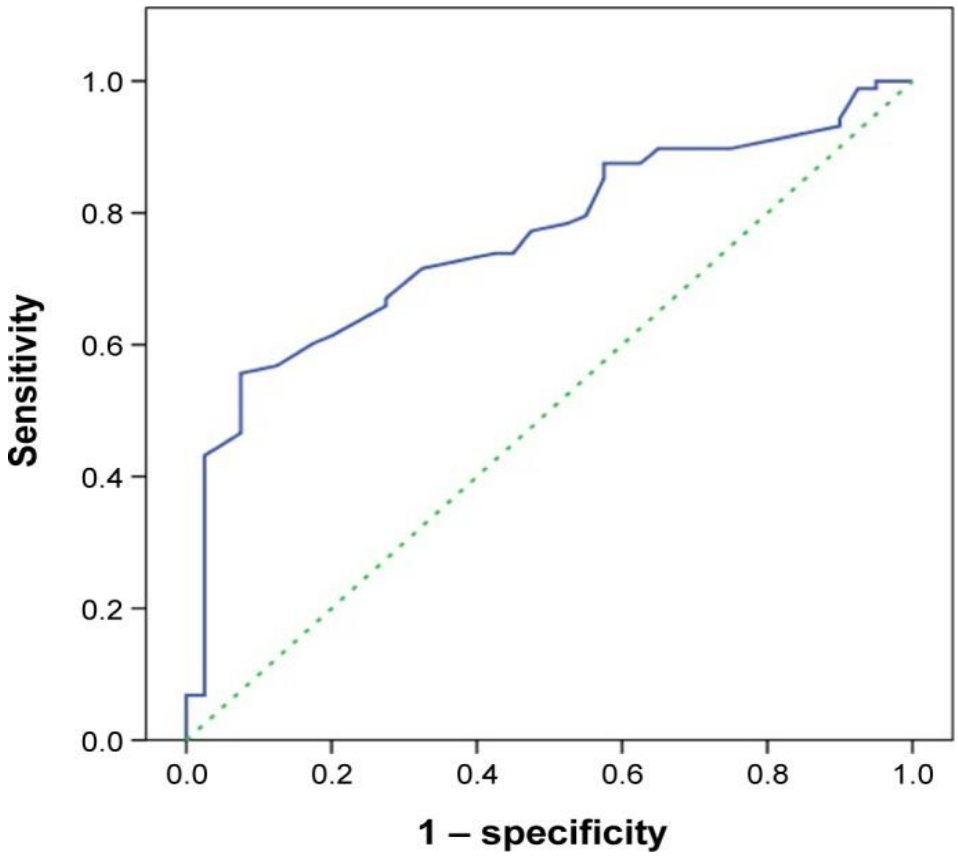


Figure 4-5: Receiver operating characteristic curve for lymphocyte (%) to predict fever.

Lymphocytes were measured using ABX Pentra 60+ auto-analyzer. The ROC curves is plot of the sensitivities and false positive (1-Specificity) probabilities and for ideal tests, a rectangular plot passing from the origin at the bottom left hand corner towards top left hand corner and progress to the top right hand corner is obtained. Since the ROC curve for lymphocyte arches towards the upper left hand corner away from the diagonal, it is indication of a good test. The cut-off point for the AUC in deciding between good and poor test was arbitrarily selected from the ROC curve such that a larger values obtained the more positive test. The AUC for the lymphocyte evaluated was 0.78.

Table 4-11: Predictive performance of white blood cell parameters as predictors of fever

Parameter	Febrile Children N=795		Afebrile Children N=713		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Positive predicted value (%) (95% CI)	Negative predicted value (%) (95% CI)	Odds ratio (95% CI)	Area under ROC (95% CI)
	Num. Pos. (TP)	Num. Neg (FN)	Num. Pos (FP)	Num Neg. (TN)						
WBC total (x 10^9/L)	159	636	79	634	20.0 (17.3-23.0)	88.0 (86.4-91.1)	66.8 (60.4-72.8)	49.9 (47.1-52.7)	2.01(1.50-2.68)	0.58(0.55-0.62)
LYM (%)	518	277	233	480	65.2 (61.7-68.5)	67.3 (63.7-70.8)	69.0 (65.5-72.3)	63.4 (59.9-66.8)	3.853.11-4.77)	0.78 (0.64-0.89)
LYM (x 10^9/L)	279	515	168	545	35.1 (31.8-38.6)	76.4 (73.1-79.5)	62.4 (57.7-66.9)	51.4 (48.4-54.5)	1.76(1.40-2.20)	0.57 (0.54-0.60)
MON (%)	206	589	100	612	25.9 (22.9-29.1)	86.0 (83.2-88.4)	67.3 (61.8-72.5)	51.0 (48.1-53.8)	2.14 (1.64-2.79)	0.59 (0.56-0.62)
MON (x 10^9/L)	138	657	136	577	17.4 (14.8-20.2)	80.9 (77.8-83.7)	50.4 (44.3-56.4)	46.8 (43.9-49.6)	0.89 (0.69-1.16)	0.49 (0.45-0.52)
GRA (%)	470	323	210	503	60.3 (55.8-62.7)	70.2 (67.1-73.9)	69.1 (65.5-72.6)	60.9 (57.5-64.2)	3.49 (2.81-4.32)	0.65 (0.63-0.67)
GRA (x 10^9/L)	132	662	129	584	16.6 (14.1-19.4)	81.9 (78.9-84.7)	50.6 (44.3-56.8)	46.9 (44.1-49.7)	0.90 (0.69-1.18)	0.90 (0.69-1.18)

WBC = white blood cell; LYM= lymphocytes; MON=monocytes; GRA= granulocytes**,** N(Num)=number of children, TP=true positive, FN=false negative, FP=false positive, TN=true negative, Num=number, Pos=positive, Neg=negative, Age-specific reference value for healthy children for each white blood cell with specific cut-off point for normal and abnormal values were used. Data was duly coded and diagt command in STATA software was employed to calculate sensitivity, specificity, positive predicted value, negative predicted value odds ratios. Receiver operating characteristic curve (ROC) obtained by plotting true positive rate on y-axis and false negative rate on x-axis. Area under the was obtained by adding a set of vertical bars with width equals to the spaces between the points on the false positive rate axis and the heights equal to the step of height on the true positive axis. The area under the curve (AUC) was used as test significant to measure predictive performance

4.9 Differential expression of cytokines among study population

Among the proinflammatory cytokines, mean concentration of IFN-γ was found to be significantly higher among the febrile compared to afebrile children (1.4 pg/mL vs. 0.7pg/mL, p=0.034, Mann-Whitney U-test) (Figure 4.5A). The dynamics of cytokine expression was showed for some cytokines as their levels correlated with increasing body temperature. The mean level of IFN-γ for children with severe fever (≥40 ^{O}C) was slightly higher (1.09 pg/mL) as compared to those with mild fever (37.5^{O}C - 39 ^{O}C) which was 0.27 pg/mL. No age association was observed in the proinflammatory cytokines evaluated.

In the case of anti-inflammatory cytokines, only IL-10 levels were significantly higher among febrile compared to afebrile children (82 pg/mL vs. 39 pg/mL, p=0.040, Mann-Whitney U-test) (Figure 4.5B). For all the cytokines analyzed among the febrile children, there were no differences in mean concentrations among male compared to females. Among the afebrile children, there were differences in mean levels of IL-6 in female (5.1 pg/mL) compared to males (4.3 pg/mL) but this was not statistically significant (p= 0.412).

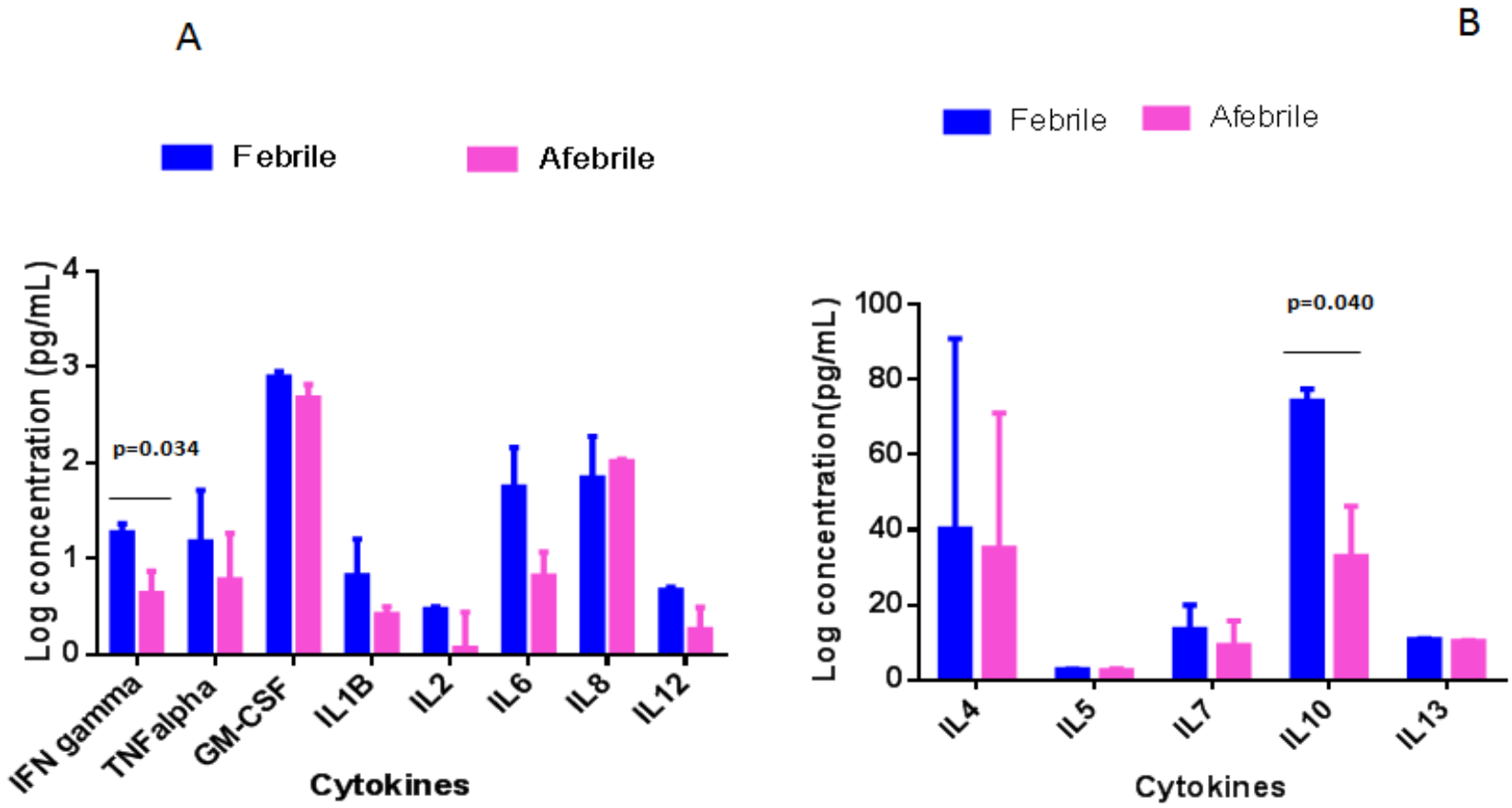


Figure 4-6: Differential expression of cytokines in febrile compared to afebrile children

The mean concentrations of the pro and anti-inflammatory cytokines were compared among children with fever and those without fever using Mann Whitney U test and data analyzed with Graph pad prison. (A) Serum levels of selected proinflammatory cytokines for the febrile and afebrile children. (B) Mean level of selected anti-inflammatory cytokine levels for febrile and afebrile children. Data presented as boxes and error bars showing standard error of mean (SEM). GM-CSF = Granulocyte-macrophage colony-stimulating factor, IFN = Interferon gamma, TNF =Tumor necrosis factor, IL= interleukin.

4.10 Cytokine expression among children with specific infection

Among children who were infected with *Plasmodium* parasites, the mean concentration of IL-10 was significantly higher among febrile children than afebrile children (2.8×10^{3}pg/mL vs. 1.6×10^{-3} pg/mL, p=0.043). However, the level of IL-10 among febrile young children aged less than 5 years with *Plasmodium* infection was not significantly different from older children aged 5 years and above (1.8×10^{-3} pg/mL vs. 1.2×10^{-3} pg/mL, p= 0.446) (Appendix-10). The differences in the mean concentration for all other cytokines examined showed no statistical differences (Figure 4.7A). In the case of bacteria infected children, the mean IL-2 concentration was significantly higher among febrile children compared to afebrile children (0.9×10^{-3} pg/mL vs. 0.6×10^{-3} pg/mL, p=0.041, Mann Whitney U-test) (Figure 4.7B). No differences in the level of IL-2 was observed for bacteria infected children in the febrile group who were aged less than 5 years compared to those aged above 5 years. Sex was not with associated the levels of all the cytokines analyzed among the bacteria infected children (Appendix-10).

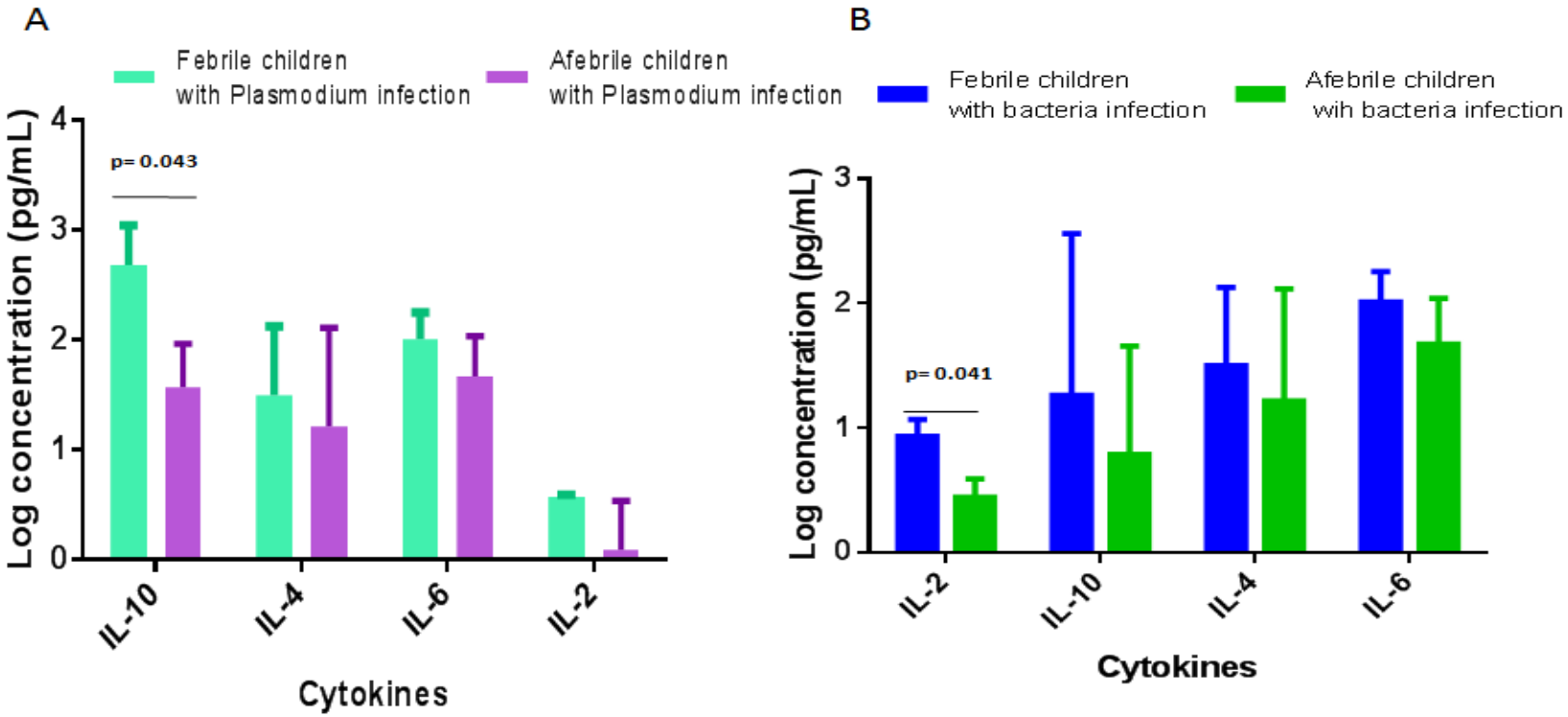


Figure 4-7: Mean cytokine levels for children with *Plasmodium* and bacterial infection

Data shows mean cytokines among febrile and afebrile children with bacteria and *Plasmodium* infections, using Mann Whitney U-test. (A) Represents comparison of mean cytokines analyzed for febrile (N=98) and afebrile (N=22) children infected with *Plasmodium* parasites and (B) Represents comparison of means of selected cytokines for febrile and afebrile children infected with bacteria. Statistical significance is indicated by the p-values. Mean cytokine present as bars and error bar indicating $\pm$ SEM. Analysis was performed with Graph pad prison. GM-CSF =Granulocyte-macrophage colony-stimulating factor, IFN = Interferon gamma, TNF=Tumor-necrosis-factor, IL=interleukin.

4.11 Diagnostic performance of serum cytokines as biomarkers of fever

Data for 97 febrile and 18 afebrile children were analyzed and serum levels of 13 cytokines among the study population are presented. The cytokines were selected based on their relevance in immune modulation in response to infection. During the analysis, all measured values with percentage coefficient variation greater than 15% were not analyzed. The combination of sensitivity and specificity were employed to determine the accuracy of the diagnostic test. The ROC analysis included the cut-off values for positive classification and therefore the larger the test results, the more positive the diagnostic test.

4.11.1 Performance of proinflammatory cytokines as fever predictor

ROC analysis showed that TNF-α [(OR):14.0], IFN-γ [(OR):4.06] and IL-6 [(OR):1.6] were significantly associated with fever. With AUC of 0.78 and a cut-off score of 22.4, TNF-α had sensitivity score of 84.4% (95% CI: 75.5-91.0) to predict fever and specificity of 72.2% (95% CI: 46.5- 90.3). The result indicates that 84.4% of children diagnosed with fever were correctly classified as having fever using TNF-α as a diagnostic test. However, only 15.6 % of the children with no fever were incorrectly classified as having fever by TNF-α. IL-6 and IFN-γ showed sensitivity of 71.9% (95% CI: 61.8-80.6) and 67.0% (95% CI: 56.7-76.2) to predict fever respectively. The proportion of children with fever that were correctly identified by GMCSF was 38.1% (95% CI: 28.5-48.6) and those without fever who were incorrectly identified was 83.3% (95% CI: 58.6-96.4). The predictive performances of other proinflammatory cytokines have been summarized in Table 4.12. The ROC curves for IL-6, IFN-γ and TNF-α, which show their fever predictive performances, have been presented in Figure 4-8.

Table 4-12: Predictive performance of proinflammatory cytokines as predictors of fever

Parameter (Pg/mL)	Febrile Children N=97		Afebrile Children N=18		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Positive predicted value (%) (95% CI)	Negative predicted value (%) (95% CI)	Odds ratio (95% CI)	Area under ROC (95% CI)
	Num Pos. (TP)	Num. Neg. (FN)	Num. Pos. (FP)	Num Neg. (TN)						
GMCSF	37	60	3	15	38.1(28.5-48.6)	83.3(58.6-96.4)	92.5 (79.6- 98.4)	20.0 (11.6- 30.8)	3.1(0.9-10.6)	0.61(0.5- 0.71)
IFN-γ	65	32	6	12	67.0(56.7-76.2)	66.7(41.0-86.7)	91.5 (82.5- 96.8)	27.3 (15.0- 42.8)	4.1(1.4-11.4)	0.75 (0.55-0.79)
IL-1β	31	65	1	17	33.0(23.8-43.3)	94.4(72.- 99.9)	97.0 (84.2- 99.9)	20.7(12.6- 31.1)	8.4 (1.0- 10.2)	0.64 (0.57-0.71)
IL-2	55	41	1	17	57.3(46.8-67.3)	94.4(72.7-99.9)	98.2(90.4- 100.0)	29.3 (18.1- 42.7)	22.8(3.7-23.9)	0.66 (0.69-0.83)
IL-8	29	67	5	13	30.2(21.3-40.4)	72.2(46.5-90.3)	85.3 (68.9- 95.0)	16.3 (8.95- 26.2)	1.1 (0.4-3.31)	0.51 (0.40-0.63)
IL-6	69	27	11	7	71.9 (61.8-80.6)	38.9(17.3-64.3)	86.3 (76.7- 92.9)	20.6 (8.7- 37.9)	1.6 (0.6-4.5)	0.80 (0.43-0.68)
IL-12	10	87	2	16	10.3(5.06-18.1)	88.9(65.3- 98.6)	83.3 (51.6- 97.9)	15.5 (9.15- 24.0)	0.9 (0.2-0.2)	0.50 (0.42-0.58)
TNF-α	81	15	5	13	84.4(75.5-91.0)	72.2(46.5- 90.3)	94.2 (87.0- 98.1)	46.4 (27.5- 66.1)	14.0(4.5-43.6)	0.89 (0.67-0.90)

IL-interleukin, TNF=tumor nucleosis factor, N,Num=number of children, Pos=positive, Neg=negative, FN=false negative, FP=false positive, TN=true negative. Age-specific reference value for healthy children for each cytokine with specific cut-off point for normal and abnormal values were used. Data was duly coded and diagt command in STATA software was employed to calculate sensitivity, specificity, positive predicted value, negative predicted value odds ratios. Receiver operating characteristic curve (ROC) obtained by plotting true positive rate on y-axis and false negative rate on x-axis. Area under the was obtained by adding a set of vertical bars with width equals to the spaces between the points on the false positive rate axis and the heights equal to the step of height on the true positive rate axis. The area under the curve (AUC) was used as test significant to measure predictive performance

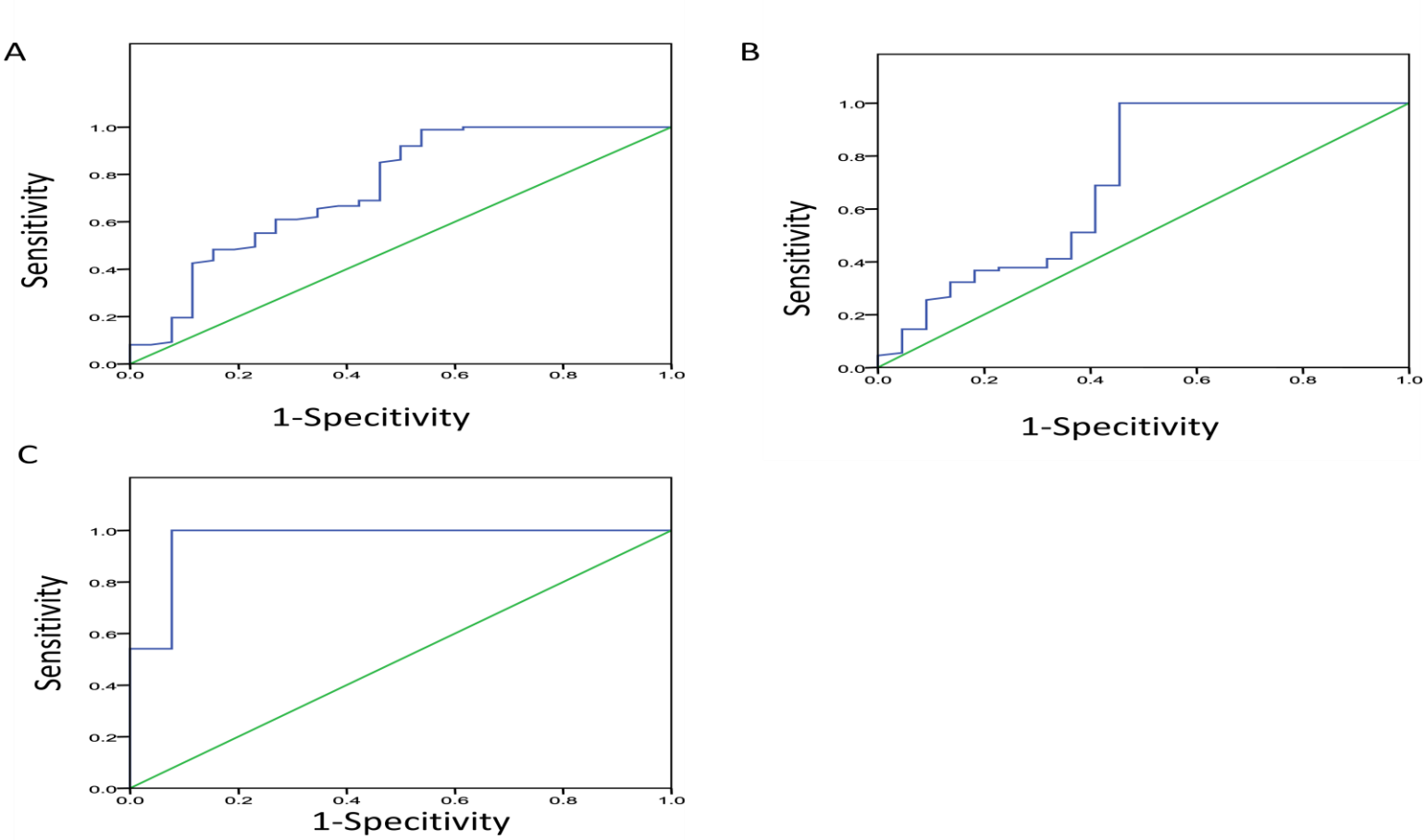


Figure 4-8 Receiver operating characteristic curve curves for three proinflammatory cytokines to predict fever
Sensitivity and specificity were determined and used to create the ROC curves in STATA software. The curves were obtained by plotting the sensitivity against 1-Specificity (false positives rate).The AUC was obtained by using the trapezoidal method which uses average of a number of small trapezoidal segments from the ROC curve. A) ROC curve for IL-6 with AUC of 0.80, B) ROC curve for IFN-γ with AUC of 0.75 and C) ROC curve for TNF-α with AUC of 0.89. AUC>0.9 is good one, AUC<0.5 is a random or poor one.

4.11.2 Performance of anti-inflammatory cytokines as predictors of fever

The anti-inflammatory cytokines evaluated for their diagnostic potential to predict fever included IL-4**,** IL-5, IL-7, IL-10 and IL-13. Among these, statistically significant level with the chosen optimum cut-off for sensitivity and specificity was found in only IL-10. It showed highest sensitivity of 76.3% and specificity of 83.3% with a cutoff 14.6 pg/mL to predict fever with positive predictive value of 96.1%. The ROC curve which confirms the diagnostic potential of IL-10 is presented in Figure 4-9**.** Additionally, IL-4 gave sensitivity score of 26% and specificity of 83% to predict fever with AUC of 0.55% with a cut-off value of 30.0 pg/mL.

Table 4-13: Predictive performance of anti-inflammatory cytokines as predictors of fever

Parameter	Febrile Children N=97		Afebrile Children N=18		Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Positive predicted value (%) (95% CI)	Negative predicted value (%) (95% CI)	Odds ratio (95% CI)	Area under ROC (95% CI)
	Num Pos. (TP)	Num. Neg. (FN)	Num Pos. (FP)	Num. Neg. (TN)						
IL-4	25	71	3	15	26.0 (17.6-36.0)	83.3 (58.6-96.4)	89.3 (71.8- 97.7)	17.4 (10.1-27.1)	1.76 (0.50-6.13)	0.55 (0.45-0.65)
IL-5	21	75	6	12	21.9 (14.1-31.5)	66.7 (41.0-86.7)	77.8 (57.7-91.4)	13.8 (7.34-22.9)	0.56 (0.19-1.61)	0.44 (0.32-0.56)
IL-7	2	94	1	17	2.08 (0.25-7.32)	94.4 (72.7-99.9)	66.7 (9.43-99.2)	15.3(9.18-23.4)	0.36 (0.04-1.30)	0.48 (0.43-0.54)
IL-10	74	23	3	15	76.3 (66.6-84.3)	83.3 (58.6-96.4)	96.1(89.0-99.2)	39.5 (24.0-56.6)	16.1(4.52-56.30)	0.80 (0.70-0.90)
IL-13	7	90	4	14	7.22 (2.95-14.3)	77.8 (52.4-93.6)	63.6 (30.8-89.1)	13.5 (7.56-21.6)	0.27(0.07-0.98)	0.43 (0.32-0.53)

IL- interleukin, N,Num=number of children, Pos=positive, Neg=negative, FN=false negative, FP=false positive, TN=true negative. Age-specific reference value for healthy children for each cytokine with specific cut-off point for normal and abnormal values were used. Data was duly coded and diagt command in STATA software was employed to calculate sensitivity, specificity, positive predicted value, negative predicted value odds ratios. Receiver operating characteristic curve (ROC) obtained by plotting true positive rate on y-axis and false negative rate on x-axis. Area under the was obtained by adding a set of vertical bars with width equals to the spaces between the points on the false positive rate axis and the heights equal to the step of height on the true positive rate axis. The area under the curve (AUC) was used as test significant to measure predictive performance

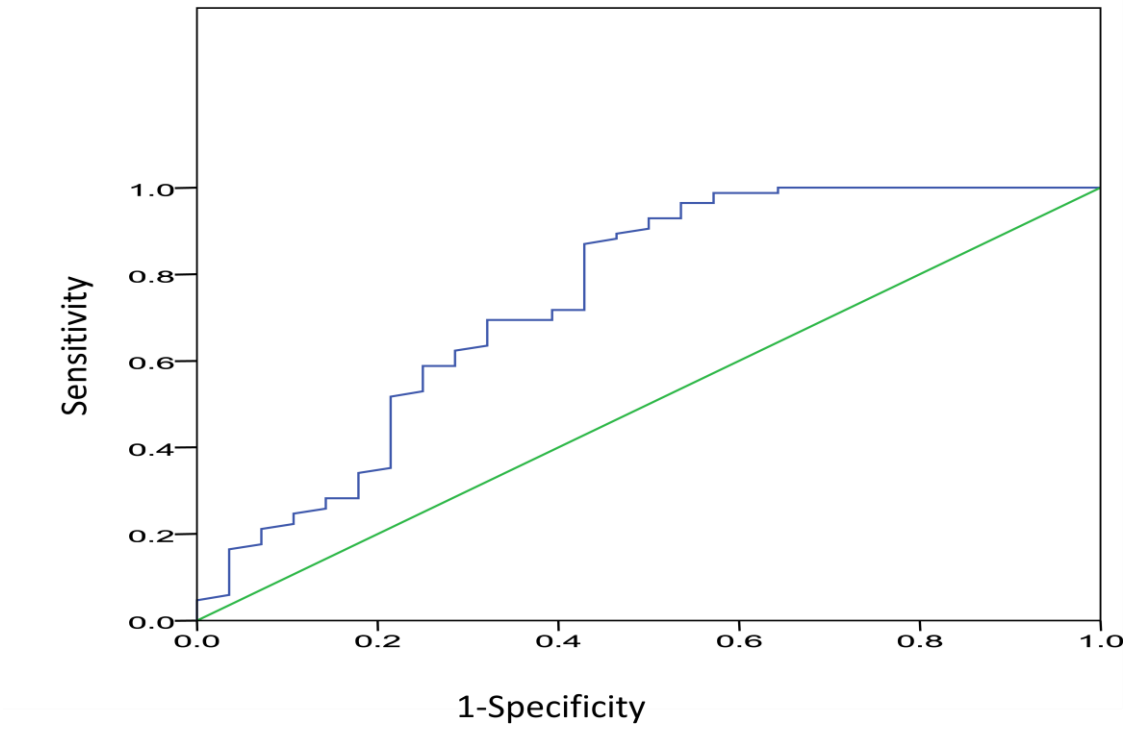


Figure 4-9 Receiver operating characteristic curve for IL-10

The ROC curve for IL-10 was obtained by calculating the sensitivity and specificity of every observed value, and then plotting sensitivity (Y-axis) against 1- specificity (X-axis). The diagonal straight line represents a test that does not discriminate between children with fever and those without fever. All points on the straight line represent a 1:1 ratio of true to false positives for IL-10. An ideal test would give a rectangular plot passing from the origin at the bottom left corner towards top left corner at first and then to the top right corner. Cut-off point for deciding between normal and abnormal values was selected arbitrarily where the ROC curve changes direction from being vertical to horizontal. The more the ROC curve arches into the upper left corner away from the diagonal, the better the test. IL-10 had AUC of 0.80

4.12 Selection of classification models to predict probability of infection

Data from 795 febrile and 713 afebrile children were used to model the probability of infection. Whilst blood culture, urine culture and TAC-PCR were classified as variables with "small datasets", RDT and microscopy were classified as variables with "large datasets" based on the numbers of observations contributed to the analysis.

Among the three models compared and basing on ROC performance, Naïve Bayes classifier incorporating only clinical signs and symptoms (designated as "S") gave the most decent performance, as ROC curves cluster away from the diagonal line, signifying a good classification (Figure 4.10A). The ROC performance for Lasso Regression and Random Forest when only clinical signs and symptoms were modeled proved less appreciative as the ROC curves rather approached or crossed the diagonal line indicating a poor performance. However, the inclusion of all other parameters into the Naïve Bayes model deteriorates ROC performance with blood culture being the most affected (Figure 4.10B). Contrary to Naïve Bayes, the inclusion of blood parameters into Lasso Regression and Random Forests improved the ROC performance for only RDT, microscopy and TAC-PCR, but not for blood or urine culture tests (Figure 4.10A and B). Thus, the ROC values for blood and urine cultures declined with the inclusion of blood parameters into the models.

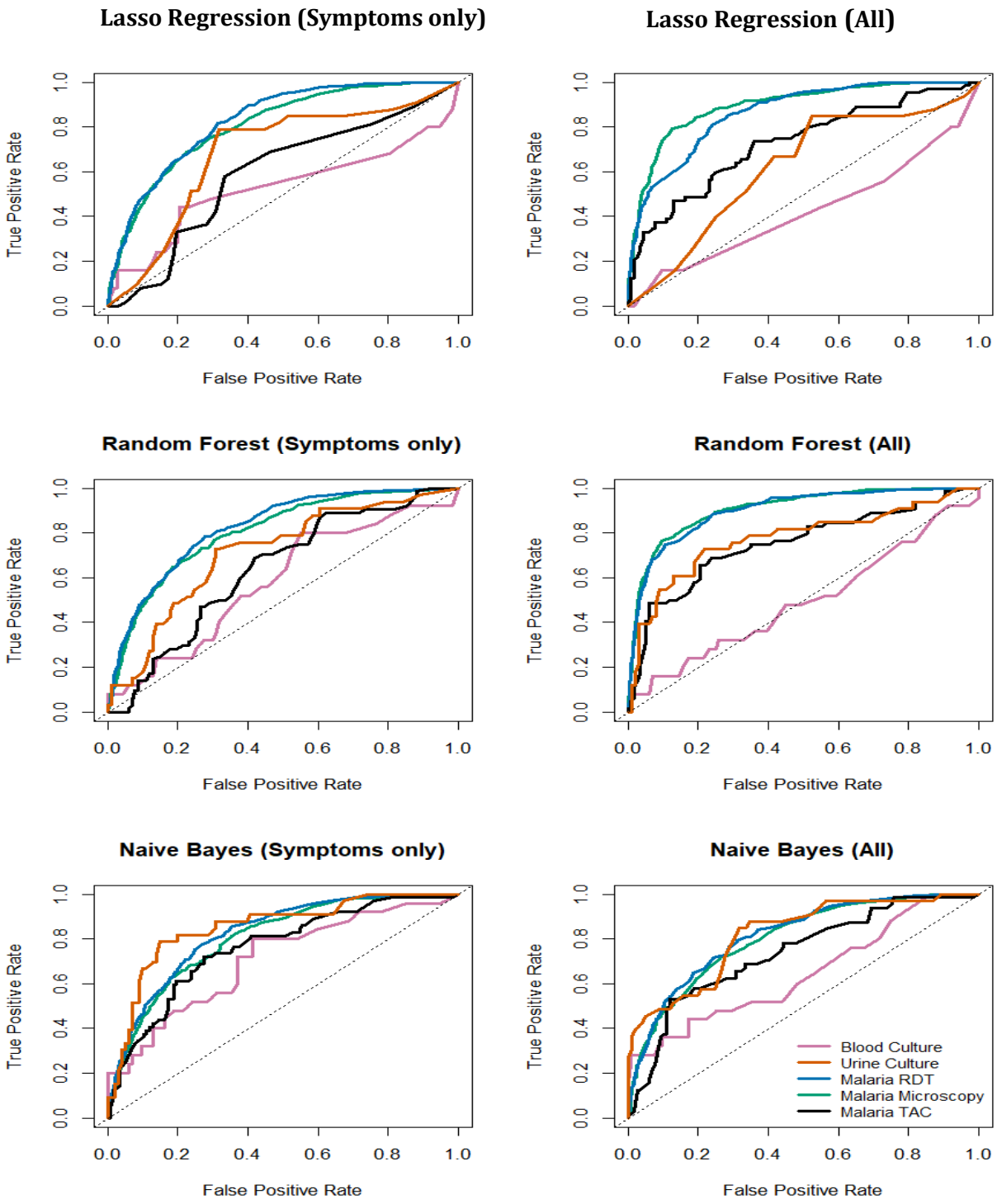


Figure 4.10A Figure 4.10B

Figure 4-10: Receiver operating characteristic curves for diagnostic test outcomes
The ROC analysis simultaneously evaluated blood culture, urine culture malaria RDT, malaria microscopy and malaria TAC following modeling with Naïve Bayes, Lasso regression and Random Forest. True positive rate = sensitivity, false positive rate = 1-Specificity

When the AUC for the models was evaluated using small dataset for instance, the AUC for Naïve Bayes was superior for blood and urine cultures compared to Lasso Regression and Random Forest (Table 4.14). The outcomes for the three models were similar for TAC-PCR with incorporation of hematological parameters but Random Forest and Lasso Regression performed worse with hematological parameters. Interestingly, without hematological parameters, Naive Bayes improved in terms of AUC performance. For the larger datasets (RDT and microscopy), Lasso Regression and Random Forest performed far better with incorporation of hematological parameters. However, without the hematological parameters, we observed similar AUC patterns for Lasso Regression and Random Forest whereas no substantial changes occurred for Naïve Bayes (Table 4.14).

Table 4-14: Validation of model performance using area under the curve scores and misclassification rate

Test	Measurement	Lasso (S)	Lasso (A)	RF (S)	RF (A)	NB (S)	NB (A)
Blood Culture	Misclassification	0.177	0.177	0.163	0.170	0.191	0.135
	AUC	0.529	0.422	0.585	0.496	**0.701**	0.646
Urine Culture	Misclassification	0.246	0.246	0.261	0.187	0.172	0.164
	AUC	0.682	0.621	0.716	0.777	**0.952**	0.921
RDT	Misclassification	0.211	0.179	0.210	0.139	0.236	0.208
	AUC	0.827	**0.964**	0.822	**0.901**	**0.823**	0.815
Microscopy	Misclassification	0.212	0.151	0.206	0.134	0.228	0.202
	AUC	0.807	**0.994**	0.902	**0.907**	**0.904**	0.903
Malaria-TAC	Misclassification	0.354	0.326	0.387	0.271	0.276	0.243
	AUC	**0.589**	**0.727**	**0.637**	**0.746**	0.751	**0.737**

NB: Bolded values are best among the six models. The higher AUC and lower misclassification rate, the better the results. S = Symptoms only, A = All covariates (Symptoms + Blood parameters), RF = Random forest, NB = Naïve Bayes, Lasso=Lasso Regression.

4.13 Online tool to predict the probability of infections of patients

A prototype decision support tool has been created to demonstrate the advantage of using a predictive model to determine infection status of AFI patients as compared to conventional laboratory diagnostic tests. It is available at http://kokbent.shinyapps.io/AFI_test/ and has demonstrated how empirical data when translated into a model can effectively communicate to healthcare practitioners for patient management. It has a baseline probability for the listed test (blood culture, urine culture, microscopy, RDT and malaria TAC) which is updated on selection of temperature, clinical symptoms, location or hematological test performed. The updated probability as compared with the baseline probability, in the form of bar charts provides information about the chances of a patient being positive for a given test. The model was trained using data from both febrile and afebrile patients and can provide probability about afebrile individuals as well. The uncertainties of the tests are represented as error bar for all tests with similar probabilities, the one with small error chosen. The tool is quick to operate and provides results immediately.

CHAPTER FIVE

5 DISCUSSION

5.1 General discussion

In most countries in Africa, the problem of attributing every febrile illness to malaria has been widely acknowledged, but data that comprehensively describe the causes of non-malaria febrile illnesses are limited (Amexo et al., 2004; Bisoffi & Buonfrate, 2013; Prasad et al., 2015; Reyburn et al., 2004). Causes of acute febrile illnesses have primarily focused on hospital inpatient and high-risk groups including children less than 5 years of age and HIV patients (Mahende et al., 2014; Prasad et al., 2015). This study examined the etiology of AFI at the outpatient setting of two hospitals at different locations in Ghana where there is scarcity of data on microbial causes of fever in children aged 1-15 years.

This study has demonstrated that malaria remains a major contributor to fever among Ghanaian children despite the recent reports on decreasing malaria incidences in some sub-Saharan African countries (Bhatt, et al., 2015). Malaria parasites were detected in 38.6% of the febrile and 10.4% of afebrile children examined. We have also presented evidence of bacterial zoonoses resulting from Q fever, rickettsioses and dengue virus infection among Ghanaian febrile children. Dengue and *Coxiella burnetti* detected in this study are not traditionally recognized as causes of febrile disease in Ghana. Using a combination of a clinical, microbiological and molecular methods, the etiology of 45.3% of outpatient children reporting with AFIs were determined; this figure is low, compared to other findings from studies in Kumasi, Ghana and Tanzania where the causes of fever in 90% (Hogan et al., 2018a) and 97% (D'Acremont et al., 2014) of children were identified. The

difference could be attributed to our inability to analyze other samples for pathogen such as the nasopharyngeal sample collected due to limited logistical and financial constraints.

5.2 Prevalence of bacteremia

In this cohort, 17.9% of the children had bacteremia. *Staphylococcus aureus*, *Escherichia coli*, *Group D streaptococcus, Pseudomonas aeruginosa*, non-typhoidal Salmonella were predominant organisms isolated. The prevalence of bacteremia observed in this study is higher than that reported in a cohort of febrile Tanzanian children (9.8%) (Crump et al., 2013). Other studies have reported bacteremia in the range of 11% to 19% among febrile children (Blomberg et al., 2007; Elfving et al., 2016; Nadjm et al., 2010; Were et al., 2011) and much higher rates of 25% in areas of high malaria endemicity (Biggs, 2013).

S. aureus emerged as the most common Gram–positive isolate affecting about half of the children aged less than 5 years of age. The dominance of *S. aureus* may not necessarily be attributed to malaria endemicity but instead confirms its critical involvement in community-acquired bacteremia. This is consistent with other studies in Ghana where *S. aureus* was the major etiological agent among children with bacteremia (Evans et al., 2004; Nielsen et al., 2012). A study in Nigeria also showed that *S. aureus* (48.7%) was the most frequent cause of bacteremia in infants and young children (Meremikwu et al., 2005). These findings suggest that infections by *S. aureus* constitute a major threat to childhood survival in these areas.

Several investigations have also implicated many Gram-negative organisms as prominent causes of bloodstream infections in children (Archibald et al., 2003; Ayoola et al., 2003; Gordon et al., 2002). *Escherichia coli* was the commonest Gram-negative bacteria isolated

in this study, confirming that it remains a major clinical problem in the health care system in Ghana (Obirikorang et al., 2012; Opintan & Newman, 2017). Among the organisms isolated, *E. coli*, *Group D Streptococcus, Pseudomonas aeruginosa*, and non-typhoidal *Salmonella* have been reported elsewhere as clinically important urine pathogens associated with about 90% of both community- and hospital-acquired infections (Linhares et al., 2013). These Gram-negative isolates belong to the group of bacteria known as *Enterobacteriacae* that usually colonize the gut but can progress to cause systemic infection in severe conditions. In the Gambia, community acquired Gram-negative bacteremia were found to be higher in young children as compared to adults (Were et al., 2011). A similar study at a rural hospital in Mozambique showed an increased risk of community acquired Gram-negative bacteria in children less than 3 years of age (Sigauque et al., 2009). Contrary to the current findings; other studies reported non-typhoidal *Salmonella* as the commonest pathogen isolated among children with bloodstream infections (Evans et al., 2004; Kariuki et al., 2006; Were et al., 2011). In those studies, major predisposing factors such as lack of personal hygiene and malnutrition were identified among children living in those settings.

The low prevalence of bacteremia observed in our study cohort could partly be associated with prior treatment at home. Over 73% of the children from Kintampo and 34% from LEKMA had received some form of medication including antibiotic treatment at home before seeking medical care at the hospital. Prior antibiotic usage affects the efficiency of recovery of viable microbial pathogens and this might have influenced the detection of bacteria in this study (Ndhlovu et al., 2015; Yusuf et al., 2010). The use of drugs few days before visiting the hospitals as reported by some caregivers reflects inappropriate home

treatment of bacteremic children. We found association between caregiver's educational level and occupation with infection. Although most of the caregivers have had secondary education (Table 4.4), the risk of infection was associated with level of education. Caregivers with lower levels of education were more likely than those with higher education to have their children sick and present them for treatment in the hospitals. Those caregivers were more likely to delay treatment and choose to first manage fever at home prior to seeking attention at the hospital. These observations suggest a critical need for targeted educational programs to improve care-seeking behaviors for caregivers, particularly, for those with low level of education and unemployed parents who sometimes cannot afford medications of their sick children.

Differing care giving trajectories have been identified among caregivers and their children (Scharlach, 1994). Employed caregivers may be more effective and compassionate in their interactions with sick children and unlikely to seek financial assistance in the treatment of their children as may be the case for unemployed caregivers. In this study, children from professional parental background were less likely to get infection as they enjoy better healthcare and attention. This is supported by a study done in Iran where occupation was significantly associated with diarrhea prevention practice (χ^2= 33.5, p= 0005) (Ghasemi et al., 2013).

In this study, children whose caregivers were farmers were found to be 2- times more likely to get infections than non-farmers caregivers. Many of such children follow their parents to farms and expose themselves to pathogens through weeding and playing in the farms. This study has revealed that 20% of caregivers drink portable water. This was higher than what was found in Ethiopia, where only 4.9% of the participants had access to

portable drinking water (Getu et al., 2014). The difference might be due to socio-demographic factors, accessibility to good water supplies, and difference in study period.

5.3 Concurrent malaria and bacterial infections

Both malaria and bacteremia are common tropical diseases with similar symptoms characterized by fever, body weakness, body weight loss, anemia and sometimes stomach disturbances (Bronzan et al., 2007). The similarity in expression of disease symptoms is one of the challenges that arise in the preliminary diagnosis of febrile patients since there is few documentation from the Ministry of Health, Ghana, on the status of malaria and bacteremia co-infections in the country (Ministry of Health, 2016). Although malaria was found to be one of the leading causes of fever in this study, children with malaria and bacterial co-infections were evident, representing 3.2% of children presenting with fever (Table 4.7).

There is a report of decline in the incidence of overall bacterial infections following decline of malaria transmission in similar geographic settings (Scott et al., 2006) and this could contribute to the low prevalence of bacteremia in the current study. Even though Kintampo and LEKMA have different malaria transmission intensities, there were no significant difference in the levels of bacteremia among children in the two study areas (10.7% vs. 7.1%, p=0.051). Between 2006 and 2010, Tanzania recorded a reduction in malaria incidence among children aged less than 15 years from 504 to 106 per 100,000 patients which was associated with reductions in bacteremia incidence from 82 to 7 per 100,000 patients (Graham et al., 2000; Nadjm et al., 2010). Bacteremia has been recognized as a major factor complicating malaria severity. Recent studies have reported, that patients with acute malaria may have increased risk of bacterial infections, with

accompanying clinical complications (Church & Maitland, 2014; Lundgren et al., 2015; Nielsen et al., 2012). Gram-negative bacteria in particular NTS have been reported to co-infect with malaria but the exact nature of the interaction remain unclear (Park et al., 2016). Wilairatana and colleagues reported that *P. falciparum* patients may show high gut permeability (Wilairatana et al., 1997) which could facilitate bacterial translocation from the intestinal lumen to the general circulatory system. Gómez-Pérez proposed that malaria could lead to a mal-functional spleen in young children, which could predispose them to bacterial bloodstream infection (Gómez-Pérez et al., 2014).

In the current study, only 1 out of the 5 NTS-positive children was co-infected with malaria. The isolation of only 5 NTS-positive cases seems to contradict the high prevalence recorded in other studies that have associated the susceptibility of patients with NTS to malaria infection (Biggs, 2013; Graham et al., 2000). Both NTS and malaria infections have been rated as very common in the tropics and thus easily subjected to misdiagnosis (Evans et al., 2004; Nielsen et al., 2012). The small number of NTS obtained in our study may be as a result of the small number of samples screened for bacteria. Furthermore, our results do not suggest a reversing trend in associating NTS co-infections with malaria as previously demonstrated by other investigators (Bronzan et al., 2007; Evans et al., 2004). It is however suggest that low cases of bacteremia could be associated with reduced malaria parasitemia in the present study. Simultaneous diagnosis of bacteremia and malaria did not allow the attribution of symptoms, such as fever to either of them or to the combination of both diseases. There are reports of viral suppression of malaria in children who are infected by measles or influenza (Rooth & Bjorkman, 1992).

Therefore, there is the likelihood that bacterial infection may also be suppressing malaria but it is not conclusive from this study and more research needs to be done in this area.

Malaria parasites were detected in two out of five *Rickettsia felis*-positive children, and all the two affected children were more than 10 years old. This is consistent with similar findings from Senegal, where co-infections mostly occurred in older children (Socolovschi et al., 2010). Many of the *Plasmodium*-positive children also tested positive for bloodstream invasive bacteria such as *S. aureus*, which also demonstrated the complex nature of the causes of AFI in high malaria transmission areas. This co-infection is consistent with a recent investigation in Kumasi where 51% of febrile children diagnosed with malaria were co-infected with more than one pathogens (Hogan et al., 2018a).

5.4 Zoonotic bacterial infections

The current study confirmed presence of *Rickettsia felis* and *Coxiella burnetti* as important zoonotic pathogens causing non-malarial fever in both Kintampo and Teshie communities. These bacteria usually require more elaborate diagnostic techniques for their detection in blood and therefore escape detection by the traditional culture methods despite their public health importance. Most studies have implicated domestic and wild animals as the major reservoirs for these bacteria, and infections have often been linked with contaminated food, either at home or in the food industries (Angulo et al., 2000). However, very little is known about the source and transmission dynamics of those bacteria and one could only speculate person-to-person transmission, and sporadic food borne outbreaks, may play an important role in community-acquired bacteremia among patients. Three of the children who were infected with *Rickettsia felis* lived in houses where both cat and dog were kept as pets. One can therefore assume that those animals could be potential source of infection,

assuming they were infected. The source of infection for the other two children could not be directly linked to the presence of domesticated animals as they did not keep any domestic animals.

Animals can be important family members. Keeping of animal as pets can be very beneficial to children by serving as companionship. However the role played by domestic animals in the building of human pathogen diversity have been hypothesized many years age (McNeill, 1976). Among species described as human pathogen, over 60% are from animal sources (Woolhouse & Gowtage-Sequeria, 2005). In the current study, the risk of getting infection was more pronounced when both cat and dogs were kept as pets than when there was just a single animal. The reason for this trend is not immediately known. Therefore, domestic animals should be regularly inspected by veterinary officers to ensure that they do not carry any disease. Children must wash their hands every time they touch, feed or clean up an animal. For their safety, children should be taught not to handle unfamiliar animals either wild or domesticated, even if they appear friendly.

In recent times, *Rickettsia felis* has been found to be potentially widespread across Africa, where infection can be up to 15% among febrile patients as reported in Kenya and Gabon (Angelakis et al., 2016; Maina et al., 2012; Mediannikov et al., 2013). These bacteria have also been reported in patients from Mali and Senegal where up to 24%-30% of febrile patients have been tested PCR positive (Labruna & Walke, 2014; Socolovschi et al., 2010). A study in Kumasi, Ghana recently detected *Rickettsia felis* in 1.5% of febrile pediatric patients (Sothmann et al., 2017). *Rickettsia felis* has been shown to produce symptoms similar to murine typhus and was implicated as the causative agent of vesicular fever in Senegal (Mediannikov et al., 2013). However, the pathogenesis of *Rickettsia felis*

infections is not very clear and its role as a true pathogen is still not fully elucidated. As a result, the clinical significance of *Rickettsia felis as* human pathogen has been controversially discussed (Angelakis et al., 2016; Sothmann et al., 2017).

In this study, all children from whom *Rickettsia felis* were detected were febrile, but additional symptoms were non-specific. Besides fever, cough and diarrhea were reported with no skin rashes which were consistent with reports from other studies in Africa (Angelakis et al., 2016). Remarkably, some authors have detected *Rickettsia felis* DNA in blood samples of afebrile individuals and in the feces of certain vectors such as *Ctenocephalides felis* and *Liposcelis bostrychophila* (Brown et al., 2016). This makes it very unclear, to which extent these findings may be influenced by skin contamination.

In Ghana, standard antibiotic regimens usually do not cover *Rickettsia* spp infections and so none of the *Rickettsia felis*-positive children received empirical antimicrobial treatment for *Rickettsia* spp. The course of illness resulting from *Rickettsia* infections could not be monitored as children were managed and treated on outpatient basis without follow up visits. The lack of appropriate tool at the hospital setting to diagnose the presence of *Rickettsia* spp is a challenge to the management of the disease. Therefore management will require specific patient tallied algorithms that would help clinicians in their empirical management of suspected *Rickettsia* patients. The cross-sectional nature of this study did not allow a definitive determination of *Rickettsia felis* as the disease-causing agent and it is unclear whether *Rickettsia* co-infection with malaria alleviated or aggravated the course of disease in these children. The detection of *Rickettsia felis* in our study has added to the increasing evidence for its widespread occurrence in Africa.

5.5 Arbovirus infections

TAC-PCR analysis led to the detection of dengue virus from two female children age 3 and 14 years from Teshie. Previous investigations conducted between, 2011-2014 among malaria-positive children showed dengue antibodies in 21.6% of the study children (Stoler et al., 2015). However, that study did not detect the presence of dengue virus in the blood of any of the children, suggesting previous exposure rather than acute dengue infections. Although there has been suspected dengue transmission long after the isolation of the virus in two foreigners from Finland who visited Ghana during 2000–2005 (Huhtamo et al., 2008a), there has not been any report of dengue virus detection among indigenous Ghanaians. This study has provided new evidence that the dengue virus is actively circulating in Ghana.

The caregivers of the dengue infected children in this study said their children had not traveled outside Ghana before and this clearly indicates that the infection have been acquired locally. Convalescent-phase blood samples taken one month from the dengue positive children tested positive for dengue-specific IgG and one child was also positive for dengue IgM. Although antibodies of some flavivirus infections such as Japanese-encephalitis and Lassa fever virus and non-flavivirus infections including leptospirosis could cross-react with the dengue ELISA assay, the sensitivity of the dengue-specific IgG test was high, ranging from 83.5% to 96.8%. Moreover, detection of the virus was confirmed by PCR assay as dengue-2 serotype.

Given the small number of samples screened (due to insufficient funds to purchase the TaqMan array cards) that resulted in the detection of the two dengue virus in Ghana, it is more likely that this virus may be one of the important causes of non-malarial febrile

disease in wider Ghanaian population. Generally dengue infections have been regarded as an urban disease because most cases have been reported from large cities around the globe (Mondiniet al., 2007). The occurrence of dengue in big towns and cities might be caused by poorly planned urbanization, which creates environmental conditions that support increased vector reproduction, virus propagation and expose susceptible populations (Stoler et al., 2015). The main dengue virus vectors, *Aedes.aegypti* and *Aedes. albopictus*, are predominantly urban and peri-urban in nature and therefore may be limited to towns and cities as supported by published entomologic data from the study areas (Dery et al., 2010). Future studies should focus on febrile patients of all age groups in Kintampo which has become urban by recent demographic data (KHRC, 2017). Teshie, where the infected children reside is a peri-urban area in the Greater Accra Region of Ghana that has over 171,875 residents with the challenge of rapid urbanization and insanitary conditions in many areas (web-based technical appendix figure available at https: // wwwnc. gov/ EID/article/24/8/18-0341-Techapp1.pdf) and this goes to confirm the dengue as urban disease.

5.6 Cytokines as biomarkers of fever

Causes of fever have been investigated based on a combination of clinical and laboratory parameters. PCT and CRP are the most recommended biomarkers of fever-associated pathogens but not practically used. This is because of the uncertainties surrounding their superiority to already existing microbiological test and sensitivity issues (Meisner et al., 1999b; Reinhart et al., 2011). Both PCT and CRP are known to be synthesized in response to cytokines stimulation after infections (Selberg et al., 2000; Whicheret al., 2001).

A previous study showed that cytokines are better prognostic biomarkers of infections than CRP in individuals diagnosed with febrile neutropenia (von Lilienfeld-Toal et al., 2004). In this study, we observed differences in expression levels of key pro-inflammatory and anti inflammatory cytokines among febrile and afebrile children. Three pro-inflammatory cytokines namely IL-6, IFN-γ and TNF-α, demonstrated the potential to be better predictors of fever with sensitivity above 60% at 95 % confidence (Table 4.12). The remaining cytokines had sensitivity less than 50% and could not be regarded as good predictors of fever. TNF-α, with sensitivity of 84.4% was the best predictor of fever having AUC of 0.89. IL-6 was the second best fever predictor with a sensitivity of 71.9% and AUC of 0.80%. The least performing pro-inflammatory cytokine was IL-12 with a sensitivity of 10.3%, specificity of 88.9% and a ROC of 0.50%.

Infections with Gram-negative bacteria, has been known to cause the release of lipopolysaccharides which stimulate the production of TNF-α and IL-2, and subsequently IL-6 by immune cells particularly macrophages (Bouwmeesteret al., 2004; Marchant et al., 2013). These cytokines are produced in large amounts during acute bacterial infections and in smaller amounts in chronic infections (Geiler et al., 2011). The systemic outpouring of these cytokines are known to be responsible for fever and other symptoms that characterize febrile illness (Wennerås et al., 2014). IL-6 and TNF-α share many pro-inflammatory properties and TNF-α is reported to be one of the first cytokines that are secreted during fever onset (Kokkonen et al., 2010). Elevated levels of TNF-α have been reported in febrile patients with sepsis and septic shock (Zanotti & Kumar, 2002) and has displayed the potential to predict fever in this present study. In addition, correlation between elevated levels of TNF-α and the severity of disease has been established (Bustinduy et al., 2015).

The potential of TNF-α to induce fever has been demonstrated in animal models where it was involved in prostaglandin E2 (PGE2) synthesis through the stimulation of the vascular endothelium of the hypothalamus in the brain (Kokkonen et al., 2010). TNF-α is reported to exert other secondary inflammatory effects by stimulating IL-6 synthesis in many immune-cell types and mediate its own febrile effects through initiation of acute phase response (Bustinduy et al., 2015).

In this study, IFN-γ was a good predictor of fever. The predictive value as indicated by AUC was 0.75 with sensitivity of 67.0% and specificity of 66.7%. As an important immunomodulatory agent that is stimulated during infection, it aids in host defense with the onset of fever especially in sepsis and parasitic infections. Although high levels of IFN-γ can be very deleterious, it has been shown to decrease bacterial load in febrile patients and attenuates mortality (Qiu et al., 2003; Yin et al., 2005). IFN-γ, when employed as a biomarker to detect active tuberculosis, had significantly higher predictive value in patients compared to controls (Rangaka et al., 2012). IFN-γ, as an important macrophage activator, is involved in the clearance of intracellular pathogens but has not been studied as a diagnostic marker for fever. Our result has shown the potential use of IFN-γ as a biomarker of fever in children seeking treatment for febrile illness.

IL-6 is a potent acute-phase protein inducer and has been shown to elicit fever when injected into rabbits and correlated well with febrile patients with burns (Hamzic et al., 2013; Ozbalkan et al., 2004). IL-6 as biomarker of fever produced had sensitivity of 71.9% and a very good specificity (94.4%), signifying its potential to predict fever. Another study have also recorded sensitivity of 89% and negative predictive value of 91% for IL-6 in evaluating febrile response following bacterial infections in neonates (Buck et al., 1994).

In this study, the predictive value of IL-6 for fever is expected due to its potential to induce the release of other immune-cells which make it a reliable biomarker, although the release is often transient (Bhartiya et al., 2000). A high amount of IL-6 is required to stimulate prostaglandin (PG) synthesis, which is involved in the induction of fever (Netea et al., 2000). IL-6 is a known downstream mediator of fever, whose synthesis triggers the mechanisms producing a temperature enhancement through the secretion of prostaglandin E_2(PGE_2) (Netea et al., 2000). The observed high positive predictive value for IL-6 (86.3%) in this study could be due to the fact that the onset of febrile attack *in-vivo* is time-dependent. A previous study has confirmed IL-6 as a better prognostic biomarker for bacterial infections with high sensitivity than C-reactive protein in individuals reported to suffer from neutropenia (von Lilienfeld-Toal et al., 2004).

Stryjewski et al (2005) investigated IL-6, IL-8, and PCT for their ability to discriminate between bacterial infections in pediatrics and found a good discriminating power for PCT within one day after infection (Stryjewski, Nylen, & Bell, 2005). In the case of IL-8 and IL-6, the power to discriminate between infections was rated good only 48 and 24 hours after respectively, on admission, and the results was better with the combination of PCT and IL-8. In another study, IL-8 was validated as very beneficial when it was combined with clinical parameters to identify febrile patients with bacteremia (Miedema et al., 2016). The result from this study confirms the reported good performance of IL-6 to predict the possibility of infections. This could be linked to the kinetics of IL-6 which is only sustained within some hours after stimulus in the acute phase of infections and reached a plateau during the time of fever (12 and 48 hrs later) but the concentration gradually decreases when the stimulus stop (Miedema et al., 2016).

The prediction level for all the anti-inflammatory cytokines except IL-10 had sensitivity values less than 30% indicating poor diagnostic performance and did not appear to be useful biomarkers for clinical decisions to predict fever. IL-10 significantly predicted fever with a good sensitivity of 76.3% at 95% confidence. This means that in febrile patients, IL-10 could serve as biomarker of fever in the absence of positive microbiological and other laboratory investigations. Among oncology patients, IL-10 predicted the presence of the disease with a sensitivity of 73% and a specificity of 92% (cut-off:18 pg/mL) (Urbonas et al., 2012) but in our study, IL-10 predicted fever with a sensitivity of 76.3% with a cut-off of 14.6 pg/mL. An elevated level of IL-10 is known to inhibit macrophage activation and secretion of proinflammatory cytokines which restore a balance in the immune response.

Although IL-10 was a good predictor of fever, its sensitivity was less compared to that of TNF-α in the prediction of fever. It has been suggested that the combination of biomarkers may improve their performance as diagnostic test (Reinhart et al., 2012).

5.7 Hematological indices as biomarker of fever

Among the red blood cell indices examined, lymphocytes (LYM-%) were found to be the best predictor of fever with the highest sensitivity of 65.2% and specificity of 67.3%. RWD (%) with sensitivity of 57.7% was the better predictor of fever than the rest of the other hematological clotting factors evaluated. All the other red cell indices including HGB, RBC, HCV, MCH and MCHC were less than 25% sensitive to predict fever (Table 4.9). They however had high specificity 89.3% (HGB), 86.4 % (RBC), 82.0% (HCT), 96.0% (MCHC), 91.9% (MCV) and 94.5% (MCH) to predict the absence of fever.

Individuals with infections usually tend to have abnormal hematological indices (either high or low values) partly be due poor erythropoiesis. However, the association between hematological abnormalities with pathogenesis of fever remains inconclusive. A previous study showed a reduction in RBC concentrations in individuals diagnosed to have malaria parasites as compared to non-infected individuals (Kotepui et al., 2014). This is because the malaria parasites preferably target the RBCs, resulting in an accelerated breakdown and removal of both parasitized and non-parasitized RBCs by the spleen (Price et al., 2001; Kotepui et al., 2015). The levels of both MCHC and MCH are often reduced as a result of low hemoglobin and RBCs levels. The role of infection on the performance of hematological indices needs further investigations.

The diagnostic potential of platelet to predict fever was low in our study. In some studies, however, low platelet count (thrombocytopenia) has been associated with parasitic and viral infections. Whilst thrombocytopenia correlated with malaria, fever with thrombocytopenia and a negative malaria smear correlated with arboviral infection such as dengue in those studies (Epelboin et al., 2013; Kotepui et al., 2014). In India, an investigation of the clinical characteristics of dengue, malaria, and leptospirosis showed that thrombocytopenia was a better predictor of malaria with high sensitivity compared to dengue and leptospirosis (Khan et al., 2012). The low performance of platelets as a predictor of fever may be linked to reasonable high level of infections observed in the present study.

PDW as a predictor of fever had a sensitivity of 5.16%. Whilst high PDW (≥50%) levels has been associated with *P. falciparum* mono-infections, a low PDW (<36%) and thrombocytopenia (<50G/L) were significantly associated with co-infections as compared

to patients with just single infections (Sow et al., 2016). The proposed mechanisms for low PDW include enhanced intravascular coagulations and excessive removal of platelets by anti-platelet IgG in reticulo-endothelial system (Epelboin et al., 2013). Bacteremia has been showed to contribute to low PDW (Tanwar et al., 2012).

WBC total (leukocytes) was only 20% sensitive to predict fever but has specificity of 88.9% at 95% confidence. This means that leukocytes might not be useful as a triage tool for predicting fever in children with AFIs. Leukocyte alone is reported to have a poor discriminatory power for identifying either bacteremia or parasitic infections among patients presenting with fever (Brown et al., 2005). To improve the diagnostic potential of leukocytes, examination of individual components of the WBC differential count has been suggested. For instance, the use of the absolute neutrophil count (ANC) has been validated as a superior biomarker to determine the well-being of patients than using leukocyte counts alone. Some studies that examined the incidence of respiratory infection on febrile outpatients, found elevated ANC (>9500/mm^3) to be good predictors of severe respiratory infection (Kuppermann et al.,1998).

Granulocyte percent has been investigated as a biomarker of serious blood infections in adults or neonates (Borowitz, 2003; Senthilnayagam et al., 2012) but its role as fever predictor in any population has not been established. The granulocyte (%) with sensitivity of 60.3 and odds ratio of 3.49 (Table 4-11) means that for a unit rise in GRA, the likelihood of having fever is increased by 3.5 times and this makes GRA a good fever predictor. This finding emphasizes the need to evaluate host markers as predictors of diseases, many of which have fever as symptoms.

A combination of granulocyte with other markers has been suggested to increase the accuracy of prediction of severe infections characterized with fever. The predictive value of granulocyte in febrile patients was much improved when granulocyte was evaluated together with WBC and CRP to direct care among septic patients (Geest et al., 2014). Evaluation of WBC and granulocyte together would have greater potential to identify patients with serious bloodstream infection at a very early stage which could influence clinical decision making to start treatment accordingly. A study by Jukic et al (2018) found a high sensitivity of 93% when CRP, WBC and granulocyte were combined to predict bacteremia, with specificity of 86%. CRP was not determined in the current study but in future, CRP levels would be determined and analyze with hematological and cytokine profiles of febrile children for better outcome.

When absolute lymphocyte count (#) and lymphocyte (%) were compared based on their ability to predict fever in this study, lymphocyte (%) had a better predictive value to indicate the presence of fever with sensitivity of 65.2 % and specificity of 67.3%. The absolute lymphocyte only had a sensitivity of 35.1% and specificity of 76.4% to predict fever. Increased Lymphocyte has been reported to be adequate to predict patients with mild fever with a positive predictive value of 45% and negative predictive value of 77% (Jukic et al., 2015). This implies that increased lymphocytes could indicate severe illness. Hence, clinicians could use changes in Lymphocyte as a guide to treat febrile patients in the absence of more sophisticated laboratory tests.

Monocytes account for about 5% of all leukocytes in the peripheral blood and are involved in cytokine expression, antigen presentation, or phagocytosis (Wiersinga et al., 2014). In a cohort of febrile children, monocytes to lymphocyte (ML) ratios predicted high probability

of malaria infection (RR=2.3, 95% CI; 1.10-4.50, p=0.03) as compared to absence of malaria (RR=0.7, 95% CI 0.58-1.17, p=0.3)(Hensel et al., 2017). When the probability of monocyte as a hematological predictor of fever was examined, it had a sensitivity of 25.9% and specificity of 86 .0% with a ROC of 0.59%. This does not make it a good marker of fever. In terms of absolute monocyte count, the sensitivity was even poor with sensitivity of 17.4% and specificity of 80.9% to predict fever.

For the past decade, WBC total was used as independent factor for assessing major bloodstream infections. In recent times however, the focus is shifting to WBC subtypes including lymphocyte, neutrophil, and neutrophil to lymphocyte ratio (NLR) as the predictors of diseases. Among the individual subtypes, NLR is being preferred to the use of a single parameter nowadays, because under strenuous conditions such as exercise and excessive dehydration, the absolute number of neutrophils and lymphocytes may increase but NLR is less affected under such physiological circumstances. NLR is obtained from the counts of two immune-cells involved in different complementary pathways and that gives NLR a better predictive power than either parameters alone (Azab et al., 2010).

5.8 Modelling to determine infection status

The global malaria treatment policy changed from presumptive treatment based on clinical diagnosis to targeted treatment known as Test, Treat and Track (3T), following widespread implementation of microscopy or RDT for diagnosis, availability of ACTs and improved surveillance systems (WHO, 2018). Treating every febrile illness as malaria is no longer acceptable and therefore every child suspected to malaria must have malaria test done before treatment. To compliment this initiative by the health authorities in Ghana, we

modeled malaria and bacterial infection status of febrile children using test outcomes as proxy to help clinicians to manage febrile illness.

The Naïve Bayes classifier gave better predictions with variables with small datasets (blood and urine cultures, TAC-PCR) than Lasso Regression and Random Forest. On the contrary, Lasso Regression and Random Forest were good choices for modeling variables with large dataset (malaria RDT and microscopy). Incomplete laboratory data for some tests to determine the infectious status of study participants was one of the biggest challenges in this study and Naïve Bayes' ability to accommodate such missing data was clearly advantageous. Complete data used to model blood and urine cultures, TAC-PCR had only 150 observations in all, following low laboratory detection rate. By setting aside 10% of the data as test variable and omitting observations with missing data, less data remained to fit the Lasso Regression and Random Forest models which require relatively large data to run. This might have contributed to the relatively poor performances of variables with small dataset in these models. On the other hand, more than 1500 observations were available for malaria microscopy and RDT and the problem with loss of data were very minimal. The model comparison was set to choose the most suitable one to create a web-based interactive tool. Modeling with Naïve Bayes proved to be the best option to develop the interactive tool. Health practitioners are unlikely to ask and key in all the clinical symptoms of a patient, therefore the model behind the tool must accommodate missing data and small dataset that are likely to occur in practice. For Lasso Regression or Random Forest, the models would need to be retrained every time the user added a symptom for efficient running. However in the case of Naïve Bayes, all probabilities can be pre-calculated and the model will remain unperturbed with addition or removal of

symptoms. These features of the Naïve Bayer significantly improve the recovery time and ensure a smooth operation of the model. On the model operations, while the additions of blood parameters improve Lasso Regression and Random Forest, the performance of Naïve Bayes was reduced. This might be due to the unrealistic assumptions of Naïve Bayes which state that: "all symptoms and test outcomes are assumed to be independent of each other". However, it is known that certain hematological parameters may not necessarily be independent which may affect the Naïve Bayes performance as more and more correlated predictors are added to the model. Therefore prediction model will be modified in the future to select only a subset of symptoms and blood parameters that best fit particular output.

Given the quick calculation and ability to handle small dataset and strong performance in dealing with large dataset, we selected the Naïve Bayes model and created a prototype web-based tool available at "http://kokbent.shinyapps.io/AFI_test/". The tool demonstrates how models can be translated into interactive tools to offer clinicians a clear quantitative advice on patient condition based on input records to predict probability of infection for prompt treatment. Unlike the other models, the Naïve Bayes modeling also allowed the tool to display the maximum possible deviations (predictive errors) as error bars on the test output. Ultimately, it is up to the clinicians to decide, given the data-driven quantitative evidence, if they should test the patients with one of the available tests when following the test, treat and track policy. That is whether to perform a simple malaria RDT test or microbiological culture or treat patients without such test. It was observed from the operation of the tool that, for the given test outcomes, the change in probability for malaria microscopy and RDT to identify malaria parasite does not vary much with the input of

patient's characteristic or data as compared to the others such as culture and PCR. Therefore, this tool will be most useful for malaria microscopy and RDT test. This goes to confirm the preference of most health practitioner to first test febrile patients with either malaria RDT or microscopy before considering other tests that may be available.

CHAPTER SIX

6 CONCLUSIONS, LIMITATIONS, RECOMMENDATIONS AND FURTHER STUDIES

6.1 CONCLUSIONS

Our finding is consistent with other data showing that the epidemiological and clinical pattern of childhood AFI is rapidly changing in sub-Saharan Africa. Malaria was common and accounted for 38.6% of the febrile and 10.4% of afebrile children examined. Bacterial zoonoses and arbovirus infections were prevalent, yet could not be diagnosed using traditional laboratory methods and clinical presentations alone. The results highlight the need to strengthen surveillance of AFI for better estimation of non-malaria fever, as well as morbidity and mortality associated with concurrent malaria. An integrated approach is needed to improve AFIs diagnosis and to rationally target disease control efforts. Information on the usefulness of biomarkers to predict fever and infections is limited at community level in tropical and sub-tropical regions. Lymphocytes, granulocytes and TNFα have been shown to be good predictors of fever whereas IL-10 was good predictor of malaria and bacteremia. Clarification of biomarker specificity and an adaption into specific or multiplex point-of-care tests will provide great advances in case management of AFIs. Naïve Bayes modeling has provided an alternative to predict infection status of patients even with limited datasets. Requisite expertise in AFI management including modeling and careful assessment of direct and indirect costs and benefits a diagnostic tool will help determine research and development priorities. Increased investment in diagnostics for acute febrile disease will go a long way to lower mortality and improve public health in low-resource communities.

6.2 STUDY LIMITATIONS

1. Due to resource constraint, not all the tests were performed for study participants such as cultures, TAC-PCR and cytokines analysis. Whilst samples were randomly selected for analysis in the case of TAC-PCR and cytokine assays, culture, urine, stool tests were performed at the request of the attending clinician. Ideally, these tests should have been performed for all participants. This probably accounted for the small numbers obtained for bacteria and other pathogens detected. The small numbers recorded for some specific pathogens made some sub-group analysis impossible.

2. Nasopharyngeal samples of febrile and blood samples from afebrile children were not screened for viruses, parasites and bacterial by either culture or AFI TaqMan array PCR due to financial constraint to purchase them. Hence viruses and bacteria requiring elaborate cultural conditions could not be determined and this results in non-detection of important pathogens.

3. Clinician's diagnoses were assigned by a single expert clinician and this may not show equivalent sensitivity and specificity when compared to diagnoses assigned by other clinicians. Hence, there is the likelihood of incomplete concordance in final diagnoses assigned by different physicians.

4. This study examined a wide range of etiologies of AFIs but did not test for every known AFI-associated pathogen. Hence the prevalence of some infections could be underestimated.

5. A large proportion of patients were undiagnosed suggesting that potentially important pathogens were not identified. The undiagnosed group needs to be investigated further using pathogen discovery approaches.

6.2 RECOMMENDATIONS

To health practitioners

Health practitioners should adhere to follow the WHO recommendation of confirming suspected malaria cases before treatment. Other causes of fever especially from viral must be pursuit as much as possible.

To the ministry of health

Clinical management procedures for AFIs should be reviewed considering the prevailing causes of febrile illness in Ghana. Establishment AFI surveillance groups with the District health management teams (DHMT) to detect emerging pathogens will be crucial for identifying of new causes of AFI to inform strategies to prevent and manage febrile diseases. The Ministry of Health must provide resources to develop improved diagnostic, set up surveillance with effective reporting tools to better understand the distribution of pathogens causing AFI and provide guidelines to mitigate risk of spread of contagious AFI infections. The study identified dengue virus in Accra and so the risk of spread remains due to the likely presence of the vector. It will be important for the Ministry of health and Ghana Health service to identify which populations are most risk and intensify surveillance to know the magnitude of dengue infection in Ghanaian population.

To the research community

Researchers must create knowledge and awareness in the community regarding non-malaria fever and intensify afford to develop cheap, accurate, and timely point-of-care diagnostics for non-malarial causes of childhood AFI. Traditional microbiological culture methods employed to detect systemic infections are often time consuming and have

modest sensitivity. The use of molecular-based pathogen recovery techniques may be useful to complement the existing methods to accurately diagnose AFI associated pathogens. Community based studies, involving all age categories on the etiology of AFI is needed.

To the Government of Ghana/funding agencies

The government must provide resources to support research into important but neglected diseases like dengue fever, Lassa fever. Funding agencies are also encouraged to allocate a portion of their funds to support research.

To the Ghanaian population

Ghanaian populace should be educated to know more about the threat of new and emerging non-malaria febrile diseases, and practice good environmental sanitation and personal hygiene to avert the spread of such diseases.

6.3 FURTHER STUDIES

1. This study was limited to children aged 15 years and below. A larger study to determine the extent of DENV infection among persons of all ages is needed to provide answers to speculation about dengue virus circulation in Ghana that has existed for many years.

2. A study to determine the incidence of bacterial, viral and parasitic co-infections using clinical symptoms as surrogate biomarkers to evaluate impact of co-infections on childhood morbidity among children with febrile illness

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6.4 APPENDICES

APPENDIX 1: Gram Staining

Equipment: cleaned microscope slide, water, Bunsen burner

Reagents: Safranine (25%), crystal violet, Gram's iodine, acetone/ethanol 50/50, (All from BD, Maryland, USA)

Procedure

Quality Control checks: *E. coli* (ATCC 25922) as control for Gram-negative bacteria. *S. aureus* (ATCC 25923) control for Gram-positive bacteria

Steps

a. Prepared and heat fixed smear by passing it through a flame

b. Flood the slide with crystal violet for 30 - 60 seconds.

c. Pour off excess dye and wash off with clean tap water and drain the slide against a paper towel

d. Flood the slide with Lugol's iodine for 30 – 60 seconds and washed off with clean water (tap water).

e. Add a few drops of decolorizer9 with acetone/alcohol (50/50) so the solution trickles down the slide. Rinse it off with water after 5 seconds and wash off Gram decolorizer immediately with clean water

f. Counter stain with 0.25% safranin for 30 – 60 seconds.

g. Washed off safranin with clean water, drain and blot back of the slide with filter paper, and placed on a staining rack to air dry

h. Examine slide under oil immersion (X100 magnification) for bacteria and cell types present

APPENDIX 2: Coagulase Test

Principle: The enzyme coagulase is known to clot blood plasma around itself for protection from phagocytosis. The test is mostly performed on *Staphylococcus aureus* and other Gram-positive bacteria. These bacteria produce coagulase, which can clot plasma into a gel - like form in a tube or agglutinate cocci on a slide. Slide coagulase test is used to screen isolates of *S. aureus* and tube coagulase is used for confirmation. This test is also useful in differentiating *S. aureus* from other coagulase-negative *staphylococci*.

Quality Control Used: *S. aureus* (ATCC 25923) as positive control and *S. epidermidis* (ATCC 12228) as negative control

Procedure:

Using the slide method to detect bound coagulase

1. Put a drop of physiological saline on two separate relabeled slides (slide 1 and slide

2. Emulsify two to three pure colonies of the suspected organism on both slides.

3. Add a drop of rabbit plasma to one of the suspensions on the slide 1 and gently mixed and observe clumping within 10 second

5. Observe the suspension on the slide labeled 2 for any granular appearance. This step is used to differentiate any granular appearance of the organism from the true coagulase clumping.

6. Clumping within 10 seconds is interpreted as bound coagulase produced by *S. aureus*.

7. No clumping within 10 seconds is interpreted as coagulase negative *Staphylococcus* or no bound coagulase produced.

APPENDIX 3: Catalase Test

Principle: Catalase is an enzyme is produced by bacteria to neutralize toxic forms of oxygen metabolites (H_2O_2). Many aerobic and anaerobic neutralize the bactericidal effects of hydrogen peroxide for protection but anaerobes generally lack the catalase enzyme.

Quality Control checks: *Staphylococcus aureus* (ATCC 25923) was used as a positive control *and S. epidermidis* (ATCC 12228) was used as a negative control.

Procedure:

Pick desired colony of a primary culture and transfer onto a clean glass slide. Add a drop of 3% hydrogen peroxide (BD Catalase Reagent Droppers) to the smear on the slide and examine slide immediately for the rapid production of gas bubbles.

Results interpretation;

A rapid and sustain appearance of bubbles is considered as a positive test. Few tiny bubbles forming after 20-30 seconds is considered as a negative test and if no bubble formed, the test is considered a negative.

APPENDIX 4: Preparation of Inoculums

Steps

a. Suspend one to two pure colonies of the test organism in a test tube containing 2ml sterile physiological saline by touching the surface of the desired colonies with a sterile straight loop.

b. Obtain a homogeneous mixture by touching the inner wall of the test tube containing the saline with a loopful of the test organism to make a paste. Swirl the test tube to mix the saline and paste together.

c. Compare the turbidity of the prepared inoculums using a 0.5 McFarland standard solution.

d. Check the turbidity by using a black cardboard at the background.

d. Adjust the turbidity of the inoculums until it reaches the 0.5 McFarland standards.

APPENDIX 5: Triple Sugar Iron (TSI) Agar Fermentation

Principle: TSI Agar is used to determine carbohydrate fermentation and hydrogen sulfide production in the identification of Gram-negative bacilli. TSI Agar consist of three sugars (dextrose, lactose and sucrose), phenol red for the detection of carbohydrate fermentation and ferrous ammonium sulfate to detect hydrogen sulfide production (indicated by black coloration in the butt of the tube).Carbohydrate fermentation is detected by the presence of gas and a color change from red to yellow. The production of hydrogen sulfide is indicated by the presence of a precipitate that blackens the medium in the butt of the tube.

Procedure:

1. Prepare medium based on the manufacturer's instructions.

2. Allow rubes to attain room temperature and always use pure cultures for inoculation.

3. Pick a single isolated colony and stab to the bottom of the tube and lightly streak surface of the slant afterwards.

4. Cap the tube loosely and incubated inoculated tubes for 18 to 24hrs at 35°C in an ambient condition.

5. Use the chat provided to interpret the results

APPENDIX 6: MIO (Motility Indole Ornithine**) Test**

Principle: MIO test is used for identification of *Enterobacteriaceae* on the basis of their motility, indole production and ornithine decarboxylation in a single test tube, where motility is observed as a diffuse zone of growth flaring out from the line of inoculation. The Indole test is used to determine organism's ability split indole from tryptophan by the tryptophanase enzyme. Upon reacting with Kovacs reagent, indole combines with the color in the alcohol layer, to give a red ring. If the organisms have the specific decarboxylase enzyme, the ornithine is decarboxylated to putrescence, resulting in a rise in the pH of the medium. These will be a color change from purple to yellow.

Procedure:

1. Prepare MIO medium according to the manufacturer's instructions.

2. Allow tubes to attain room temperature before inoculation and always use pure cultures for the inoculation.

3. Select a single colony and stab to the middle of the tube with a stab motion using a wire.

4. Incubate Inoculated tubes for 18 to 24hrs at 35°C in an ambient condition.

5. Examined tubes at 18 - 24 hours for growth, color change, and motility.

6. Add 3 to 4 drops of Kovac's reagent to determine motility and ornithine decarboxylase reactions

Indole Test

Appearance of a pink or red color indicates an Indole positive and formation of yellowish ring or no color change indicates a Indole negative test

Motility Test

Motility is indicated by turbidity of the medium or growth extending from inoculating stab line.

Ornithine Production

A purple color throughout the medium shows a positive ornithine reaction.

A yellow color change in the medium shows a negative reaction.

Note: Indole is detected when Kovac's Reagent is added to the surface of the medium and a pink or red color indicates an indole-positive culture.

APPENDIX 7: Cytochrome Oxidase Test

Principle: Cytochrome oxidize is found in most bacteria that transfers electrons to oxygen in the electron transport chains. This test uses dyes such as p- phenylenediamine dihydrochloride that substitute for oxygen as artificial electron acceptors. P-phenylenediamine dihydrochloride is colorless in its reduced state but in the presence of cytochrome oxidase and oxygen it becomes oxidized, forming indophenol blue.

Procedure:

Indirect paper strip technique was used.

1. Add two to three drops of the reagent to a filter paper strip.

2. With the aid of plastic loop, smear pure colony of the test organism onto the area on the filter paper containing the reagent.

3. Bacterial colonies will develop a deep blue color at the inoculation site within 10 seconds when they have cytochrome oxidase activity. The test organism is therefore positive for oxidase test.

APPENDIX 8: The Analytical Profile Index (API 20E)

Procedure

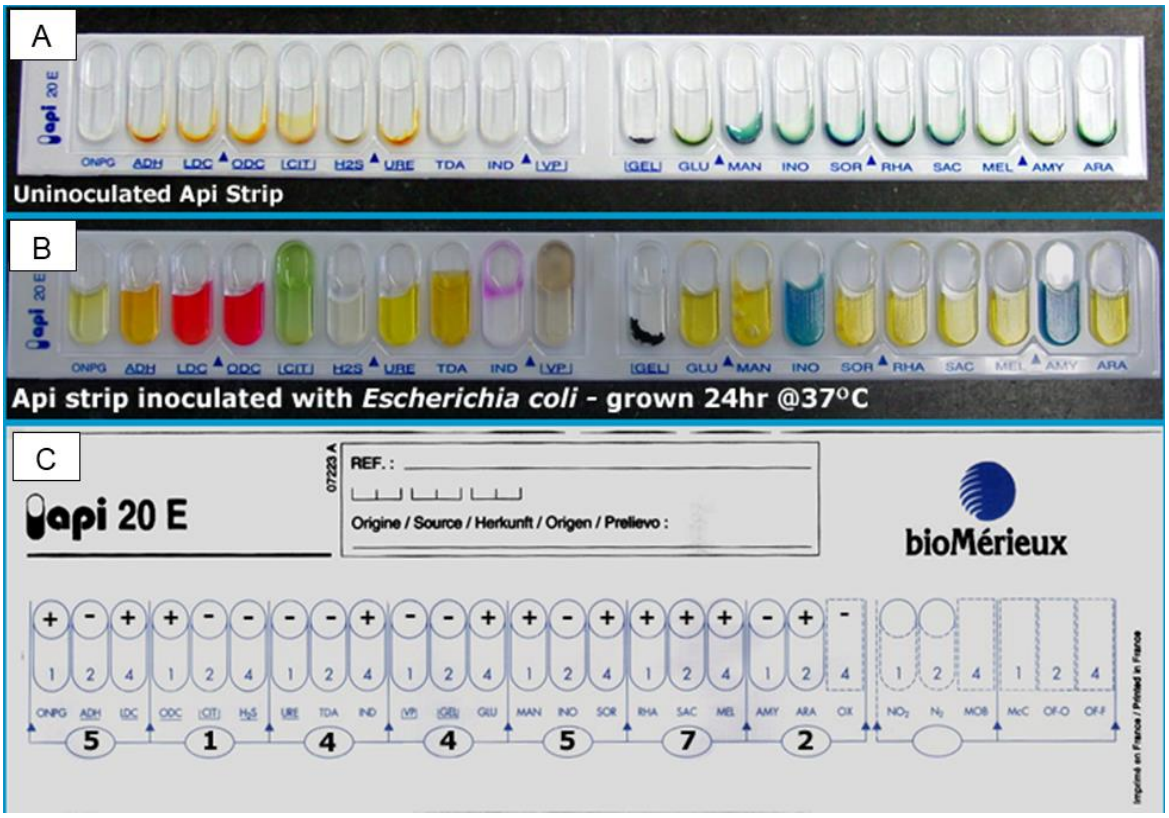
1. Bacterial suspension (0.5 McFarland standard equivalents) was made from 1-2 colonies of pure culture and inoculated into each well of a strip containing the dehydrated substrates for 20 different biochemical tests using sterile Pasteur pipette according to the manufacturer's instructions.

2. Drops of sterile mineral oil were added to those well containing LDC, ODC, ADH, H_2S, and URE to create anaerobic conditions whilst wells labeled CIT, VP, and GEL having boxes around them were filled up to the top with bacterial suspension only .

3. The API strip was placed into the incubation chamber or tray which was filled with water to prevent dehydration. The whole set up was incubated at 37° C for 18-24 hours after which it was examined for color changes.

4. The appropriate reagents were added to their respective compartments including one drop of Kovac's to the IND and reading taken within a couple of minutes and one drop of Barrett's reagent A and B to VP which was read 10 minutes later. Also, one drop of $FeCl_3$ was added to TDA and read immediately.

5. On the report sheet, the tests are separated into groups of three and a number allocated for each test. A positive test results were scored as a 7-digit numbers and the identity of the organism was derived from the database with the relevant cumulative number or profile in the API 20 E analytical coded book.



Analytical Profile Index (API) depicting miniaturised panel of different 20 biochemical assays. (A) Un-inoculated API test strip, (B) inoculated API test strip showing distinct bacterial metabolic activity marked by color change (C) Sample result sheet for recording individual biochemical test results which becomes the code for bacterial identification in the API catalogue

1. ONPG: test for bete-galactosidase enzyme by hydrolysis of the substrate o-nitro phenyl-b-D-galactopyranoside

2. ADH: decarboxylation of the amino acid arginine by arginine dihydrolase

3. LDC: decarboxylation of the amino acid lysine by lysine decarboxylase

4. ODC: decarboxylations of the amino acid ornithine by ornithine decarboxylase

5. CIT: utilization of citrate as only carbon source

6. H_2S: production of hydrogen sulphide

7. URE: test for the enzyme urease

8. TDA: (Tryptophan deaminase): detection of the enzyme trptophan deaminase

9. IND: indole Test-production of indole from tryptophan by the enzyme tryptophanase

10. VP: the Voges-Proskauer test for the detection of acetone (acetyl methylcarbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway

11. GEL: test for the production of the enzyme gelatinase which liquefies gelatin

12. GLU: fermentation of glucose (hexose sugar)

13. MAN: fermentation of mannose (hexose sugar)

14. INO: fermentation of inositol (cyclic polyalcohol)

15. SOR: fermentation of sorbitol (alcohol sugar)

16. RHA: fermentation of rhamnose (methyl pentose sugar)

17. SAC: fermentation of Sucrose (disaccharide)

18. MEL: fermentation of mellbiose (disaccharide)

19. AMY: fermentation of amygdaline (glycoside)

20. ARA: fermentation of amygdalin (pentose sugar)

APPENDIX 9: Cytokine Bead Array Protocol

Preparation of samples for immunoassay

When using frozen samples, thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.

Preparation of reagents for immunoassay

A. Preparation of Antibody-Immobilized Beads

1. Sonicate the premixed bead bottle 30 seconds and then vortex for 1 minute before use.

2. Add 60 µL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0 mL with bead diluent. Vortex the mixed beads well. Unused portion may be stored at 2-8 ^{O}C for up to one month.

B. Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 ml deionized water. Invert the vial several times to mix and vortex. Allow the vial to sit for 5-10 minutes and then transfer the controls to appropriately labeled polypropylene micro centrifuge tubes. Unused portion may be stored at -20 ^{O}C for up to one month.

C. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 30 mL of 10X Wash Buffer with 270 mL deionized water. Store unused portion at 2-8 ^{O}C for up to one month.

D. Preparation of Serum Matrix

Add 1.0 mL deionized water to the bottle containing lyophilized Serum Matrix. Mix well and allow at least 10 minutes for complete reconstitution. Leftover reconstituted Serum Matrix should be stored at -20 ^{O}C for up to one month.

E. Preparation of Human Cytokine Standard

1.) Prior to use, reconstitute the Human Cytokine Standard with 250 µL deionized water to give a 10,000 pg/mL concentration of standard for all analytes. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes and then transfer the standard to an appropriately labeled polypropylene micro centrifuge tube. This will be used as the 10,000 pg/mL standard; the unused portion may be stored at -20 ^{O}C for up to one month.

2). Preparation of Working Standards

Label five polypropylene micro centrifuge tubes 2,000, 400, 80, 16, and 3.2 pg/mL. Add 200 µL of Assay Buffer to each of the five tubes. Prepare serial dilutions by adding 50 µL of the 10,000 pg/mL reconstituted standard to the 2,000 pg/mL tube, mix well and transfer 50 µL of the 2,000 pg/mL standard to the 400 pg/mL tube, mix well and transfer 50 µL of the 400 pg/mL standard to the 80 pg/mL tube, mix well and transfer 50 µL of the 80 pg/mL standard to 16 pg/mL tube, mix well and transfer 50 µL of the 16 pg/mL standard to the 3.2 pg/mL tube and mix well. The 0 pg/mL standard (Background) will be Assay Buffer.

Procedure

1. Add 200 µL of Wash Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25 ^{O}C).

2. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.

3. Add 25 µL of each Standard or Control into the appropriate wells. Use assay buffer for 0pg/mL as Background.

4. Add 25 µL of Assay Buffer to the sample wells.

5. Add 25 µL of Serum Matrix solution to the background, standards, and control wells.

6. Add 25 µL of serum Sample into the appropriate wells.

7. Vortex Mixing Bottle and add 25 µL of the Premixed Beads to each well. Shake bead bottle intermittently during adding to avoid settling.

8. Seal the plate with a plate sealer and wrap the plate with foil and incubate with agitation on a plate shaker overnight at 4^{O}C.

9. Gently remove well contents and wash plate 2 times following instructions listed in the **PLATE WASHING** section**.**

10. Allow the Detection Antibodies to warm to room temperature and add 25 µL of Detection Antibodies into each well.

11. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25 ^{O}C). **DO NOT ASPIRATE AFTER INCUBATION.**

12. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.

13. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20- 25 ^{O}C).

14. Gently remove well contents and wash plate 2 times following instructions listed in the **PLATE WASHING** section**.**

15. Add 150 µL of Sheath Fluid to all wells. Resuspend the beads on a plate shaker for 5 minutes.

16. Run plate on Luminex 200™, MAGPIX® with xPONENT software.

17. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic curve-fitting method for calculating cytokine concentrations in samples. (Note: For diluted samples, multiply the calculated concentration by the dilution factor.

APPENDIX 10: Cytokine levels and specific infections

Association of IL-10 and IL-2 levels and demographics of children with *Plasmodium falciparum*

	IL-10 (x10^{-3}/pg/mL)	p-value	IL-2 (x10^{-3}/pg/mL)	p-value
STUDY GROUP				
Febrile	*2.8*	***0.043***	***0.9***	***0.041***
Afebrile	*1.6*		*0.6*	
AGE (YEARS)				
<5	1.8	0.446	1.6	0.127
≥5	1.2		1.9	
SEX				
Male	2.8	**0.**043	0.9	0.127
Female	1.6		1.1	

Association of IL-10 and IL-2 levels and demographics of children with Bacteria infection

	IL-10 (x10^{-3}/pg/mL)	p-value	IL-2 (x10^{-3}/pg/mL)	p-value
STUDY GROUP				
Febrile	*0.9*	*0.041*	*0.9*	*0.041*
Afebrile	*0.6*			
AGE GROUP (YEARS)				
<5	2.1	0.880	2.1	0.631
≥5	1.8		2.9	
SEX				
Male	2.9	0.432	1.8	0.544
Female	2.4		2.7	

PARENTAL CONSENT FORM

Project title: Microbial etiology of acute febrile illness in children presenting to hospitals in Ghana (A study to find out the main causes of acute fever children**)**

Research Student: Mr. Nicholas Amoako
Address: Department of Biochemistry, Cell and Molecular Biology, University of Ghana, P. O. Box LG54 Legon, Accra. Telephone: 0243059105.
Email: amnick71@yahoo.co.uk

General Information and Purpose of Research

We are inviting your child to participate in a research study to investigate the main diseases that cause acute fever. We know that malaria is one possibility. However, we have seen in Ghana and other parts of Africa that malaria is not the most frequent cause of fever in people who attend our hospitals. So it is important to know which other diseases are causing fever, in order to give an appropriate treatment and provide adequate counseling. Therefore, the aim of this study is to identify all germs that cause diseases in people with acute fever including those we may not be aware of but circulate in our environment. So we want to collect samples from people having acute fever like your child to do these investigations. We are recruiting children from the LEKMA Hospital, Accra, Kintampo Municipal Hospital, Kintampo, and War Memorial Hospital, Navrongo. Your child's participation in the study will take one day.

Procedures

If you or your child agrees to participate, we would like your permission to:

1. Take about a teaspoonful of blood (5ml), nasopharyngeal sample and ask you to bring your child's stool, urine only once. From these samples, we will test for the presence of

disease causing germs using the procedures the hospitals normally follow (conventional laboratory test).

2. We will take your child's body temperature, weight, height, demographic and clinical data and ask some questions on your socio-economic status.

3. If the laboratory test initially performed at the hospital are not able to detect the germs that may be causing the fever ,some of the samples collected will be taken to the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon so that we can use some special machines to test them.

4. Some of these tests may take a long time, so we may keep the samples for up to two years. This will allow enough time to perform all the tests we want to do and repeat them whenever necessary. Any sample remaining at the end of the holding period will be destroyed. The 5ml blood samples would be enough to conduct many laboratory tests we want do. These investigations will not include testing related to your child's genes or hereditary characteristics. Your transport fare will be refunded to you if you are asked by our study doctor to come back to the hospital at a later date for a convalescent blood sample to be taken (0.5ml) for antibody test or for any test to be repeated during our investigations period.

Possible Risks and Discomforts

Blood to be collected from your child will be drawn from a vein in the arm for the detection of the germs. There is a rare risk of fainting or infection from having blood drawn. Discomfort, bleeding, or bruising at the spot where the needle enters the body, and swelling in the area are some common risks that may occur. In addition, infection may occur if the skin is not properly cleaned before blood is drawn but all these will be minimized as

qualified personnel will perform such task. The amount of blood to be collected will not pose a health risk, but the needle can cause minor pain and discomfort when entering the vein. There will be a doctor available at all times, so in case of any serious effects during the blood draw, the medical doctor will be called in immediately.

Possible Benefits

We will use the information from this study to develop a disease predicting model (a step by step procedure of knowing the cause of a disease) that will help doctors to predict which disease is causing the acute fever based on the person's sign and symptoms and demography. The studies will yield information that will be very important in helping us know other diseases apart from malaria that cause acute febrile illness which will aid in diagnosis and treatment. All participants will benefit from the laboratory test of the study free of charge and those without health insurance cards will be assisted to get appropriate drugs as long as the disease diagnosed is within our investigations. There are technicians at the hospital who are assigned to the project, so high quality, rapid, diagnostic services will be available to your child for early treatment and care.

Confidentiality

There will be no breach of confidentiality. We will ensure confidentiality as part of the acceptable standard practice in the Ghana Health Service using non-identifiable study identification codes**.** Your child will not be identified by name in any publication, meeting, abstract, or report derived from the study results or information collected. Records of volunteer names and study numbers will be stored in both a locked file and secured computer files, and accessible only to key investigators. Individual laboratory results will be made available to clinical personnel involved in the care of patients, the patients

themselves, and caregivers of the patient. No information on your child's genes or heredity will be attributable to him/her. All information in paper form will be destroyed after five years holding period.

Compensation

Your child will be given a cake of soap and biscuit to compensate for his/her time and participation in the study. No money will be given to you or your child for participating in this study.

Voluntary Participation and Right to Leave the Research

Participation of your child in this study is completely voluntary and may withdraw at any time during the study, without any penalties. You are free not to answer any question to feel uncomfortable.

Your Child's Rights as a Participant

This research has been reviewed and approved by the Institutional Review Committee of KHRC and Ghana Health Service ERC. If you have any questions about your rights as a research participant you can contact the IEC Office between the hours of 8am-5pm through the landline 0302916438 or contact the chairman of the KHRC Institutional Ethics Committee, Dr. Eyison on 0352092035

Persons to contact

If at any time you have questions related to this study, you may contact Mr. Nicholas Amoako on telephone number 0243059105 or write to him through this address: Department of Biochemistry Cell, and Molecular Biology of the University of Ghana, Legon, Post Office Box LG54, Legon, Accra. If you have questions regarding your rights as a study participant, please contact the chairman of the Ghana Health Service ethical review

committee. You may also contact Mrs. Hannah Frimpong of Ghana Health Service Ethical Review Committee on 0243235225 or 0507041223 for further information.

Contacts for Additional Information

1). Mr. Rupert Delimini, Kintampo Health Research Centre, and Mobile: 0203534137

2). Dr. Lucas Amenga- Etego, Navrongo Health Research Centre, and Mobile: 0203797997

3).Dr. Juliana Ameh, LEKMA Hospital, Teshie, Mobile: 0244513263

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title "Microbial etiology of acute febrile illness among children presenting to three hospitals across Ghana" has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree that my child should participate as a volunteer.

Child's Name:_____

_____ _____

Date Name and signature or mark of parent or guardian

If volunteers cannot read the form themselves, a witness must sign here:

Declaration of witness: I declare that I have witnessed that the participant has read information given above, or the information above has been read to the participant and has apparently understood fully. The participant has been given a chance to ask questions concerning this study. The questions have been answered apparently to his satisfaction.

The Participant has been told that participation in the study is voluntary and that he/she has the right to withdraw from this study at any time with or without giving any reason (s).

_____ _____

Date Name and signature of witness

Certification by staff or investigator: I certify that the nature and purpose, the Potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____ _____

Date Name and Signature of investigating team member

PARENTAL CONSENT FORM (AFEBRILE GROUP)

Project title: Microbial etiology of acute febrile illness in children presenting to hospitals in Ghana (A study to find out the main causes of acute fever in children**)**

Research Student: Mr. Nicholas Amoako

Address: Department of Biochemistry, Cell and Molecular Biology, University of Ghana, P. O. Box LG54 Legon, Accra. Telephone: 0243059105. Email: amnick71@yahoo.co.uk

General Information and Purpose of Research

We are inviting your child to participate in a research study to investigate the main diseases that cause acute fever. We know that malaria is one possibility. However, we have seen in Ghana and other parts of Africa that malaria is not the most frequent cause of acute fever in people who attend our hospitals. So it is important to know which other diseases are causing fever, in order to give an appropriate treatment and provide adequate counseling. Therefore, the aim of this study is to identify all germs that cause diseases in people with acute fever including those we may not be aware of but circulate in our environment. So we want to collect samples from people having fever and those who do not have fever but are sick like your child to do these investigations. The information we will get from children with fever and those without fever will help us to make a good comparison in a disease model we intend to develop at the end of the study. We are recruiting children from the LEKMA Hospital, Accra, Kintampo Municipal Hospital, Kintampo, War Memorial Hospital, and Navrongo. Your child's participation in the study will take one day.

Procedures

If you or your child agrees to participate, we would like to have your permission to:

1. Take about a teaspoonful of blood (5ml), nasopharyngeal sample and ask you to bring your child's stool, urine only once. From these samples, we will test for the presence of disease causing germs in sick people without fever like your child using the procedures the hospitals normally follow (conventional laboratory test).

2. We will take your child's body temperature, weight, height, demographic and health data and ask some questions on your socio-economic status.

3. If the laboratory tests to be performed at the hospital are not able to detect the germs that are making your child sick, some of the samples collected will be taken to the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon so that we can use some special machines to test them.

4. Some of these tests may take a long time, so we may keep the samples for up to two years. This will allow enough time to perform all the tests we want to do and repeat them whenever necessary. Any sample remaining at the end of the holding period will be destroyed. The 5ml blood samples would be enough to conduct many laboratory tests we want do. These investigations will not include testing related to your child's genes or hereditary characteristics. Your transport fare will be refunded to you if you are asked by our study doctor to come back to the hospital at a later date for a convalescent blood sample to be taken (0.5ml) for antibody test or for any test to be repeated during our investigations period.

Possible Risks and Discomforts

Blood to be collected from your child will be drawn from a vein in the arm for the detection of the germs. There is a rare risk of fainting or infection from having blood drawn. Discomfort, bleeding, or bruising at the spot where the needle enters the body, and swelling

in the area are some common risks that may occur. In addition, infection may occur if the skin is not properly cleaned before blood is drawn but all these will be minimized as qualified personnel will perform such task. The amount of blood to be collected will not pose a health risk, but the needle can cause minor pain and discomfort when entering the vein. There will be a doctor available at all times, so in case of any serious effects during the blood draw, the medical doctor will be called in immediately.

Possible Benefits

We will use the information from this study to develop a disease predicting model (a step by step procedure of knowing the cause of a disease) that will help doctors to know which disease is causing the acute fever based on the person's sign and symptoms, as well as the age and sex. The studies will yield information that will be very important in helping us know other diseases apart from malaria that cause acute febrile illness which will aid in diagnosis and treatment. If we found out during our laboratory investigation that your child has any germs that cause disease, you will be notified and our study doctor will attend to your child and you will not be asked pay for laboratory test. All participants without health insurance cards will be assisted to get appropriate drugs as long as the disease diagnosed is within our investigations. There are technicians at the hospital who are assigned to the project, so high quality, rapid, diagnostic services will be available to your child for early treatment and care.

Confidentiality

There will be no breach of confidentiality. We will ensure confidentiality as part of the acceptable standard practice in the Ghana Health Service using non-identifiable study identification codes**.** Your child will not be identified by name in any publication, meeting,

abstract, or report derived from the study results or information collected. Records of volunteer names and study numbers will be stored in both a locked file and secured computer files, and accessible only to key investigators. Individual laboratory results will be made available to clinical personnel involved in the care of patients, the patients themselves, and caregivers of the patient. No information on your child's genes or heredity will be attributable to him/her. All information in paper form will be destroyed after five years holding period.

Compensation

Your child will be given a cake of soap and biscuit to compensate for his/her time and participation in the study. No money will be given to you or your child for participating in this study.

Voluntary Participation and Right to Leave the Research

Participation of your child in this study is completely voluntary and may withdraw at any time during the study, without any penalties. You are free not to answer any question to feel uncomfortable.

Your Child's Rights as a Participant

This research has been reviewed and approved by the Institutional Review Committee of KHRC and Ghana Health Service ERC. If you have any questions about your rights as a research participant you can contact the IEC Office between the hours of 8am-5pm through the landline 0302916438 or contact the chairman of the KHRC Institutional Ethics Committee, Dr. Eyison on 0352092035

Persons to contact

If at any time you have questions related to this study, you may contact Mr. Nicholas Amoako on telephone number 0243059105 or write to him through this address: Department of Biochemistry Cell, and Molecular Biology of the University of Ghana, Legon, Post Office Box LG54, Legon, Accra. If you have questions regarding your rights as a study participant, please contact the chairman of the Ghana Health Service ethical review committee. You may also contact Mrs. Hannah Frimpong of Ghana Health Service Ethical Review Committee on 0243235225 or 0507041223 for further information.

Contacts for Additional Information

1). Mr. Rupert Delimini, Kintampo Health Research Centre, and Mobile: 0203534137

2). Dr. Lucas Amenga-Etego, Navrongo Health Research Centre, and Mobile: 0203797997

3).Dr. Juliana Ameh, Lekma Hospital, Teshie, Mobile: 0244513263

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title "Microbial etiology of acute febrile illness among children presenting to three hospitals across Ghana" has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree that my child should participate as a volunteer.

Child's Name:_____

_____ _____

Date Name and signature or mark of parent or guardian

If volunteers cannot read the form themselves, a witness must sign here:

Declaration of witness: I declare that I have witnessed that the participant has read information given above, or the information above has been read to the participant and has apparently understood fully. The participant has been given a chance to ask questions concerning this study. The questions have been answered apparently to his satisfaction. The Participant has been told that participation in the study is voluntary and that he/she has the right to withdraw from this study at any time with or without giving any reason (s)

_____ _____

Date Name and signature of witness

Certification by staff or investigator: I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____ _____

Date Name and Signature of investigating team member

CHILD ASSENT FORM

Title: Microbial etiology of acute febrile illness in children presenting to hospitals in Ghana (A study to find out the main causes of acute fever in children**)**

Note: This form should be used only for children who are 10-15 years old

Introduction

My name is …… and I am part of a team from University of Ghana. We are conducting a research study entitled "Microbial etiology of acute febrile illness among children presenting to three hospitals across Ghana". We are asking you to take part in this research study because we want to find out about the main causes of acute febrile illness in Ghana, particularly among children. Your participation in the study will take one day.

General Information

If you agree to be part of this study, you will be asked a few questions about how you are feeling and ask you to donate small amount of blood (about teaspoonful or 5ml), stool, urine and any other sample samples that will be necessary in our investigation. These samples will be tested to find out the germs or agents that cause fever. Your samples will be given a code so that it does not directly identify you. We will take your body temperature, weight, height and ask your parents or guardian some few questions on your socio-economic and demographic status. All the samples will be tested using laboratory machine or methods available at the hospitals and other special machines at the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon**.** Some of these tests may take some time, so we may keep the samples for up to two years to allow ample time to do all the tests and also repeat them whenever necessary. Any

sample remaining at that time will be destroyed. Your transport fare will be refunded to you if you are asked by our study doctor to come back to the hospital at a later date for a convalescent blood sample to be taken (0.5ml) for antibody test or for any test to be repeated during our investigations period.

Possible Benefit

Your participation in this study will help us to know which other germs besides malaria parasite cause fever, in order to give an appropriate treatment and provide adequate counseling. We hope to develop a simple disease model (a step by step procedure of knowing diseases' cause) that can be used by doctors to predict acute febrile illness at the point of cure in future. If we found out during our laboratory investigation that you have any germs that cause disease, our study doctor will attend to you and you will not be asked to pay for laboratory test. All participants without health insurance cards will be assisted to get appropriate drugs as long as the disease diagnosed is within our investigations.

Possible Risk and Discomfort

The possible risks associated are the discomfort and pain that you may feel during the blood draw. The small amount of blood required will not pose any risk to your health. However, if there any problem with your health, a doctor will be called immediately to attend to you. These tests will not include testing related to your genes' hereditary characteristics.

Voluntary Participation and Right to Leave the Research

You can terminate your participation at any time if you feel uncomfortable. No one will be angry with you if you do not want to participate. You are free not to answer any question to feel uncomfortable.

Confidentiality

There will be no breach of confidentiality. No one will be able to know how you responded to the questions and your information will not be made known to anybody as part of the acceptable standard practice in the Ghana Health Service by using non-identifiable study identification codes. Your samples will be given a code so that they do not directly identify you and information on your genes or heredity will not be attributable to you. You will not be identified by name in any publication, meeting, abstract, or report derived from the study results or information collected. Records of your name and study number will be stored in both a locked file and secured computer files, and accessible only to key investigators.

Contacts for Additional Information

You may ask me any questions about this study or you can also contact the leader of this study, who is Mr. Nicholas Amoako from the Department of Biochemistry, Cell and Molecular Biology at the University of Ghana, Legon. You can call him at any time on his mobile phone at 0243059105. You may also contact Mrs. Hannah Frimpong of Ghana Health Service Ethical Review Committee on 0243235225 or 0507041223 for further information. Please talk about this study with your parents before you decide whether or not to participate. I will also ask permission from your parents before you are enrolled into the study. Even if your parents say "yes" you can still decide not to participate.

Your rights as a Participant

This research has been reviewed and approved by the Institutional Review Committee of KHRC and Ghana Health Service ERC. If you have any questions about your rights as a research participant you can contact the IEC Office between the hours of 8am-5pm through

the landline 0302916438 or contact the chairman of the KHRC Institutional Ethics Committee, Dr. Eyison on 0352092035

VOLUNTARY AGREEMENT

By making a mark or thumb printing below, it means that you understand and know the issues concerning this research study. If you do not want to participate in this study, please do not sign this assent form. You and your parents will be given a copy of this form after you have signed it.

This assent form which describes the benefits, risks and procedures for the research titled "Microbial etiology of acute febrile illness among children presenting to three hospitals across Ghana has been read and or explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate.

Child's Name: _____ Child's signature/Thumbprint_____

Date: _____

If volunteers cannot read the form themselves, a witness must sign here:

Declaration of witness: I declare that I have witnessed that the participant has read information given above, or the information above has been read to the participant and has apparently understood fully. The participant has been given a chance to ask questions concerning this study. The questions have been answered apparently to his/her satisfaction. The Participant has been told that participation in the study is voluntary and that he has the right to withdraw from this study at any time with or without giving any reason (s).

_____ _____

Date Name and signature of witness

Certification by staff or investigator: I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____ _____

Date Name and Signature of investigating team member

UNIVERSITY OF GHANA ACUTE FEBRILE ILLNESS (AFI) STUDY **ENROLMENT FORM**	Form Number

Child's hospital folder number: ……

1. BASIC INFORMATION

1. Study id number ……

2. Date of visit (dd/mm/yy)……

3. Name of Participant……

4. Initials (first two letters of first and last names)……

5. Gender…… 1.Male 2.Female

6. Date of birth (dd/mm/yy)……

7. Age (completed years)……..

8. Community of residence ……

9. Contact No./Compound ID……_____

10. Ethnicity

1. Akan (Bono, Ashanti etc	2. MO	3. Dagarti, Frafra, Kusasi	4. Bimoba,Chokosi
5. Fulani	6. Kokomba/Basare	7. Sisala, Wala	8. Zambrama
9. Ga,Adangbe	10. Gonja, Dagomba,Mamprusi	11. Ewe	14.Other specify:

2. Socio-Economic Background

1 Educational Back ground of child's parent/guardian

1.None	2.Primary	3.Middle school, JSS
4.Post JSS (Commercial, Technical, Apprenticeship	5.SSS	6.Post SSS (Commercial Technical, Apprenticeship
7.Post SSS (Teacher, Nursing)	8. University, Polytechnic	14.Others specify:

2. Occupation of child's parent/guardian

1. Farmer	2. Hair dressing	3.Trader
4.Carpentery ,Masonry	5.Tailoring	6.Teacher
7.Health worker	9. Unemployed	14.Others specify; ……

3. What type of house do you live in?

1. Cement block with Aluminium/Zinc roof	2.Sans crate &plaster with aluminium roof	3.Mud wall with thatch roof
4.Mud Wall with Bamboo/palm roof	5.Mud wall with aluminium roof	6.Sand crate &plaster with thatch roof
7.kiost /Container	8.Open floor	14. others specify: ……

4. Indicate which of the following is owned either by child's parent/guardian

1.Cement block house	2.Thatched house	3.Car(s)
4.T.V/Radio Set/	5.Motor bike	6.Farm
7.Fridge	8.Store	14. others specify:

5. Vegetation around your house/community

1. Forest	2. Savanna	3.Forest-savanna	14. Others…….	15.NA

6. Water body around your house/community

1. River	2. Lake	14. other……	15.NA

7. Source of drinking water

1. Pipe borne	2. River	3.Well	4.Bottled water	5.Sachet water	14. Others…….

8. Do you (child) sleep under bed net?..... | 1. Yes | 2.No |

9. Have you or child had contact with any animal (alive or dead) for the past two weeks? | 1.Yes | 2.No |

10. Mention animal type | 1.Cat | 2.Dog | 3.Sheep | 14. Other…… | 15.NA |

11.Mention the type of food eaten for the past 2 days

1. Rice 2. Fufu 3. TZ
15. Others……

3. SYMPTOM REPORT:

Does the patient report of any of the following?

1. Fever in last 48 hrs	1.Yes	2.No	16.NK	FEVER	1a.Duration (days)	
2.Muscle pain…..	1.Yes	2.No	16.NK	MYAL	2a.Duration(days)	
3. Loss of Appetite.	1.Yes	2.No	16.NK	ANOR	3a.Duration(days)	
4. Joint pain……	1.Yes	2.No	16.NK	JPAIN	4aDuration(days)	
5. Chills……	1.Yes	2.No	16.NK	CHILL	5a.Duration(days)	
6. Headache……	1.Yes	2.No	16.NK	HACH	6a.Duration(days)	
7. Nausea……	1.Yes	2.No	16.NK	NSEA	7a.Duration(days)	
8. Vomiting……	1.Yes	2.No	16.NK	VOMIT	8a.Duration(days)	
9. Diarrhea……	1.Yes	2.No	16.NK	DIRHEA	9a.Duration(days)	
10. Cough……	1.Yes	2.No	16.NK	COUGH	10a.Duration(days)	
11.Dizziness ….	1.Yes	2.No	16.NK	DIZY	11a.Duration(days)	
12. Sore throat……	1.Yes	2.No	16.NK	SORE	12a.Duration(days)	
13. Convulsion…	1.Yes	2.No	16.NK	CONUL	13a.Duration(days)	
14. Jaundice……	1.Yes	2.No	16.NK	JAUN	14a.Duration(days)	
15. Skin rash….	1.Yes	2.No	16.NK	SKINR	15a.Duration(days)	
16. Running nose…	1.Yes	2.No	16.NK	RUNNO	16a.Duaration(days)	
17. Mouth sore….	1.Yes	2.No	16.NK	MOUSO	17a Duration(days)	
18. Boil……	1.Yes	2.No	16.NK	BOIL	18a.Duration(days)	
19. Ear discharge…	1.Yes	2.No	16.NK	EARDIS	19aDuration(days)	
20. Eye discharge	1.Yes	2.No	16.NK	EYEDIS	20a.Duaration(days)	
20. Swollen tonsil	1.Yes	2.No	16.NK	SWOTON	20a.Duartion(days)	

21. Others……. | | OTHER | 21a.Duaration(days) | |

4. HISTORY OF HEALTH AND GENETIC DISEASE

1. Have you or the child been transfused with blood recently? …… | 1.Yes | 2.No |

2. When did the transfusion take place (mm/yy)… | | | | | 15.NA |

3. Have you or the child taken drug for the current illness?..... | 1.Yes | 2.No |

4. Do you know of any genetic disease in your child's family…… | 1.Yes | 2.No |

5. Mention the type of genetic disease

1.Sickle cell disease	2.G6PD Deficiency	3.Thalassaemia	4.Diabetes
16.NK	14.Others		15.NA

5. ANTHROPOMETRY/OTHER EXAMINATIONS

1. Weight (kg)…… | | | . | |

2. Height (cm)…… | | | . | |

3 .MUAC (cm) if 5years and below…… | | | . | |

4. Axillary Temperature (^{O}C)…… | | | . | |

5. Respiratory system (observes and circle)…… | 1.Normal | 2.Abnormal |

6. LABORATORY SAMPLES

1. Blood samples taken into EDTA and SST microtainers ?…… | 1.Yes | 2.No |

2. Stool sample taken for R/E…….. | 1.Yes | 2.No |

3. Urine sample taken for R/E…… | 1.Yes | 2.No |

4. Nasopharyngeal (NP) sample taken into tube with UTM…… | 1.Yes | 2.No |

5.Swab-taken?-(underline)
(Nasal/throat/skin/Ear/Eye/Vaginal etc) | 1.Yes | 2.No |

6. Request made for microbial culture?..... | 1.Yes | 2.No | 15.NA |

7. Sample taken for culture (specify).	1.blood	2.stool	3.urine	14. others
8. Other laboratory request made	1.(Specify) ……			15. NA
9. Full blood count (FBC) performed……	1.Yes			2.No
10. If FBC is not performed, give reason……	1.			15. NA

7. CLINICAL DIAGNOSIS

1. Evidence of blood stream infection for the current illness?	1.Yes	2.No
2. Circle the suspected infection	1.viral 2.bacteria 3.fungi 4.protozoan	15.NA

3. Record the DIAGNOSIS made and TREATMENT given (table below)
CLINDIA

Name or code of attending clinician……		

4. Name or code of interviewer**……** | | |