

**DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY
UNIVERSITY OF GHANA**

**ERYTHROCYTE INVASION MECHANISMS OF *PLASMODIUM FALCIPARUM*
CLINICAL ISOLATES FROM GHANAIAN CHILDREN**

A dissertation submitted to the Board of Graduate Studies, University of Ghana,



**In partial fulfilment of the requirements for the award of the Master of Philosophy degree
in Biochemistry**

By

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DECLARATION

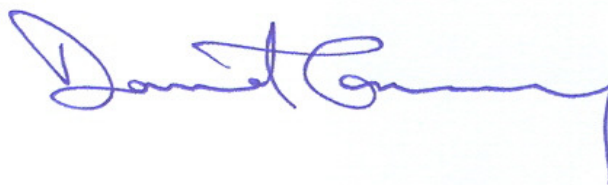
The experimental work presented in this thesis was done by me, Henrietta, E. Mensah-Brown, at the Department of Biochemistry, Cell and Molecular Biology and Noguchi Memorial Institute for Medical Research under the supervision of Dr. Gordon A. Awandare (Department of Biochemistry, Cell and Molecular Biology, University of Ghana) and Prof. David Conway (London School of Hygiene and Tropical Medicine).

All references have been duly cited.



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DEDICATION

To God be the Glory. Thank you for Your mercies.



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LIST OF ABBREVIATIONS

ACTs	Artemisinin Combination Therapies
AMA-1	Apical Membrane Antigen-1
BSA	Bovine Serum Albumin
BSG	Basigin
CPM	Complete Parasite Medium
CR1	Complement Receptor 1
CSP	Circumsporozoite Protein
DBL	Duffy Binding Ligand
DDAO-SE	7-hydroxy-9 <i>H</i> -(1,3-dichloro-9,9-dimethylacridin-2-one) succinimidyl ester
DNA	Deoxyribonucleic Acid
EBA	Erythrocyte Binding Antigen
EBL	Erythrocyte Binding Ligand
FITC	Fluorescein Isothiocyanate
GPI	Glycosylphosphatidylinositol
GYP	Glycophorin
HSPG	Heparan-Sulphate Proteoglycans
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
IRS	Inside Residual Spraying
ITNs	Insecticide Treated Nets
LLITNs	Long Lasting Insecticide Treated Nets
MSP-1	Merozoite surface protein 1
PE	Phycoerythrin

PfRh	<i>P. falciparum</i> Reticulocyte-like binding Homologue
PVM	Parasitophorous Vacuolar Membrane
PWM	Parasite Wash Medium
SA	Sialic Acid
sCR1	Soluble Complement Receptor 1
SEM	Standard Error of the Mean

ABSTRACT

Clinical manifestations of Plasmodium falciparum infections are caused by invasion of erythrocytes by malaria parasites, a process mediated by multiple receptor-ligand interactions. Antibodies against some parasite ligands significantly inhibit parasite growth in vitro, demonstrating that these interactions may be good targets for the development of an effective blood stage vaccine. This study was aimed at investigating the erythrocyte receptors used by P. falciparum isolates in Ghana. P. falciparum field isolates were collected from children aged 2-6 years attending hospitals in three ecologically distinct zones in Ghana: Accra, Navrongo, and Kintampo. Erythrocyte invasion assays were performed on eighteen isolates to test the ability of the parasites to invade erythrocytes treated with neuraminidase, trypsin and chymotrypsin which selectively remove receptors from the erythrocyte cell surface. In addition, antibodies against two recently identified receptors, basigin (BSG) and complement receptor 1 (CR1) were used to determine the dependence of the isolates on these pathways. One to three assays were performed on each isolate. The majority of field isolates tested were capable of invading neuraminidase-treated erythrocytes with greater than 50% invasion efficiencies relative to untreated erythrocytes, suggesting that these field isolates have sialic acid (SA)-independent invasion phenotypes. Invasion efficiency in trypsin-and chymotrypsin-treated erythrocytes varied between 20 to 75% relative to untreated erythrocytes. In all field isolates tested, antibodies against CR1 significantly inhibited invasion of neuraminidase-treated erythrocytes. However, anti-BSG antibodies significantly inhibited invasion in both untreated and neuraminidase-treated erythrocytes to a similar extent. This may suggest that the interaction between basigin and its parasite ligand PfRh5 may be upstream of interactions involving glycophorins and CR1.

CHAPTER ONE

1.0. INTRODUCTION

Malaria is an infectious disease known to affect more than half of the world's population. In 2012, the World Health Organization (WHO) estimated that malaria caused 660,000 deaths annually, of mainly children and pregnant women and mostly in sub-Saharan Africa. Malaria is the second leading cause of death due to an infectious disease in Africa and is the second leading cause of under-five mortality (WHO, 2012). It therefore continues to be a major health concern worldwide and impacts greatly on economic development of malaria-endemic countries (Snow *et al.*, 2005), the majority of which are in Africa (WHO, 2012). According to the WHO (2012), Nigeria, Democratic Republic of the Congo, United Republic of Tanzania, Uganda, Mozambique and Cote d'Ivoire have the highest malaria disease burden in Africa. In 2011, there were approximately 360,000 reported cases of malaria in Ghana, resulting in 100,000 deaths (WHO, 2012).

Malaria is caused by *Plasmodium*, a protozoan parasite belonging to phylum *Apicomplexa*. Human malaria is thought to have evolved 5000 years around the same time that man evolved from apes (Carter and Mendis, 2002). Five species of *Plasmodium* are known to infect humans: namely *P. falciparum*, *ovale*, *knowlesi*, *malariae*, and *vivax*. *P. falciparum* is responsible for the most severe forms of malaria and the majority of the disease burden associated with malaria, accounting for 90% of malaria cases (WHO, 2012). *P. falciparum* undergoes a complex life cycle consisting of an asexual stage in the vertebrate host and sexual stage within the mosquito vector. The morbidity and mortality associated with malaria is as a result of the asexual blood stages, where the parasite undergoes a cycle of invading the erythrocytes, growing and differentiating, and bursting to release merozoites,

which then invade circulating erythrocytes (Cowman *et al.*, 2012). The process of invasion is a highly sophisticated one, involving several parasite ligands that coordinate to attach and invade erythrocytes (Cowman *et al.*, 2012). Invasion involves four major steps, namely primary contact and adhesion, reorientation such that the apical portion is in direct contact with erythrocyte surface, secondary ligand interactions, and finally, entry or ingress. The secondary ligand interactions are mediated by specific parasite ligand-erythrocyte receptor interactions, which enable the parasite to invade the red blood cell. Parasite ligands that are used for invasion are from two families of proteins: erythrocyte binding-like (EBL) family, including erythrocyte binding antigen (EBA)-175, EBL-1 and EBA-140; and the reticulocyte-like binding homologue (Rh) family, including PfRh1, PfRh2a, PfRh2b, PfRh4, and PfRh5. Parasite ligands in the EBL family interact with sialic acid (SA) residues on the erythrocyte surface. These pathways are termed SA-dependent pathways, because of their dependence on SA to invade erythrocytes. Glycophorins A, B and C are known to interact with EBA-175, EBL-1 and EBA-140 respectively, in a SA-dependent manner (Maier *et al.*, 2003, Mayer *et al.*, 2009, Orlandi *et al.*, 1992, Mayer *et al.*, 2001). Other parasite ligands involved in SA-dependent invasion are EBA-181 and PfRh1, however their respective erythrocyte receptors are not yet known.

A number of laboratory-adapted strains of *P. falciparum* showed the ability to invade cells deficient of glycophorins (Hadley *et al.*, 1987, Dolan *et al.*, 1990) indicating the presence of alternate invasion pathways independent of SA. PfRh2a/b, PfRh4, and PfRh5 appear to play pivotal roles in SA-independent invasion of erythrocytes. PfRh4 has been shown to interact with complement receptor 1 (CR1) (Tham *et al.*, 2010) while PfRh5 binds to basigin (BSG) (Crosnier *et al.*, 2011). The receptors for PfRh2a/b remain unknown. Because of the extensive repertoire of ligands that the parasite possesses, EBL and Rh

proteins show functional redundancy, giving the parasite the ability to switch between SA-dependent and -independent pathways.

Antibodies against several EBL and Rh proteins, including EBA-175, EBA-181, EBA-140, have been shown to inhibit invasion significantly and confer protection against malaria (Persson *et al.*, 2008, Richards *et al.*, 2010), and making them potential candidates for a vaccine against malaria. Recent studies have shown that antibodies against recombinant PfRh5 were capable of inhibiting invasion significantly, making PfRh5 a potential vaccine target (Bustamante *et al.*, 2013). However, none of these inhibitory antibodies produced have been shown to completely abolish merozoite invasion, possibly due to the wide range of receptor-ligand interactions available to *P. falciparum* for invasion. Therefore, a vaccine which induces antibodies against a single parasite ligand will be highly ineffective, because of the multiple invasion pathways available to the parasite. A multi-component vaccine, targeting the entire array of receptor-ligand interaction available, may have a better chance of inhibiting invasion and effectively ending the life cycle of *P. falciparum*. This was clearly shown in a study conducted by Lopaticki *et al.* (2011) which showed that antibodies generated against recombinant domains from EBA-175 (RIII-V), PfRh2a/b and PfRh4 were more effective in combination relative to each antibody only.

However, the majority of these inhibition assays were conducted on laboratory-adapted strains, and may not be entirely reflective of the inhibitory activity of these antibodies on clinical isolates from malaria-endemic areas. This is especially true of EBA-175 since Persson *et al.* (2008) established that antibodies against EBA-175 were more prevalent among children in Africa than antibodies against other ligands used by *P. falciparum*. This strongly suggests that field isolates of *P. falciparum* in Africa may be using alternate pathways to invade erythrocytes in order to evade the host immune system. Therefore, a

vaccine targeting only EBA-175 will be ineffective at preventing both severe and uncomplicated malaria. It is therefore necessary to determine the invasion phenotypes of *P. falciparum* clinical isolates in endemic areas, so that these can be incorporated into a vaccine that will induce a broad spectrum of antibodies capable of blocking merozoite invasion completely.

1.1. Study Objectives

Growth inhibition assays designed to study invasion phenotypes used by *P. falciparum* clones utilize enzymes and antibodies that selectively cleave and block specific receptors. Several of such studies have been conducted across sub-Saharan Africa, South-East Asia and South America to determine the invasion phenotypes of field isolates (Awandare *et al.*, 2011, Baum *et al.*, 2003, Deans *et al.*, 2007, Gomez-Escobar *et al.*, 2010, Lopez-Perez *et al.*, 2012, Jennings *et al.*, 2007, Lobo *et al.*, 2004, Okoyeh *et al.*, 1999). These studies show that *P. falciparum* uses a wide range of ligands to invade erythrocytes, mainly EBL and Rh proteins, similar to those observed in laboratory-adapted strains.

Recent studies on field isolates in Kenya (Deans *et al.*, 2007) and across South America (Lopez-Perez *et al.*, 2012) show that *P. falciparum* isolates rely on neuraminidase resistant receptors for invasion, implying the presence of parasites in endemic populations using receptors that are independent of SA for invasion. Indeed, Awandare *et al.* (2011) showed that Kenyan field isolates used CR1, as a major SA-independent receptor for erythrocyte invasion. This is consistent with a study by Persson and co-workers in 2008, which showed the prevalence of high levels of antibodies against ligands of SA-dependent pathways in African children, suggesting that field isolates in Africa may be under an immunological pressure to switch to SA-independent phenotypes in order to survive. Apart from studies

conducted to determine invasion phenotypes of Gambian field isolates (Gomez-Escobar *et al.*, 2010, Baum *et al.*, 2003), no studies have been conducted in other West African countries. The study conducted here sought to test hypothesis that *P. falciparum* field isolates from malaria endemic areas such as Ghana will invade erythrocytes using receptor-ligand interactions that are SA-independent. Furthermore, the study sought to test the hypothesis that CR1 would be the major receptor for SA-independent invasion in Ghanaian field isolates, as was the case in Kenya.

1.1.1 Aim

To determine the mechanisms of invasion of *P. falciparum* field isolates from Ghana.

1.1.2. Specific Objectives

- To determine the ability of *P. falciparum* field isolates in Ghana to invade erythrocytes treated with neuraminidase, trypsin and chymotrypsin which specifically disrupt specific receptor-ligand interactions
- To determine the contribution of SA- independent pathways to invasion in field isolates in Ghana by using antibodies against CR1 and BSG to inhibit invasion.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. The Burden of Malaria

Malaria still continues to be a major health challenge, which also affects the economy and productivity in endemic areas. Malaria is endemic in mainly sub-Saharan Africa, South America and parts of Asia (Figure 2.1). The World Malaria Report 2012 estimates that approximately 80% of malaria cases in 2010 occurred within the African region. More than 90% of global deaths also occurred within Africa and children under five years represented 91% of deaths within the Africa region (Table 2.1). Approximately 90% of malaria cases worldwide are caused by *P. falciparum* (WHO, 2012). Table 2.1 estimates the global morbidity and mortality caused by malaria by region, and shows that the greatest mortality due to malaria occurs in Africa.



Figure 2.1. Global distribution of malaria transmission. (Source: World Health Organization., 2012)

Table 2.1. WHO estimates of malaria cases and deaths in 2010 by geographical region (Adapted from World Malaria Report, 2012)

WHO Region	Malaria Cases (%)	Malaria Deaths (%)	Under 5 years Deaths (%)*
African	79.5	90.3	91
Region of the Americas	0.5	0.2	29
Eastern Mediterranean	4.7	2.3	70
Europe	0	0	–
South East Asia	14.6	6.5	32
Western Pacific	0.8	0.6	41

*% Under 5 years deaths is a percentage of total deaths within the WHO region.

The burden of malaria is particularly hard to estimate especially in low income countries due to incomplete reports from health facilities, under-diagnoses, over-diagnoses and lack of hospitals or health facilities in many rural areas (Autino *et al.*, 2012b). Estimates by the WHO are based on data from the Ministries of Health of endemic countries. This is highly inadequate since they lack basic logistics to properly ascertain the prevalence of malaria country-wide.

The economic burden of malaria on sub-Saharan Africa is unmistakable. The poorest countries in the world are mainly in sub-Saharan Africa where malaria is endemic. Sachs and Malaney (2002) stated that the global distribution of per-capita gross domestic product shows a striking correlation between malaria and poverty, and malaria-endemic countries also have lower rates of economic growth than non-endemic countries. According to Asante and Asenso-Okyere (2003), the impact of malaria on the growth in real GDP in Ghana is negative and decreases by 0.41% for every 1% increase in the malaria morbidity rate.

2.2. The Life Cycle of *Plasmodium falciparum*

The life cycle of *P. falciparum* is complex occurring in the *Anopheles* mosquito vector and within the vertebrate host.

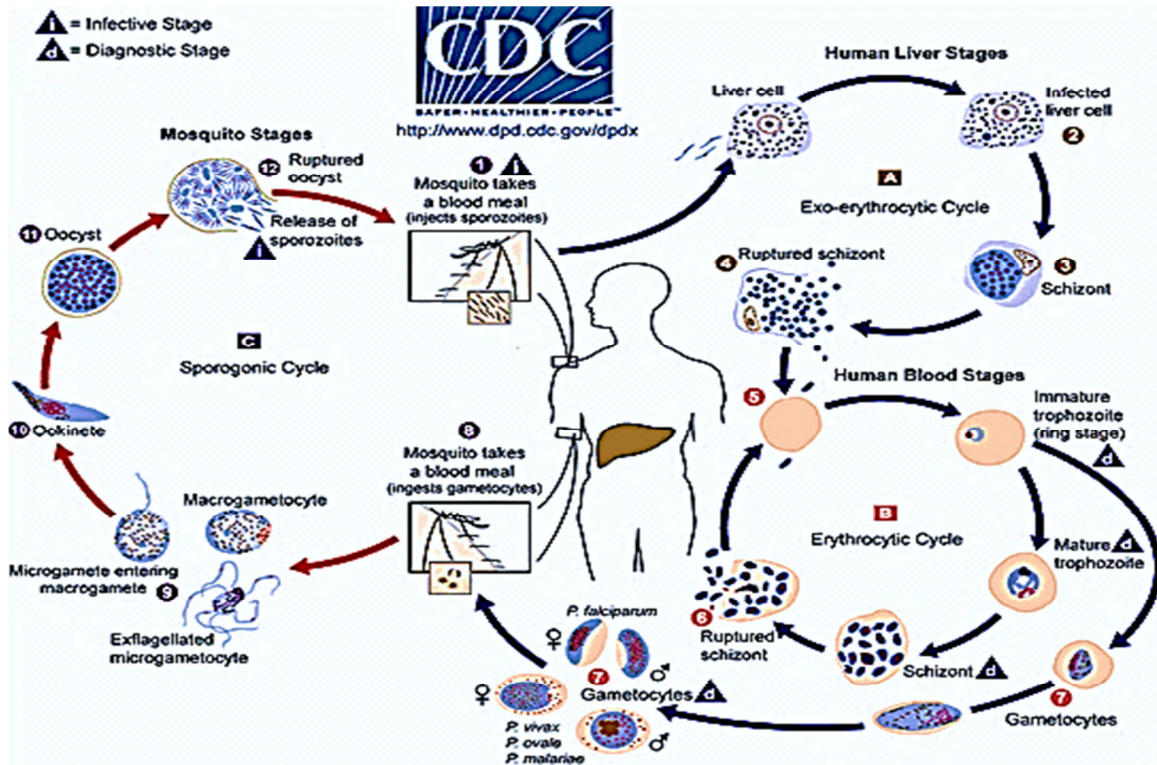


Figure 2.2. The life cycle of *P. falciparum*.

When the female *Anopheles* bites a human host, sporozoites are injected into the bloodstream and enter the hepatocytes. There, they differentiate into merozoites that invade erythrocytes and grow to form trophozoites and schizonts. Schizonts burst to release merozoites that reinfest circulating erythrocytes. Some merozoites differentiate into gametocytes beginning the sexual phase of the life cycle. Gametocytes are taken up into the gut of a feeding mosquito and mature to form male and female gametes. The fertilized zygote develops to an ookinete and an oocyst and finally sporozoites that migrate to the salivary glands to begin the cycle once more (Source: the Center for Disease Control & Prevention, Division of Parasitic Disease).

The female *Anopheles* mosquito is the vector for malaria transmission. While the male *Anopheles* mosquito feeds on plants and nectar, the female *Anopheles* mosquito requires a blood meal to facilitate egg development. The life cycle begins when an infected female *Anopheles* mosquito bites a vertebrate host (Figure 2.2.). In order to prevent the blood from

clotting, the mosquito injects an anti-coagulation factor Xa (Stark and James, 1996), together with *Plasmodium* sporozoites present in its salivary glands. After injection into the skin of the host, the sporozoites then transmigrate through the endothelia of the skin into the blood. Sporozoites circulate through the blood, until they reach the liver, where they adhere to the endothelium and bind to highly sulfated heparin sulfate proteoglycans (HSPGs) (Coppi *et al.*, 2007) which are presented by hepatocytes through small channels in endothelial cells called fenestrae (Figure 2.2). This triggers a signaling cascade within the parasite that activates calcium-dependent protein kinase 6, which causes the cleavage of circumsporozoite protein (CSP) and causes the sporozoites to switch to an invasive phenotype (Coppi *et al.*, 2007). However, the sporozoites do not remain in the first hepatocyte it invades but begins a cycle of transmigration through several hepatocytes before finally invading and differentiating in one hepatocyte (Mota *et al.*, 2001). Though several theories have been postulated, the mechanism and reason for transmigration is not known.

After invading the hepatocyte, the sporozoite forms a parasitophorous vacuolar membrane (PVM) around itself, which contains a parasite vacuole (Graewe *et al.*, 2012). The sporozoite then transforms into a small trophozoite, and begins active differentiation into a schizont (Graewe *et al.*, 2012). The differentiation of sporozoites from trophozoites to schizonts usually takes 5-10 days for human malaria parasites. The mature merozoites must now egress from the hepatocyte into the bloodstream to infect erythrocytes, whilst evading sessile macrophages called the Kupffer cells in the liver. The process of egress begins with the disruption of the PVM (Graewe *et al.*, 2011), leading to the release of merozoites into the host cytoplasm. This is promptly followed by host cell death. However, death of infected hepatocytes is unlike apoptosis or necrosis since the host cell membrane is not

only maintained (Wirth and Pradel, 2012), but is characterized by cell detachment from the liver tissue, condensation of the nucleus and loss of the mitochondrial membrane potential (Sturm *et al.*, 2006, Sturm *et al.*, 2009). In addition, caspases are not involved in this type of programmed cell death. Though the exact mechanism of egress of liver stage parasites is not known, cysteine proteases of the serine repeat antigen (SERA) family have been implicated in PVM breakdown, degradation of mitochondria, cell detachment and merosome formation (Sturm *et al.*, 2006). The merosomes, which consists of parasites enclosed in the host cell membrane, are released into the bloodstream by squeezing between two adjacent cells of the fenestrated endothelium of the liver sinusoid (Wirth and Pradel, 2012). The merosomes then travel through the bloodstream to the pulmonary capillary system where the parasites are injected into the bloodstream through a mechanism that is not yet fully understood (Baer *et al.*, 2007). This marks the beginning of the asexual blood stage of the life cycle. The merozoite enters the erythrocyte to form the ring stage parasite, forming a parasitophorous vacuole membrane (PVM) around itself using part of the erythrocyte membrane and lipids expelled from its own secretory organelles (Ward *et al.*, 1993), and this serves as a membrane through which nutrients are exchanged between the parasite and the host cell. As the parasite develops from the ring stage into the trophozoite stage, its metabolism increases and it forms a specialized organ called the food vacuole. The food vacuole is highly acidic and contains several proteases for the digestion of haemoglobin. The parasite digests about 60-80% of erythrocytic haemoglobin, deriving amino acids from the globin chain (Krugliak *et al.*, 2002). The proteases falcipain 2, falcipain 2^l, plasmepsin II, plasmepsin IV, histo aspartic protease (HAP) and *Plasmodium* haem degradation protein (HDP) form a complex for the digestion of globin and the degradation of haem to haemozoin (Chugh *et al.*, 2013). The trophozoite

undergoes deoxyribonucleic acid (DNA) replication and forms a schizont which divides into 16-32 merozoites.

Occasionally, some of the rings in the asexual blood stage differentiate to form gametocytes that ensure the continuity of the life cycle of the *Plasmodium* parasite. The process of gametocytogenesis takes about two weeks, after which the female *Anopheles* mosquito takes up the gametocytes during a blood meal. The gametocytes are activated by the drop in temperature, change in pH and contact with xanthurenic acid when moved from a mammalian host to the midgut of the mosquito (Wirth and Pradel, 2012). For a zygote to form from the fusion of the male and female gametes, the activated gametes must first egress from the red blood cell in which they are. Egress of gametocytes is a process involving several proteases, which begins with the rupture of the PVM followed later by the rupture of the erythrocyte cell membrane (Sologub *et al.*, 2011, Torres *et al.*, 2005). The male gamete undergoes three rounds of replication to produce eight microgametes in a process known as ex-flagellation (Guinet *et al.*, 1996). The female gamete forms a large macrogamete that is fertilized by one of the male microgametes to form diploid zygote in the midgut of the mosquito. The zygote undergoes further development to form a motile ookinete that crosses into the midgut epithelium. The parasite settles between the basal epithelial surface and the basal lamina where it develops into an oocyst after meiosis. The oocyst attaches to the midgut epithelium and stays there for about 10 days whilst it undergoes mitosis to form haploid sporozoites. The sporozoites are then released from the oocyst into the open haemolymph circulation of the mosquito and travel to the mosquito salivary gland where they invade it. When the infected female *Anopheles* mosquito takes a blood meal, it injects the sporozoites into the vertebrate host and the cycle begins once more.

2.4. Pathogenesis of Malaria

The clinical manifestations of malaria are associated with the asexual stages of the life cycle of *P. falciparum*. General symptoms of malaria include fever, headaches, vomiting, etc. However, more severe forms of malaria such as severe malarial anaemia (SMA) and cerebral malaria (CM) are associated with hypoglycaemia, haemoglobinuria, acute respiratory distress syndrome (ARDS), renal failure, and coma. Severe manifestations of malaria are caused by a phenomenon called parasite sequestration (Grau and Craig, 2012), which enables parasites to avoid the host immune system. As infected erythrocytes mature from the ring stage to the young trophozoites, they adhere to human cells within the microvascular circulatory system (Autino *et al.*, 2012a). Sequestration of infected erythrocytes occurs in three main forms:

- a) Cytoadherence when mature infected erythrocytes adhere to endothelial cells in vascular organs (Udeinya *et al.*, 1981)
- b) Rosetting where infected erythrocytes adhere to uninfected erythrocytes (Udomsangpetch *et al.*, 1989) and
- c) Platelet-mediated clumping when infected erythrocytes bind to platelets (Pain *et al.*, 2001).

P. falciparum erythrocyte Membrane Protein 1 (PfEMP-1) is the major parasite adhesion protein involved in cytoadherence (Su *et al.*, 1995), and plays a crucial role in malaria virulence and pathogenesis (Kraemer and Smith, 2006). The extracellular portion of PfEMP-1 contains multiple adhesion domains that bind to host receptors causing sequestration. Though parasite sequestration is crucial for pathogenesis, host factors contribute to the severity of malaria disease. Malarial anaemia for instance is associated

with suppression of erythropoiesis, as well as splenic clearance and phagocytosis of infected erythrocytes and uninfected erythrocytes. Sequestered erythrocytes cause vaso-occlusion, which causes hypoxia. Hypoxia causes tissue death, oedema, and hypertension. Tissue death within the brain leads to cerebral malaria which results in coma. These are major causes of mortality due to malaria.

2.5. Malaria Control

The control of malaria is classified into three major groups: vector control, the use of prophylactics and curative drugs, and the development of vaccines.

2.5.1. Vector Control

Vector control is an integral component of the control of malaria today. Eradication of malaria in various parts of the world was achieved through vector elimination. The WHO currently has two major strategies to control the mosquito vector, namely indoor residual spraying (IRS) and the use of long-lasting insecticide treated nets (LLITNs).

IRS is one of the most effective means of vector elimination and has been in use for over 60 years. IRS involves the application of insecticides on the wall and roofs of houses. It has been widely successful and responsible for the reduction in disease incidence during the early twentieth century, including elimination of malaria from many countries at the edges of the global malaria distribution, especially in Europe, the Americas and parts of South Africa (Karunamoorthi, 2011, Trigg and Kondrachine, 1998, Shiff, 2002). According to the WHO (2012), 153 million people were protected against malaria through IRS in 2011, including 77 million people in Africa. Di-chloro-di-phenyl-trichloro-ethane (DDT) and pyrethroids are the main insecticides used for IRS. Though IRS has been widely successful, its use as a means of malaria control is on the decline. This is due to

environmental concerns, emerging resistance and the lack of funds from governments to sustain the program (World Health Organization., 2006). Furthermore, mosquito resistance to at least one insecticide used in malaria control has been reported in 64 countries around the world according to the World Malaria Report (2012), which is a major concern for the World Malaria Control Program.

Another major initiative in the control of malaria is the use of LLITNs to replace the insecticide treated nets (ITNs). LLITNS have proved to be even more widely successful than IRS, since it is more sustainable financially and in terms of logistics. LLITNs are impregnated with pyrethroids, which have low mammalian toxicity and last for about 3-4 years. Randomized controlled trials in Kenya, Ghana, The Gambia and Burkina Faso have demonstrated that wide-scale use of ITNs can reduce child mortality by approximately 20%, saving an average of six lives for every 1000 children aged 1–59 months protected every year (Lengeler, 2004). Both LLITNs and IRS are dependent on one class of insecticides, pyrethroids, leading to widespread incidence of resistance in endemic countries.

Chemical larvicides are also used to eliminate the larvae and pupae. Paris green (cupric acetoarsenite) was used initially to eliminate larvae and pupa. However, its use has been discontinued due to toxicity, despite the fact that it is highly effective and inexpensive. More recently, insect growth regulators (IGRs) are being used to control mosquitoes. Typically, these substances are homologues of juvenile hormones, enabling them to bind to juvenile hormone receptors in the immature form of an insect, preventing the development of the insect (Karunamoorthi, 2011). Other methods of larvae control employ the use of biological agents. Two bacterial species are widely effective against several species of

mosquito larvae. These are *Bacillus thuringiensis israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*). The mechanism of action is through protoxins found in parasporal crystals and the spore coat of these bacteria, which act as stomach poisons in the mosquito larval midgut (Karunamoorthi, 2011). The advantages of larval biological control far outweigh larval chemical control. Biological control methods have lower toxicity to animals and humans, can be used in low doses and the possibility of resistance development is highly reduced. The one major downside though is the multiple widespread breeding grounds of mosquitoes.

2.5.2. Therapeutic Drugs and Drug Resistance

Quinine, the first widely used antimalarial, was first discovered in the 17th Century. It was extracted and purified from Cinchona bark by Pierre Pelletier and Joseph Caventou in 1820 (Meshnick, 2001). By the mid-nineteenth century quinine had gained widespread use and was highly effective at reducing mortality due to malaria (Carter and Mendis, 2002). Quinine is used today to cure severe malaria and malaria during pregnancy.

Chloroquine was discovered over 60 years ago during World War II. It became the drug of choice for treating malaria in the WHO Global Eradication Programme in the mid twentieth century. Other derivatives of chloroquine such as primaquine were used as prophylaxis to prevent malaria. Chloroquine inhibits a major metabolic process in the asexual blood stages: the detoxification of haem. Haem, a by-product of haemoglobin digestion, is toxic to the parasite and is therefore converted to haemozoin crystals. Chloroquine binds to the haem degradation protein, preventing the formation of a haem detoxification complex in the food vacuole (Chugh *et al.*, 2013). Widespread use of

chloroquine has led to the appearance of resistant strains of *Plasmodium* in many endemic countries, and therefore its use has been discontinued in most countries.

Sulfadoxine-pyrimethamine (SP/Fansidar®) is another widely used drug which targets the folate synthesis pathway in the parasite. Pyrimethamine binds to the enzyme dihydrofolate reductase, which reduces dihydrofolate to tetrahydrofolate, an essential cofactor in nucleic acid synthesis. Sulfadoxine targets another stage of the pathway by inhibiting dihydropteroate synthase (DHPS). SP was highly effective against malaria, especially in areas where chloroquine resistance was prevalent, but resistant strains emerged not long after its introduction and it quickly spread in endemic areas (Mita *et al.*, 2009, Wongsrichanalai *et al.*, 2002)

Currently, artemisinin based drugs are used in the treatment of malaria, though *P. falciparum* strains resistant to artemisinin have also emerged in South East Asia (WHO, 2012). Artemisinin based drugs are used in combination therapy and are collectively called ACTs. The common ACTs used as first line treatment for uncomplicated malaria in endemic countries are artemether-lumefantrine, artemether-amodiaquine, and artesunate-mefloquine. Though *P. falciparum* resistance has been reported in Cambodia, Myanmar, Thailand and Viet Nam (WHO, 2012), ACTs still remain highly effective in other endemic countries, and are still the recommended first line treatment for uncomplicated malaria.

Malaria control strategies in Ghana include IRS, the use of LLITNS, the use of ACTs as first line treatment and the use of SP to prevent malaria in pregnancy (WHO, 2012).

2.5.3. Vaccine Development

Elimination and eradication of malaria globally is one of the main objectives of the World Malaria Program. There is a general consensus that the current strategies against malaria

such as IRS and the use of LLITNs may not be enough to eradicate malaria. The development of a highly efficacious vaccine may help block transmission of malaria, enabling its subsequent eradication globally. There are currently no vaccines against malaria. However, the fact that people living in malaria endemic areas acquire immunity against severe malaria first and later clinical malaria, provides evidence that humans can be vaccinated against malaria. *P. falciparum* is highly immune-evasive, and shows a lot of antigenic variation. This makes it particularly difficult to develop a vaccine with 50-80% efficacy against severe and clinical malaria that can last for 4 years, which is the goal stated in the Malaria Vaccine Technology Roadmap in 2006. Nevertheless, as stated previously, an efficacious vaccine must include all possible antigens because of the high antigenic variation exhibited by *P. falciparum*. Currently, several vaccines are in clinical trials and these are documented in the WHO Rainbow tables for malaria vaccines. These vaccines are designed to target the pre-erythrocytic stage, the asexual blood stage, and gametocytogenesis (transmission blocking vaccine). Arguably, a blood stage vaccine would be the most effective since it is during this stage that most antigens are exposed to the immune system. The major challenge facing the development of a blood stage vaccine is antigenic polymorphism. Many of the potential blood vaccines currently in clinical trials target apical membrane antigen-1 (AMA-1), which is involved in tight junction formation during invasion. However, AMA-1 is highly polymorphic and producing a vaccine that can induce broad-spectrum antigen specificity will be difficult. Clinical trials on another potential blood stage vaccine, EBA- 175 RII, on semi-immune adult volunteers was recently completed in Ghana (NIH, 2013) . There is no data on this trial; however, this vaccine is unlikely to work, since it targets an antigen that is redundant in the mechanism of invasion.

The most advanced vaccine candidate, RTS, S/AS01, which targets the pre-erythrocytic stage of the life cycle of *P. falciparum*, is currently in Phase 3 clinical trials. It is a subunit vaccine containing circumsporozoite protein (CSP) and Hepatitis B surface antigen (HBsAg) formulated with an adjuvant AS01. In recent Phase 3 trials, the vaccine showed an efficacy of 20-50% against clinical and severe malaria (Agnandji *et al.*, 2012). The recent results suggest that RTS, S/AS01 may not be very effective at preventing both clinical and severe forms of malaria.

2.6. The Mechanism of Invasion

The invasion of erythrocytes by the merozoites released from schizonts is essential for the survival of the *Plasmodium* parasite within the vertebrate host. Invasion is a multistep process involving four major steps after the parasite has egressed from the erythrocyte: primary contact, reorientation, secondary ligand interactions, and entry/ingress.

2.6.1. Egress and Primary Contact

Merozoites egress from erythrocytes in a multistep process involving the destabilization of the host cell cytoskeleton and breakdown of the PVM (Wirth and Pradel, 2012). This is followed by the host cell membrane turning inside out and rapidly vesiculating to release the merozoites. However, the process of invasion begins before the merozoite is released into the blood stream (Cowman *et al.*, 2012). Proteins involved in cytoadherence are 'primed' through a series of enzymatic reactions involving subtilisin 1 (PfSUB1) and subtilisin 2 (PfSUB2). These proteins are glycosylphosphatidylinositol (GPI)-anchored

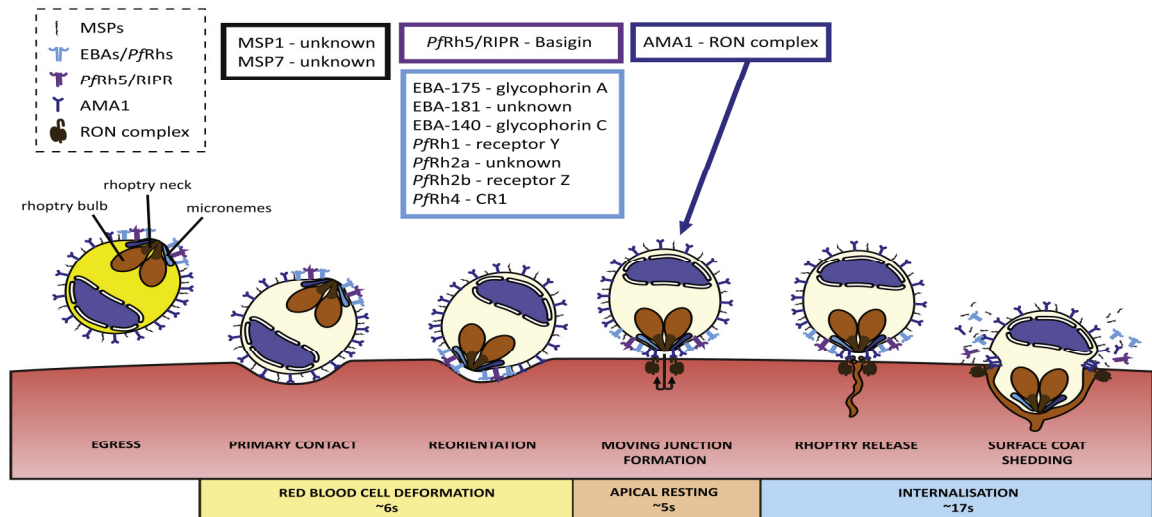


Figure 2.3. The stages of merozoite invasion of erythrocytes.

Parasite encoded proteins are featured in the key. Egress: low environmental potassium triggers a cytosolic calcium increase (yellow) and release of microneme proteins onto the merozoite surface. Primary contact: merozoites attach reversibly to an erythrocyte possibly via glycosylphosphatidylinositol-anchored merozoite surface proteins (black) Reorientation: primary contact causes deformation of the erythrocyte, resulting in reorientation of the parasite. This enables the merozoite to form an irreversible attachment through Erythrocyte binding-like (EBL) proteins and *P. falciparum* reticulocyte binding-like (PfRh) proteins by binding to specific erythrocyte receptors. Moving junction formation: parasites remain apically attached to allow translocation of the rhoptry neck protein complex (brown) into the erythrocyte and apical membrane antigen (dark blue) to collect at the apical tip. AMA1–RON complex binding triggers formation of the moving junction apposition between the merozoite and host cell through which the parasite invades. Entry: during host penetration surface ligands are shed and the parasite invades into the nascent parasitophorous vacuole created by secretion of the rhoptries into the host cell. (Source: Harvey *et al.*, 2012)

proteins on the surface of the merozoite, and merozoite surface protein-1 (MSP-1) is the most abundant of them.

After the merozoite is released from the erythrocyte, PfSUB1 proteolytically processes full length MSP1 into MSP183, MSP130, MSP138, and MSP142, whilst PfSUB2 further fragments MSP142 into MSP-1-19 and MSP-1-33 (Barale *et al.*, 1999, Child *et al.*, 2010, Koussis *et al.*, 2009). These fragments form a non-covalent macromolecular complex (Koussis *et al.*, 2009) ,with the MSP119 fragment acting as an anchor in the merozoite surface. PfSUB1 and PfSUB2 are also involved in the proteolytic processing of other

surface proteins including the SERA proteins, though the specific functions of these in invasion are not known (Child et al., 2010). Upon release from the erythrocyte, the merozoite encounters low potassium levels which signals the release of calcium, causing an increase in the concentration of cytosolic calcium which further triggers the secretion of adhesins and invasins from the micronemes (Singh *et al.*, 2009, Srinivasan *et al.*, 2011, Treeck *et al.*, 2009) (Figure 2.3). When the merozoite encounters an erythrocyte, it attaches reversibly to the erythrocyte by the binding of the GPI-anchored proteins to unknown erythrocyte receptors (Figure 2.3).

2.6.2. Reorientation of the Merozoite

The merozoite then undergoes a reorientation process such that the apical portion is in direct contact with the erythrocyte surface (Figure 2.3). This enables the merozoite to now form an irreversible attachment through Erythrocyte binding-like (EBL) proteins and *P. falciparum* reticulocyte binding-like (PfRh) proteins by binding to specific erythrocyte receptors (Figure 2.3).

2.6.3. Secondary Ligand Interactions

The secondary ligand interactions are thought to mediate commitment to invasion and trigger subsequent events leading to entry (Riglar *et al.*, 2011, Srinivasan *et al.*, 2011, Singh *et al.*, 2009). The secondary ligand interactions are followed by the formation of a tight junction. Tight junction formation begins with the formation of a complex consisting of RON2, 3, 4 and 5, with RON2 inserting into the erythrocyte membrane to anchor the complex. AMA-1 then binds to this complex, forming a bridge between the parasite and the erythrocyte membrane (Riglar *et al.*, 2011). Functional knockouts of EBL and PfRh proteins have shown that they play a role in attachment initiate an internal signal within the

merozoite that triggers the release of the rhoptry contents for establishment of the parasitophorous vacuole as the parasite moves into the host cell (Riglar *et al.*, 2011). Though the erythrocyte receptors for many of these receptors have been identified, a few still remain unknown

2.6.4. Ingress

Ingress into the erythrocyte is driven by an actomyosin motor. This is supported by studies which have shown that the actin inhibitor, cytochalasin D and a myosin ATPase inhibitor prevent ingress of *P. falciparum* merozoites (Farrow *et al.*, 2011).

2.7. Erythrocyte Invasion Receptors and Their Ligands

Several studies showed that an erythrocyte membrane binding protein called Duffy-associated chemokine receptor (DARC) or glycoprotein D that carried the Duffy blood group antigen epitopes (Hadley, 1986, Nichols *et al.*, 1987), was crucial for erythrocyte invasion in both *P. vivax* and *P. knowlesi* (Barnwell *et al.*, 1989, Miller *et al.*, 1976, Miller *et al.*, 1975). At the time, these results indicated that DARC may also be involved in erythrocyte invasion of *P. falciparum*. However, this was not the case as *P. falciparum* was shown to use erythrocyte membrane glycoproteins that contained SA as a major receptor for invasion (Miller *et al.*, 1977, Pasvol *et al.*, 1982a, Pasvol *et al.*, 1982b, Perkins, 1984b, Perkins, 1984a, Perkins and Rocco, 1988). A 175- KDa parasite ligand, named erythrocyte binding antigen 175, was shown to bind erythrocytes and this binding was sensitive to neuraminidase, which is an enzyme that cleaves SA (Camus and Hadley, 1985, Mayer *et al.*, 2001). EBA-175 was later shown to bind specifically to glycophorin A (Dolan *et al.*, 1994, Orlandi *et al.*, 1992). Genetic analysis identified that the gene encoding EBA-175, *eba-175*, was similar to that for the Duffy binding proteins in *P. vivax* as well as other

paralogues in *P. falciparum*; these were collectively named erythrocyte-binding like (EBL) proteins. Several paralogues of EBA-175 have since been identified along with their erythrocyte receptors. EBA-140 was shown to bind to glycophorin C (Maier *et al.*, 2003) in a neuraminidase -sensitive manner, similar to EBA-175, whilst EBL-1 was shown to bind erythrocytes using glycophorin B as a receptor (Mayer *et al.*, 2009). EBL-1 binds to glycophorin B in a neuraminidase-sensitive manner, similar to another paralogue EBA-181 that does not bind glycophorin B (Gilberger *et al.*, 2003). The receptor for EBA-181 still remains unknown.

Erythrocyte invasion assays on different *P. falciparum* isolates showed differences in dependence on SA (Hadley *et al.*, 1987, Mitchell *et al.*, 1986, Perkins and Holt, 1988), indicating that invasion was not completely mediated by EBL proteins. This indicated that *P. falciparum* used multiple or alternative pathways that were independent to invade, with some isolates showing the ability to switch between SA-dependent and independent pathways (Dolan *et al.*, 1990). It became apparent that apart from using glycophorins, *P. falciparum* isolates also used a receptor that was neuraminidase resistant and trypsin sensitive, named receptor X. Receptor X was identified as complement receptor 1 (CR1) (Spadafora *et al.*, 2010), and shown to bind to a parasite ligand from the PfRh family of proteins. Six proteins have been identified in this family, namely PfRh 1, 2a, 2b, 3, 4 and 5. Transcriptional upregulation of PfRh4 was associated with a switch of the erythrocyte invasion pathway of the Dd2 clone of *P. falciparum* from SA-dependent to a SA-independent. PfRh4 has been since shown to bind to CR1 (Tham *et al.*, 2010), and appears to play significant roles in invasion in both laboratory strains and field isolates (Awandare *et al.*, 2011). PfRh1, 2a, 2b and 5 have also been shown to play roles in invasion. PfRh1, is located at the apical end during merozoite invasion and binds to a SA-dependent, trypsin

and chymotrypsin resistant receptor known simply as receptor Y (Gao *et al.*, 2008). Disruption of the gene encoding PfRh1 indicates that this ligand is required for SA-dependent invasion, and expression of this ligand is associated with differences in invasion pathways (Cowman and Crabb, 2006). PfRh2a and 2b share a similar gene sequence and are a result of gene duplication. Disruption of both genes indicated that these proteins played a role in SA-independent invasion (DeSimone *et al.*, 2009) by interacting with a chymotrypsin sensitive receptor (Tham *et al.*, 2012). PfRh2a and b were shown to bind erythrocytes with different binding specificities, binding in both SA-dependent and -independent manners (Sahar *et al.*, 2011, Triglia *et al.*, 2011). Observations by Gunalan *et al.* (2011) showed that PfRh2a was processed into fragments, and the different binding specificities were as a result of this processing.

PfRh5 slightly differs from the other PfRh proteins because it does not possess a transmembrane domain (Tham *et al.*, 2012). Chen *et al.* (2011) showed that PfRh5 interacts with an EGF-like protein called Rh5 interacting protein, PfRipr. However, PfRipr also does not contain a transmembrane domain, suggesting that PfRh5 and PfRipr interact with another protein, with a transmembrane domain, before binding to the erythrocyte receptor for PfRh5, basigin (Crosnier *et al.*, 2011). PfRh3 is a pseudo gene, which is transcribed but not translated (Cowman and Crabb, 2006) and has not been shown to play any role in invasion so far.

Antibodies raised against EBL and PfRh proteins have been shown to inhibit merozoite invasion significantly, making them potential vaccine candidates. For examples, antibodies against PfRh4 (Tham *et al.*, 2009), PfRh5 (Bustamante *et al.*, 2013), and PfRh2 (Gunalan *et al.*, 2012) all inhibit invasion *in vitro*. Antibodies against region II of EBA-175 from

individuals in malaria-endemic areas also showed the ability to inhibit invasion (Ohas *et al.*, 2004), indicating that EBL and PfRh proteins may play important roles in immunity and may be the keys to finding a highly efficacious vaccine against malaria. However, EBL and PfRh proteins appear to work co-operatively in order to invade erythrocytes, and thus targeting one ligand only in a vaccine may be ineffective at providing protection against malaria. Disruption of PfRh1 ablates SA-dependent invasion, while PfRh2a, PfRh2b and PfRh4 have been shown to act together for invasion to occur (DeSimone *et al.*, 2009). Furthermore, disruption of EBA-181 from some parasites strains appears to reduce the ability of antibodies against PfRh2 to inhibit invasion (Tham *et al.*, 2012). Therefore, any potential vaccine must be able to target all relevant parasite antigens in order to be effective.

2.8. Approaches to Studying Erythrocyte Invasion Mechanisms

Various approaches have been used to study erythrocyte receptors and parasite ligands involved in invasion by *P. falciparum*. The earliest approach used to determine the receptor-ligand interactions that occur during invasion by *P. falciparum* was the use of naturally-occurring erythrocyte mutants which were devoid of a specific receptor. Glycophorin A null cells (Facer, 1983, Miller *et al.*, 1977, Pasvol and Jungery, 1983, Pasvol *et al.*, 1982b), glycophorin B null cells (S-s-u) (Pasvol *et al.*, 1982b; Facer, 1983; Pasvol and Jungery, 1983), glycophorin C mutant and null cells Gerbich and Leach (Serjeantson, 1989, Lobo *et al.*, 2003, Maier *et al.*, 2003) were used to determine the dependence of *P. falciparum* on glycophorins in merozoite invasion. However, this approach cannot be used to determine dependence on SA-independent invasion since there are no naturally occurring CR1 null or BSG null erythrocytes.

Erythrocyte invasion mechanisms can be also studied by selectively removing specific receptors from the erythrocyte surface using enzymes. The most commonly used enzyme for this purpose is a surface antigen of the influenza virus, neuraminidase. Neuraminidase hydrolyses the SA residues of cell surface glycoproteins which helps reveal the cell surface receptors required for cell invasion in the influenza virus and also in *Vibrio cholera* (Moustafa *et al.*, 2004). In contrast, SA is an essential residue for merozoite invasion using certain erythrocyte receptors. Glycophorins A, B and C are receptors for members of the EBL family and these interactions are abolished by neuraminidase. Some erythrocyte receptors are not sensitive to neuraminidase since they lack SA residues. Invasion pathways have therefore been classified into two major groups; SA-dependent and -independent invasion pathways (Table 2.2). Trypsin and chymotrypsin are endopeptidases which have been used to further characterize the receptors for merozoite invasion. Trypsin hydrolyses peptide in which the C-terminal end is a basic amino acid. Glycophorin A, C and CR1 are all sensitive to trypsin (Table 2.2). Chymotrypsin on the other hand hydrolyses peptide in which the C-terminal end is an aromatic amino acid; CR1, glycophorin B and receptor Z are sensitive to chymotrypsin (Table 2.2).

Based on Table 2.2, erythrocyte invasion mechanisms of parasites can be studied by performing erythrocyte invasion assays using enzyme-treated erythrocytes and this will give a fair indication of the receptors used by a majority of the parasite population.

A study in the Gambia indicated that most field isolates were unable to invade erythrocytes treated with neuraminidase and trypsin (Baum *et al.*, 2003, Gomez-Escobar *et al.*, 2010). This suggested that Gambian field isolates utilize a receptor that was sensitive to neuraminidase and trypsin but resistant to chymotrypsin, which is characteristic of

glycophorin A and C (Table 2.2). Similar studies by Lobo et al. (2004) suggested the use of glycophorin A and C by field isolates in Brazil which is consistent with recent studies by Lopez-Perez et al. (2012). Lopez-Perez et al. (2012) also established that majority of field isolates in Columbia showed an atypical invasion profile which was resistant to all enzyme treatments, indicating the use of the PfRh5-basigin interaction to invade erythrocytes. This was supported by the elevated levels of PfRH5 expression in these isolates (Lopez-Perez et al., 2012). Deans et al. (2007) showed evidence that Kenyan field isolates utilise a receptor which is neuraminidase-resistant but trypsin and chymotrypsin-sensitive, indicating the use of the parasite ligand PfRh4.

Table 2.2. A summary of merozoite – erythrocyte ligand interactions and their sensitivity to neuraminidase, trypsin and chymotrypsin.

	SA-Dependent Pathways					SA-Independent Pathways		
RBC Receptor	GYP A	GYP B	GYP C	E	Y	Z	CR1	BSG
Merozoite Ligand	EBA-140	EBL-1	EBA-140	EBA-181	PfRh1	PfRh2a/b	PfRh4	PfRh5
Neuraminidase	S	S	S	S	S	S/R	R	R
Trypsin	S	R	S	R	R	R	S	R
Chymotrypsin	R	S	R	S	R	S	S	R

S = sensitive; R = resistant; GYP = glycophorin. EBA = erythrocyte binding antigen. EBL = erythrocyte binding ligand, CR1 = complement receptor 1 BSG = Basigin. (Rodriguez, *et al.* 2008; Mayer, *et al.* 2004; Tham, *et al.* 2009; Baum, *et al.* 2002; Cowman, *et al.* 2006; Maier *et al.* 2002; Duraisingh, *et al.* 2003; Tham, *et al.* 2010, Crosnier *et al.*, 2011, Sahar *et al.*, 2011).

Antibodies against erythrocyte receptors and soluble receptors are also used as an approach to study dependence on specific receptors by inhibiting merozoite invasion. For example, antibodies against glycophorin A were shown to inhibit invasion, however this result was possibly due to membrane rigidity caused by high concentration of antibodies (Pasvol *et al.*, 1989). It is for this reason that the use of neuraminidase-treated erythrocytes is preferred in the study of SA dependence. However, antibodies against CR1 and BSG have

been successfully used to inhibit merozoite invasion mediated by these receptors (Spadofora *et al.*, 2010, Crosnier *et al.*, 2011). Awandare *et al.* (2011) confirmed the use of the PfrRH4-CR1 interaction by field isolates using antibodies against CR1 and soluble CR1 to inhibit invasion. Crosnier *et al.* (2011) used both monoclonal antibodies against BSG and pentamerized BSG to show that BSG was an important receptor in invasion in Senegalese field isolates.

In this study, erythrocyte invasion assays using enzyme-treated erythrocytes were used to determine receptor ligand interactions based on the criteria in Table 2.2. In addition, the dependence on specific SA-independent receptors was investigated using antibodies against the two known SA-independent receptors to inhibit invasion in the presence and absence of SA.

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Ethical Statement

The research presented here was approved by the Ethics Committees of the Ghana Health Service, Navrongo Health Research Centre, Kintampo Health Research Centre and Noguchi Memorial Institute for Medical Research, Legon. All samples were collected with the consent of the parent/guardian of the child after an explanation of what the study entails.

3.2. Study Sites and Sample Collection

Three study sites in different ecological zones of Ghana, with different transmission intensities were chosen for the collection of blood samples: Kintampo Health Research Centre in Kintampo (Brong Ahafo Region), Navrongo Health Research Centre in Navrongo (Upper East Region), and Ledzokuku-Krowor Municipal Assembly (LEKMA) Hospital in Accra (Greater Accra Region). Samples were collected between August 2012 and July 2013. Kintampo is in a holoendemic region where malaria transmission is high all year round, whereas Navrongo has seasonal high transmission of malaria in the rainy season and Accra is a relatively low transmission region. Blood samples were collected during the rainy season between July and November, when malaria transmission is high in Ghana. Children between the ages of 2-14 years presenting with symptoms of malaria were diagnosed by a clinician at the hospital and screened for malaria using rapid diagnostic tests (RDTs). Children who tested positive for malaria were further confirmed with malaria using microscopy, by viewing thick and thin smears of their blood on slides, which is the

gold standard for malaria diagnosis. After obtaining consent from the parent/guardian of the child, approximately 5ml of venous blood was collected from children who tested positive for both tests into sterile heparinized Vacutainers® by hospital staff. Parasitaemia, i.e. number of parasites/ μ L of blood, was estimated by counting the number of parasites per 200 white blood cells (WBCs) on a thick smear of blood stained with Giemsa. Parasitaemia was then calculated using the following formula based on the WBC count of the patient obtained from a haematology analyzer.

$$\text{Parasite}/\mu\text{L} = \frac{\text{number of parasites counted}}{200} \times \text{WBC}/\mu\text{L}$$

Parasites were transported in an ice chest to the laboratory, where the sample was processed.

3.3. Cryopreservation of Clinical Parasite Isolates

The blood was centrifuged and separated into erythrocytes, buffy coat, and plasma. Parasitized erythrocytes from Navrongo and Kintampo were cryopreserved in liquid nitrogen using Glycerolyte® (Fenwal Inc., USA). The volume of the sample was measured and a volume of Glycerolyte® equal to one-third of the sample volume was added dropwise. After incubating for 5 minutes at room temperature, 1.33 times the sample volume of Glycerolyte® was added drop wise, whilst gently swirling the tube. The samples were then stored at -80°C for initially before being transferred into liquid nitrogen, where they were stored until use.

3.4. Thawing of Cryopreserved Clinical Isolates

Cryopreserved samples from the various study sites were removed from cold storage and thawed at 37°C for 1 minute. The volume of the sample was measured and $10\mu\text{L}$ aliquots

of 12% NaCl (Sigma Aldrich) were added for every 100 μ L of the sample volume. After incubating for 5 minutes at room temperature, ten times the sample volume of 1.6% NaCl (Sigma Aldrich) was added drop wise, whilst gently swirling the tube. The sample was again centrifuged at 1500 rpm for 5 minutes at room temperature. The supernatant was removed and the erythrocytes were resuspended with complete parasite medium (RPMI 1640 containing 5 mg/mL Albumax II, 10 μ g/mL gentamycin, 0.2 μ g/mL hypoxanthine, 2 mM L-glutamine, 25 mM HEPES, 23.8 mM NaHCO₃) supplemented with 2% inactivated human AB⁺ serum. The sample was then centrifuged at 1500 rpm for 5 minutes at room temperature (20°C). The supernatant was removed and the parasitized erythrocytes were maintained in O⁺ erythrocytes at approximately 4% haematocrit, a parasitaemia of less than 5% and in an atmosphere of blood gas (2% O₂, 5.5% CO₂ and 92.5% N₂). Complete parasite medium was changed daily.

3.5. Determination of Optimal Enzyme Concentrations

3.5.1. Enzymatic Treatments

To determine the optimum enzyme concentrations needed to disrupt specific erythrocyte receptors, erythrocytes were treated with each of the three enzymes at varying concentrations. Erythrocytes were treated with 250 and 500mU/mL of neuraminidase from *Vibrio cholera* (Sigma Aldrich); 1, 2, and 4mg/mL of α -chymotrypsin (pre-treated with 1-chloro-3-tosylamido-7-amino-2-heptanone, TLCK, to remove trypsin activity) from bovine pancreas (Sigma Aldrich), and 1, 2, and 10 mg/mL of trypsin from bovine pancreas (Sigma Aldrich). Blood group O⁺ erythrocytes at 50% haematocrit were treated with neuraminidase, chymotrypsin, and trypsin by incubating with each enzyme for 1 hour at 37°C in a shaking incubator. The enzyme treated erythrocytes were then washed three

times with complete parasite medium and spun at 7000rpm in a microcentrifuge (Denville Scientific). The pelleted cells were resuspended to 50% haematocrit with complete parasite medium. The enzyme treated erythrocytes were labelled with 10 μ M 7-hydroxy-9*H*-(1, 3-dichloro-9, 9-dimethylacridin-2-one) succinimidyl ester (DDAO-SE) (detailed below). Erythrocyte invasion assays were performed using a laboratory strain, 7G8.

3.5.2. Efficiency of Receptor Cleavage

To determine the efficiency of receptor cleavage, neuraminidase-treated erythrocytes were tested for the presence of SA using anti-glycophorin A (GPA) monoclonal antibodies that target SA, whilst trypsin and chymotrypsin erythrocytes were tested for the presence of CR1 using anti-CR1 polyclonal antibodies.

Enzyme-treated erythrocytes were resuspended in a cold PBS containing 1% bovine serum albumin (BSA) solution. Chicken anti-human CR1 (12 μ g/mL) was added to trypsin and chymotrypsin-treated erythrocytes whilst mouse anti human GPA (10 μ g/mL, Sigma Aldrich) was added to neuraminidase-treated erythrocytes. Both were incubated at 4°C for 20 minutes. The erythrocytes were then washed twice with PBS/1% BSA solution to remove any unbound antibody. This was followed by resuspension of the erythrocytes in PBS/1% BSA solution and the addition of the secondary antibodies. Goat anti-mouse IgG antibodies (4 μ g/mL, Invitrogen) conjugated to phycoerythrin (PE) were added to neuraminidase-treated erythrocytes whilst goat anti-chicken IgG antibodies conjugated to fluorescein isothiocyanate (FITC) (10 μ g/mL, KPL Co.) were added to trypsin- and chymotrypsin-treated erythrocytes. Both were incubated at 4°C for 15 minutes. The erythrocytes were finally washed twice and resuspended in 100 μ L of PBS/1% BSA

solution. Flow cytometric analysis was performed using BD[®] FACS Calibur flow cytometer and results were analysed using FlowJo[®] (Tree Star Inc.).

3.6. Erythrocyte Labelling

The erythrocytes used as targets for invasion assays were labelled with DDAO-SE (Invitrogen), a far red cell stain, to differentiate them from the uninfected erythrocytes in the parasite inoculum. This was done to also help identify the erythrocyte population that had been treated with enzymes and measure invasion levels of that population only. Enzyme-treated type O⁺ erythrocytes at 50% haematocrit were diluted to 4% haematocrit in RPMI 1640 and centrifuged at 2000 rpm for 3 minutes. The pellet was resuspended to 4% haematocrit with DDAO-SE in RPMI 1640 (Sigma-Aldrich) and incubated for 2 hours at 37°C in a shaking incubator. Following the incubation, erythrocytes were pelleted at 2000rpm for 3 minutes. The pelleted erythrocytes were washed with pre-warmed (37°C) complete parasite medium, and then resuspended to 25% haematocrit in complete medium.

3.7. Invasion Assays

Erythrocyte invasion assays were set up using schizont stage parasites. Parasite cultures with parasitaemia between 1-5% parasitaemia of predominantly schizont stage parasites (> 90%) were used for invasion assays. These were set up by adding the parasite culture and the DDAO-SE-stained erythrocytes in a 1:1 ratio at 2% haematocrit in a 96-well titre plate. All experiments were set up in duplicate wells. The plate was incubated at 37°C for approximately 20 hours in a gas mixture of 2% O₂, 5.5% CO₂, and 92.5% N₂. SYBR Green I (Invitrogen) was used to stain parasite DNA to differentiate parasitized erythrocytes from uninfected erythrocytes. Invasion levels were then determined using flow cytometry to analyse fluorescence of the erythrocytes.

3.8. Inhibition of CR1 and Basigin

In order to determine the relative dependency of field isolates on CR1 as a receptor for invasion, chicken anti-CR1 antibodies (12µg/mL, Gallus Immunotech Inc.) and soluble CR1 (50µg/mL, Gallus Immunotech Inc.) were used to block the CR1 receptor in invasion assays. Chicken IgY antibodies (12µg/mL, Gallus Immunotech Inc.) and a serum protein, fetuin (50µg/mL, Sigma Aldrich) were used as control proteins respectively.

Monoclonal antibodies against basigin, MEM-M6/6 (10µg/mL, Sigma-Aldrich) were used to determine the contribution of the interaction between PfrH5 and basigin in invasion. Anti-mouse IgG (10µg/mL, Sigma-Aldrich) was used as an isotype control.

3.9. Data Acquisition by Flow Cytometry

A 1:50 dilution of each well was done by adding 10µL aliquots of culture from each well to 500µL of FACS Flow (BD Biosciences) in FACS tubes. The samples were acquired on a BD Calibur flow cytometer (BD Biosciences). DDAO-SE was excited by a red laser and detected by a 660/20 filter. SYBR Green I was excited by a blue laser and detected by a 530/30 filter. BD Cell Quest[®] was used to determine invasion levels for 50,000 cells per well.

CHAPTER FOUR

4.0. RESULTS

4.1. Optimization of Enzyme Treatments

To determine the optimum concentration of enzymes to be used for the investigation of invasion phenotypes in Ghana, dose-dependency experiments were performed for neuraminidase, trypsin and chymotrypsin. For neuraminidase, erythrocytes were treated with 250mU/mL and 500mU/mL concentrations of enzyme. The ability of these concentrations of neuraminidase was tested by determining the ability of the enzymes to remove SA from erythrocytes. Flow cytometric measurement of SA expression of erythrocytes treated with either concentrations of neuraminidase using a SA-specific antibody, which recognises SA, showed that SA was depleted to a similar extent by both concentrations of enzyme (Figure 4.1.A). The invasion efficiency of 7G8 was tested in erythrocytes treated with both concentrations of neuraminidase (Figure 4.2.). Invasion efficiency was approximately 70% in erythrocytes treated with 250mU/mL of neuraminidase relative to invasion in untreated erythrocytes, whereas invasion efficiency dropped to 55% in erythrocytes treated with 500mU/mL of neuraminidase. Though invasion efficiency was lower in erythrocytes treated with 500mU/mL of neuraminidase than erythrocytes treated with 250mU/mL of neuraminidase, there was background fluorescence in erythrocytes treated with the former. Therefore, 250mU/mL of neuraminidase was used to treat erythrocytes for our investigations

The concentrations of chymotrypsin and trypsin used were determined by testing the ability of varying concentrations of each enzyme to remove CR1 from the erythrocyte

surface. The concentrations of trypsin used were 1, 2, and 10 mg/mL, while 1, 2, and 4 mg/mL were used for chymotrypsin. Erythrocyte invasion assays on 7G8 showed that 2 and 4 mg/mL of chymotrypsin reduced invasion efficiency relative to untreated erythrocytes to approximately 20% (Figure 4.2.).

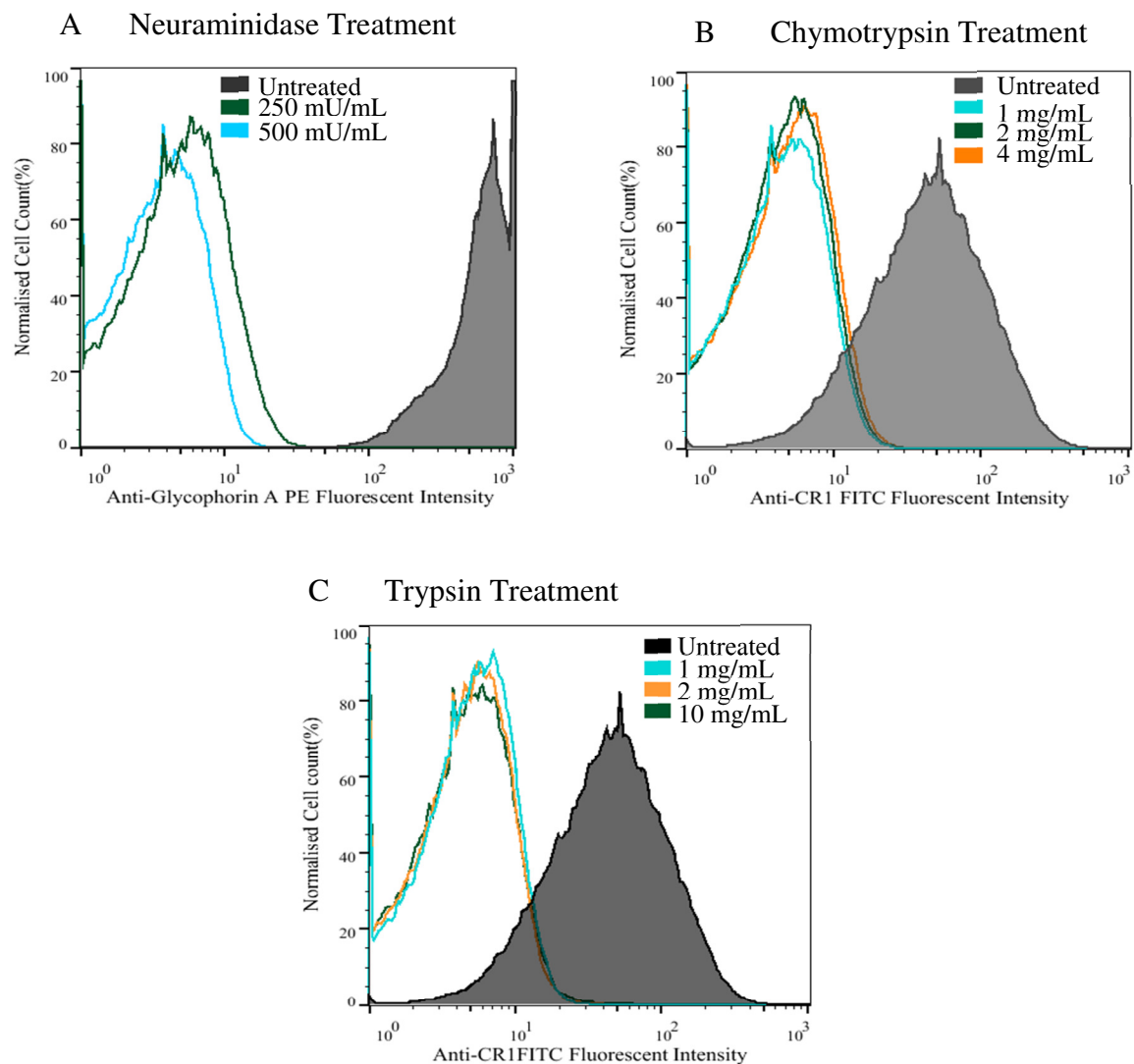


Figure 4.1. Efficacy of enzyme treatments of erythrocytes.

A) SA on the erythrocyte surface is removed by treating with 250mU/mL and 500mU/mL of neuraminidase.
B) Chymotrypsin (1, 2 and 4mg/mL) and C) trypsin (1, 2, and 10mg/mL) remove CR1 from untreated erythrocytes.

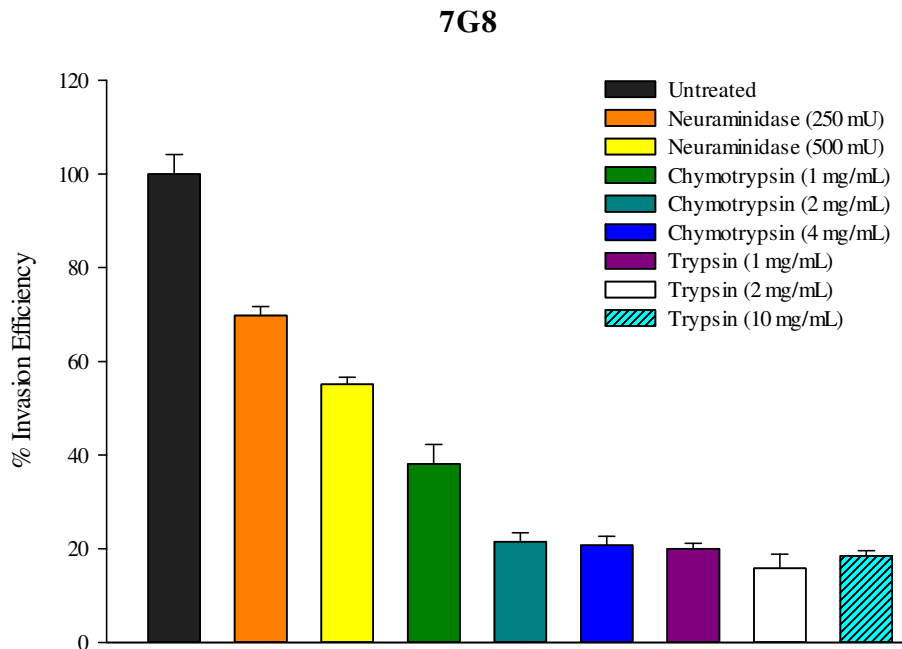


Figure 4.2. Optimization of enzyme treatments using a laboratory-adapted strain 7G8.

Invasion efficiency of 7G8 in erythrocytes treated with neuraminidase (250mU/mL, 500mU/mL), chymotrypsin (1mg/mL, 2mg/mL, 4mg/mL), and trypsin (1mg/mL, 2mg/mL, 4mg/mL). Data are expressed as a percentage relative to invasion in untreated erythrocytes.

Though 1mg/mL of chymotrypsin reduced invasion efficiency to 40%, flow cytometric analysis showed that all three concentrations of chymotrypsin were able to remove CR1 to a similar extent (Figure 4.1.B). Therefore, the concentration of chymotrypsin chosen for erythrocyte invasion assays in these studies was 1mg/mL. All three concentration of trypsin also removed CR1 to a similar extent based on flow cytometric analysis (Figure 4.1.C). Also, all three concentration of trypsin (i.e. 1, 2 and 10mg/mL) reduced invasion efficiency of 7G8 to approximately 20%. Therefore, the concentration of trypsin chosen for erythrocyte invasion assays on field isolates was 1mg/mL.

4.2. Clinical Isolates

To determine the invasion phenotypes of *P. falciparum* clinical isolates in Ghanaian children, parasitized erythrocytes from 29 participants were collected from three study sites in Ghana, namely Accra, Kintampo and Navrongo, from children diagnosed with confirmed malaria. The children were aged between 2 and 6 years (Table 4.1.). Out of these 29 samples collected, 18, representing 62% of the samples, have been successfully phenotyped. Out of the 18 samples tested overall, nine samples came from Kintampo (EIMK011, EIMK012, EIMK020, EIMK077, EIMK113, EIMK124, EIMK125 EIMK209, EIMK244), seven from Navrongo (EIMN031, EIMN062, EIMN063, EIMN079 EIMN096, EIMN110, EIMN196) and two (EIMA005, EIMA008) from Accra. With the exception of EIMN096, all participants in this study were sickling negative (Table 4.1.). The average age of the study participants from Kintampo was 3.56 ± 1.74 years, Accra donors' average age was 3.50 ± 0.71 , and Navrongo study participants were slightly older, with an average age of 4.00 ± 1.15 . However, the differences in age of donors at the three study sites were not statistically significant ($P > 0.05$). Parasitaemia of blood samples taken from participants in Kintampo were much higher than that of participants from the two other study sites. In the Kintampo samples, parasitaemia ranged between 142000 parasites/ μ L to 821000 parasites/ μ L (mean = 383476 parasites/ μ L), while Accra samples were between 31000-39000 parasites/ μ L (mean = 35131 parasites/ μ L), and Navrongo samples ranged between 14000-170000 parasites/ μ L (mean = 85905 parasites/ μ L). All participants were diagnosed with uncomplicated malaria and had had malaria at least once within the preceding year.

Table 4.1. Age, haemoglobin genotype and parasitaemia of sample donors.

Study Location	Sample ID N ^o	Age (years)	Haemoglobin genotype	Parasitaemia (parasites/ μ L)
Accra	EIMA005	4	AA	31608
	EIMA008	3	AA	38653
Kintampo	EIMK011	2	AA	257760
	EIMK012	6	AA	312000
	EIMK020	6	AA	142880
	EIMK077	2	AA	248970
	EIMK113	5	AA	213210
	EIMK124	2	AA	595700
	EIMK125	3	AA	384300
	EIMK209	4	AA	475860
	EIMK244	2	AA	820600
Navrongo	EIMN031	5	AA	83819
	EIMN062	3	AA	95370
	EIMN063	2	AA	108931
	EIMN079	4	AA	16738
	EIMN096	5	AS	14490
	EIMN196	6	AA	112811
	EIMN110	3	AA	169176

4.3. Enzyme Sensitivities of Parasite Isolates

To gain insight into the receptor preferences of Ghanaian parasite isolates, erythrocyte invasion assays were performed on parasite isolates collected from the three study sites across Ghana using enzyme-treated erythrocytes. Since invasion phenotypes of the isolates may change whilst in culture, *ex vivo* invasion assays were performed immediately after thawing where possible, using methods described by Theron *et al.* (2010). When *ex vivo* invasion assays could not be performed, the parasites were cultured for a few days prior to invasion assays. The majority of the field isolates however could not adapt to culture conditions, and therefore could not be cultured *in vitro* for more than two cycles of replication. One to three invasion assays were performed on each isolate, depending on its ability to grow in culture. In this study, strains were defined as resistant to enzyme treatment if the mean invasion efficiency in enzyme treated erythrocytes was >50% relative to invasion efficiency in untreated erythrocytes. The erythrocyte population was selected by gating according to forward scatter (size) and side scatter (granularity) (Figure 4.3 A). Based on this population, the subset of erythrocytes stained with DDAO SE was determined with a fluorescence histogram on a logarithmic scale and selected (Figure 4.3 B). DDAO-SE-positive erythrocytes that were also positive for SYBR Green I were determined with a dot plot (Figure 4.3 C) and a fluorescence histogram on a logarithmic scale (Figure 4.3 D). FlowJo was then used to analyze the data. All invasion data were expressed as a percentage relative to the invasion in untreated erythrocytes, and data for duplicate wells were averaged.

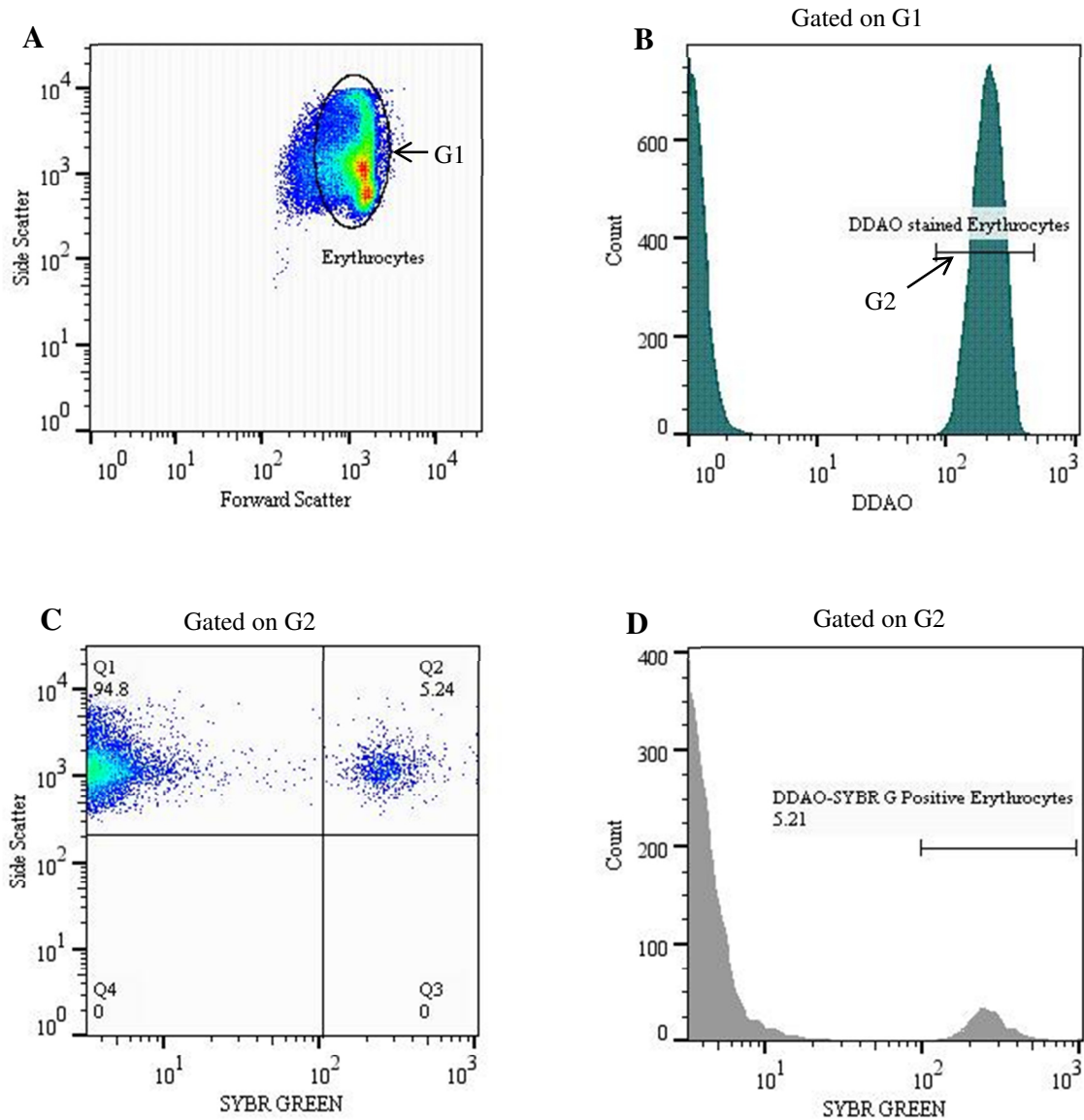


Figure 4. 3. Flow cytometric data showing parasitaemia of DDAO-stained erythrocytes.

A) Dot plot showing gated erythrocytes (G1) B) Histogram showing gated DDAO-stained erythrocytes (G2) from parent erythrocyte population. C) Dot plot of SYBR Green-positive DDAO-SE-positive erythrocytes based on G2 D) Histogram of SYBR Green positive DDAO-SE positive erythrocytes based on G2.

4.3.1. Kintampo Parasite Isolates

Out of 12 samples thawed from Kintampo, 9 were successfully characterized based on their sensitivities to neuraminidase, trypsin, and chymotrypsin. This was mainly due to the inability of some of these samples to adapt to culture conditions. The isolates that were successfully characterized were EIMK011, EIMK012, EIMK020, EIMK077, EIMK113, EIMK124, EIMK125, EIMK209, and EIMK244.

All parasite isolates tested from Kintampo showed the ability to invade neuraminidase-treated erythrocytes with efficiencies greater than 50% relative to untreated erythrocytes (Figure 4.4). This may indicate that isolates from the middle belt of Ghana do not depend heavily on glycoporphins to invade erythrocytes. In particular, 4 out of the 9 isolates tested, namely EIMK011, EIMK012, EIMK020 and EIMK077, were able to invade neuraminidase-treated erythrocytes with higher invasion efficiencies compared to the other Kintampo isolates tested, showing 70-90% invasion efficiencies. The invasion efficiency of EIMK113, EIMK124, EIMK125, EIMK209 and EIMK244 showed moderate resistance to neuraminidase, ranging between 50-65% efficiency relative to untreated erythrocytes (Figure 4.4.). The mean invasion efficiency of all the Kintampo isolates in neuraminidase-treated erythrocytes relative to untreated erythrocytes was about 70%.

All Kintampo parasite isolates except EIMK125 were sensitive to trypsin and chymotrypsin (Figure 4.4.). EIMK244 was highly sensitive to both chymotrypsin and trypsin showing an invasion efficiency of only about 3% relative to untreated erythrocytes. EIMK012 was also more sensitive to both enzymes than the other six strains, showing invasion efficiencies of 15% and 18% in trypsin and chymotrypsin respectively, relative to

untreated erythrocytes. EIMK011, EIMK020, EIMK113, and EIMK209 were moderately sensitive to both enzymes with invasion efficiencies ranging between 28% and 46%. EIMK124 was moderately sensitive to chymotrypsin (33%), but was more sensitive to trypsin (15%). However EIMK077 showed a borderline invasion efficiency of 49% in chymotrypsin-treated erythrocytes, but much lower invasion efficiency in trypsin-treated erythrocytes (23%). EIMK125 was resistant to chymotrypsin (65%) but highly sensitive to trypsin (29%). The mean invasion efficiency of isolates from Kintampo in chymotrypsin- and trypsin-treated erythrocytes were 35% and 24% respectively.

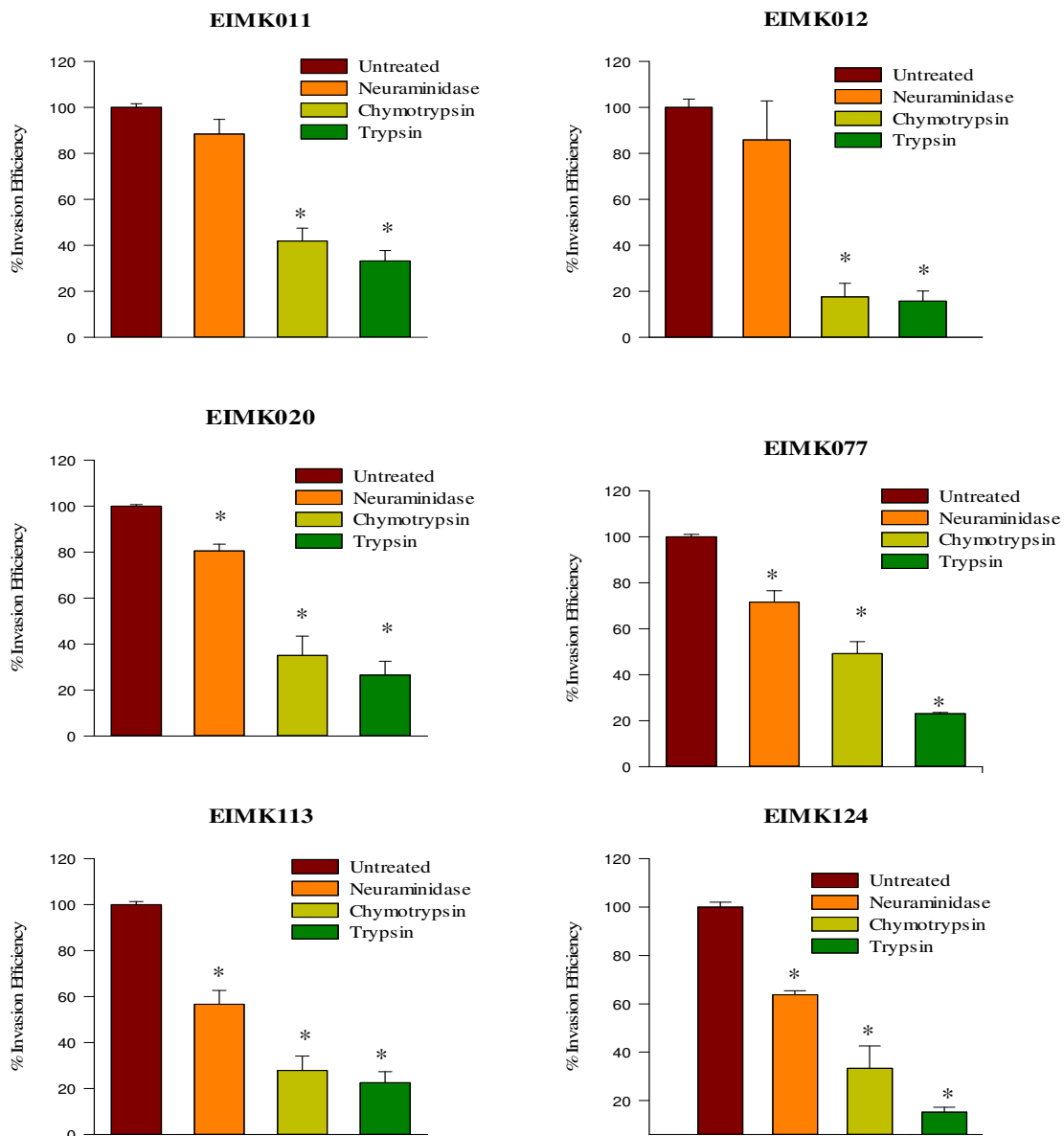


Figure 4.4. Enzyme sensitivities of *P. falciparum* clinical isolates from Kintampo. Erythrocyte invasion assays were set up in duplicate on a 96 well plate to determine the ability of parasites to invade erythrocytes treated with neuraminidase, trypsin and chymotrypsin. Parasitaemia was determined by flow cytometry. Data are presented as a percentage of invasion efficiency relative to untreated erythrocytes. Error bars represent SEMs. * Invasion inhibition by enzyme is statistically significant relative to untreated erythrocytes ($p < 0.05$, Student's t-test).

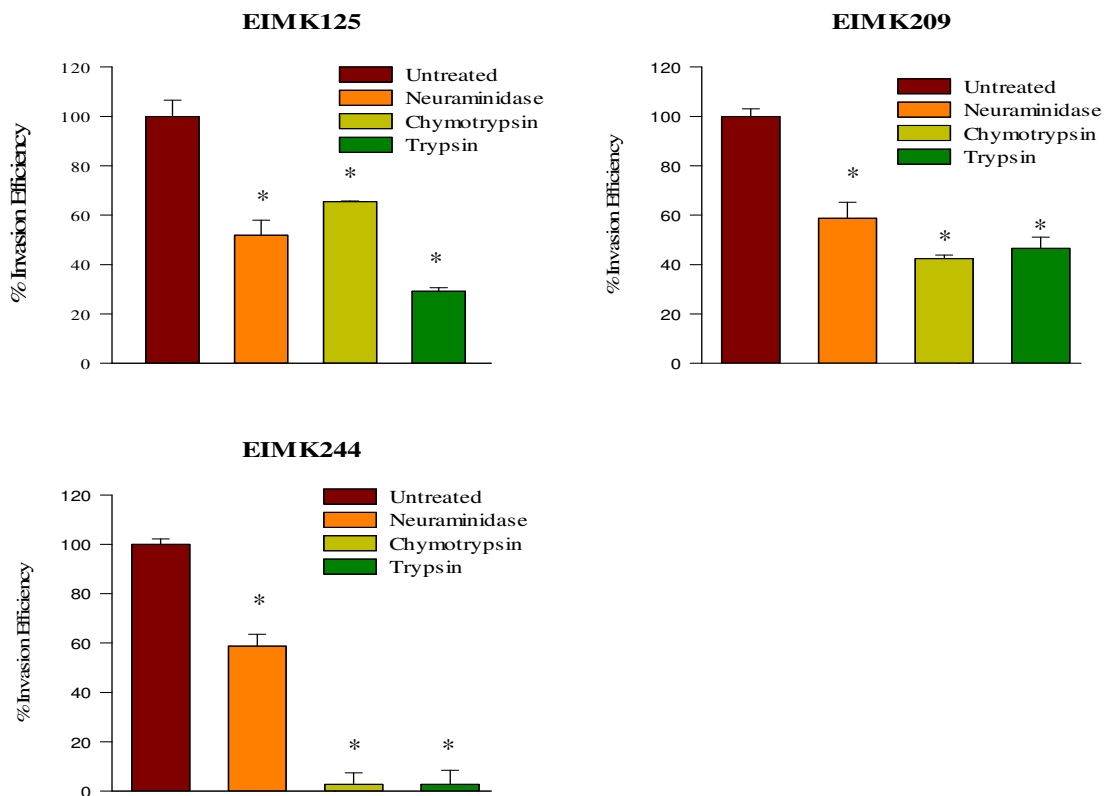


Figure 4.3 Cont'd. Enzyme sensitivities of *P. falciparum* clinical isolates from Kintampo.

Erythrocyte invasion assays were set up in duplicate on a 96 well plate to determine the ability of parasites to invade erythrocytes treated with neuraminidase, trypsin and chymotrypsin. Parasitaemia was determined by flow cytometry. Data are presented as a percentage of invasion efficiency relative to untreated erythrocytes. Error bars represent SEMs. * Invasion inhibition by enzyme is statistically significant relative to untreated erythrocytes ($p < 0.05$, Student's t-test).

4.3.2. Accra Parasite Isolates

Eight samples were collected from LEKMA hospital in Accra between October 2012 and July 2013. Out of the eight samples collected only two strains could survive culture and be tested: EIMA005 and EIMA008.

Both EIMA005 and EIMA008 were moderately resistant to neuraminidase, with invasion efficiencies of 55-60% relative to untreated erythrocytes (Figure 4.5). Also, both showed sensitivity to both trypsin and chymotrypsin (Figure 4.5). EIMA005 was more sensitive,

with invasion efficiencies below 10% in both chymotrypsin and trypsin-treated erythrocytes. Though EIMA008 showed an invasion profile similar to EIMA005, the former was moderately sensitive to chymotrypsin (31%) but highly sensitive to trypsin (18%) (Figure 4.5.). These results suggest that both isolates were neuraminidase-resistant, trypsin-and chymotrypsin-sensitive.

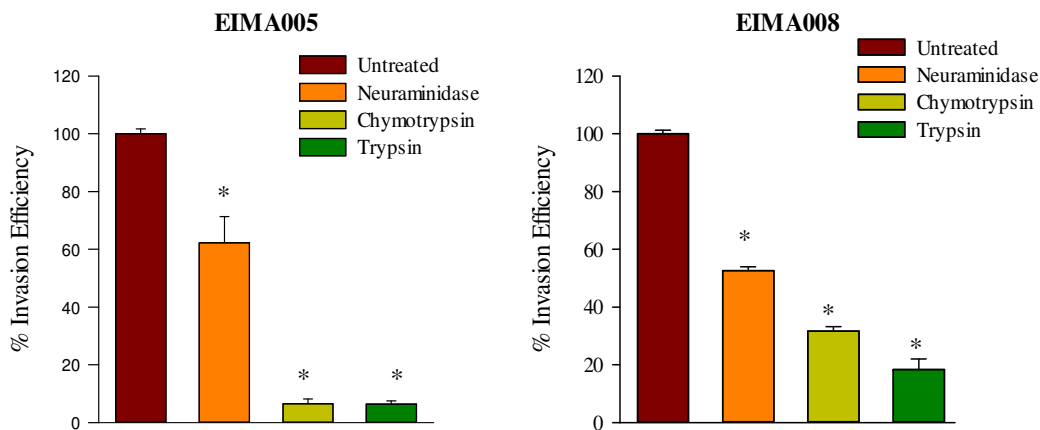


Figure 4.5. Enzyme sensitivities of *P. falciparum* clinical isolates from Accra.

Erythrocyte invasion assays were set up in duplicate on a 96 well plate to determine the ability of parasites to invade erythrocytes treated with neuraminidase, trypsin and chymotrypsin. Parasitaemia was determined by flow cytometry. Data are presented as a percentage of invasion efficiency relative to untreated erythrocytes. Error bars represent SEMs. * Invasion inhibition by enzyme is statistically significant relative to untreated erythrocytes ($p < 0.05$, Student's t-test).

4.3.3. Navrongo Parasite Isolates

Although thirteen samples from Navrongo were thawed from cold storage, only seven could be tested. This was mainly due to the inability of the other six isolates to survive in culture after thawing. The samples that were tested were EIMN031, EIMN062, EIMN063, EIMN079, EIMN096, EIMN110, and EIMN196.

Invasion efficiencies relative to untreated erythrocytes of EIMN063 (69%), EIMN031 (80%) and EIMN096 (67%) were not inhibited by the absence of SA residues (Figure 4.6.), making them SA-independent strains. EIMN079 and EIMN062 showed borderline resistance of approximately 52% invasion efficiency in neuraminidase-treated erythrocytes. EIMN110 and EIMN196 were both moderately sensitive to neuraminidase, showing invasion efficiencies in neuraminidase-treated erythrocytes of 42% and 46% respectively, relative to untreated erythrocytes. Sensitivities to trypsin and chymotrypsin also varied. EIMN031 was resistant to both trypsin and chymotrypsin with invasion efficiencies of approximately 70% relative untreated erythrocytes, (Figure 4.6.), suggesting a neuraminidase-, trypsin-, and chymotrypsin- resistant phenotype. EIMN096 was moderately sensitive to chymotrypsin (48%) but was highly sensitive to trypsin (14%) suggesting a phenotype consistent with the use of CR1 as a receptor. EIMN110 was more sensitive to chymotrypsin (24%) than to trypsin (42%), thereby indicating that this strain may be dependent on glycoporphin B and CR1. EIMN110 showed a phenotype that was sensitive to neuraminidase (42%), trypsin (42%) and chymotrypsin (24%), thereby showing a phenotype that is sensitive to all enzymes, pointing to a high dependence on glycoporphins as receptors for invasion. EIMN196, which also showed sensitivity to neuraminidase (46%), was also sensitive to trypsin (20%) and chymotrypsin (45%), indicating a phenotype similar to EIMN110, but with differing degrees of sensitivities to trypsin and chymotrypsin. EIMN062 also showed moderate sensitivity to chymotrypsin (34%) but was highly sensitive to trypsin (9%). Altogether, strains from Navrongo appeared to be more phenotypically diverse with respect to preferred invasion pathways than strains from Kintampo and Accra.

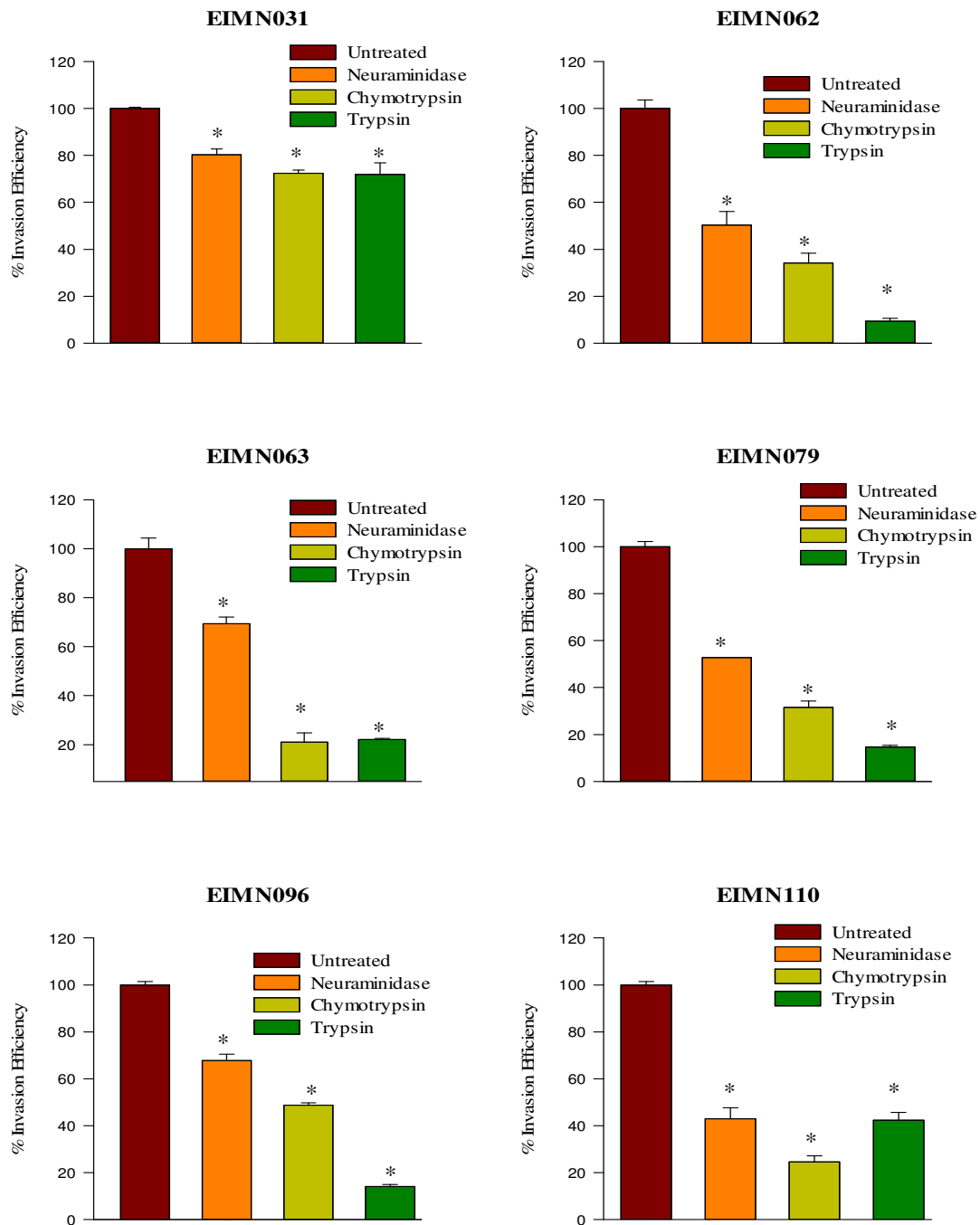


Figure 4.6. Enzyme sensitivities of *P. falciparum* clinical isolates from Navrongo. Erythrocyte invasion assays were set up in duplicate on a 96 well plate to determine the ability to invade erythrocytes treated with neuraminidase, trypsin and chymotrypsin. Parasitaemia was determined by flow cytometry. Data are presented as a percentage of invasion efficiency in untreated erythrocytes. Error bars represent SEMs. * indicates that invasion inhibition by enzyme is statistically significant relative to untreated erythrocytes ($p < 0.05$).

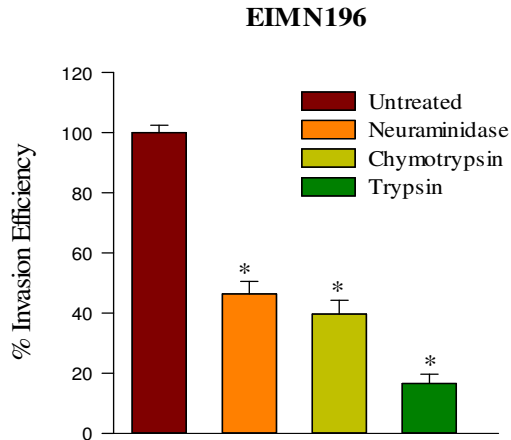


Figure 4.6 (Continued). Enzyme sensitivities of *P. falciparum* clinical isolates from Navrongo.

Erythrocyte invasion assays were set up in duplicate on a 96 well plate to determine the ability of parasites to invade erythrocytes treated with neuraminidase, trypsin, and chymotrypsin. Parasitaemia was determined by flow cytometry. Data are presented as a percentage of invasion efficiency in untreated erythrocytes. Error bars represent SEMs. * indicates that invasion inhibition by enzyme is statistically significant relative to untreated erythrocytes ($p < 0.05$).

4.4. Summary of Invasion Profiles of Ghanaian Field isolates

When all the *P. falciparum* isolates tested from across Ghana were combined, four distinct erythrocyte invasion phenotypes emerged based on their sensitivities to neuraminidase, chymotrypsin, and trypsin. Out of the 18 isolates tested, 15, representing 78% were resistant to neuraminidase but sensitive to both trypsin and chymotrypsin (NrCsTs) (Figure 4.7). A phenotype that was sensitive to all enzymes (NsCsTs) was the second most common phenotype, representing 11% of phenotypes tested. One isolate appeared to use a receptor sensitive to trypsin only (NrCrTs) representing 6%, while the last isolate was resistant to all enzymes (NrCrTr) (Figure 4.7).

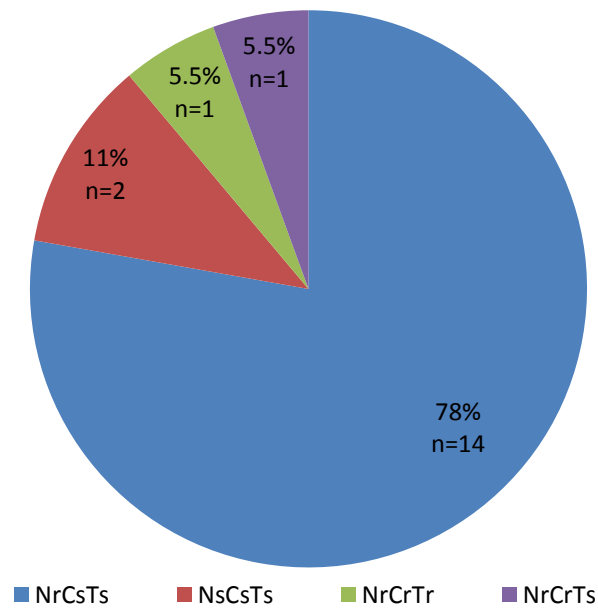


Figure 4.7. Summary of invasion profiles of 18 field isolates from Ghana.

N= neuraminidase, C=chymotrypsin, T= trypsin, s= sensitive, r=resistant

A comparison of the mean invasion efficiency of the isolates to each of the three enzymes based on the study site showed no significant difference in their sensitivities to the enzymes though samples from Navrongo showed a slightly increased sensitivity to neuraminidase whilst being slightly more resistant to chymotrypsin and trypsin, (Figure 4.8.)

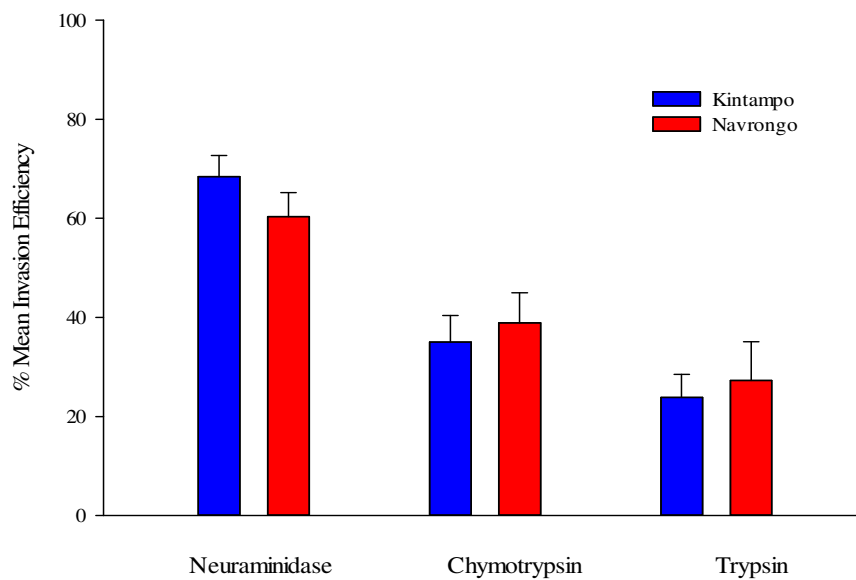


Figure 4. 8. Geographic comparison of enzyme sensitivity of *P. falciparum* isolates from Kintampo and Navrongo.

Parasite isolates from Kintampo and Navrongo were compared based on their sensitivities to neuraminidase, chymotrypsin and trypsin. Error bars represent SEMs.

Comparison of the invasion phenotypes of all 18 isolates revealed that the majority of isolates were not inhibited by neuraminidase, but were significantly inhibited by chymotrypsin and trypsin (Figure 4.9.). This suggests that the majority of Ghanaian field isolates use a neuraminidase-resistant, trypsin- and chymotrypsin-sensitive receptor to invade erythrocytes. There were four isolates that were significantly different from the others, including EIMN110 and EIMN196, which were sensitive to all three enzymes (Figure 4.9.). In addition, its resistance to chymotrypsin distinguished EIMK125, while EIMN031 was resistant to all the enzymes (Figure 4.9.).

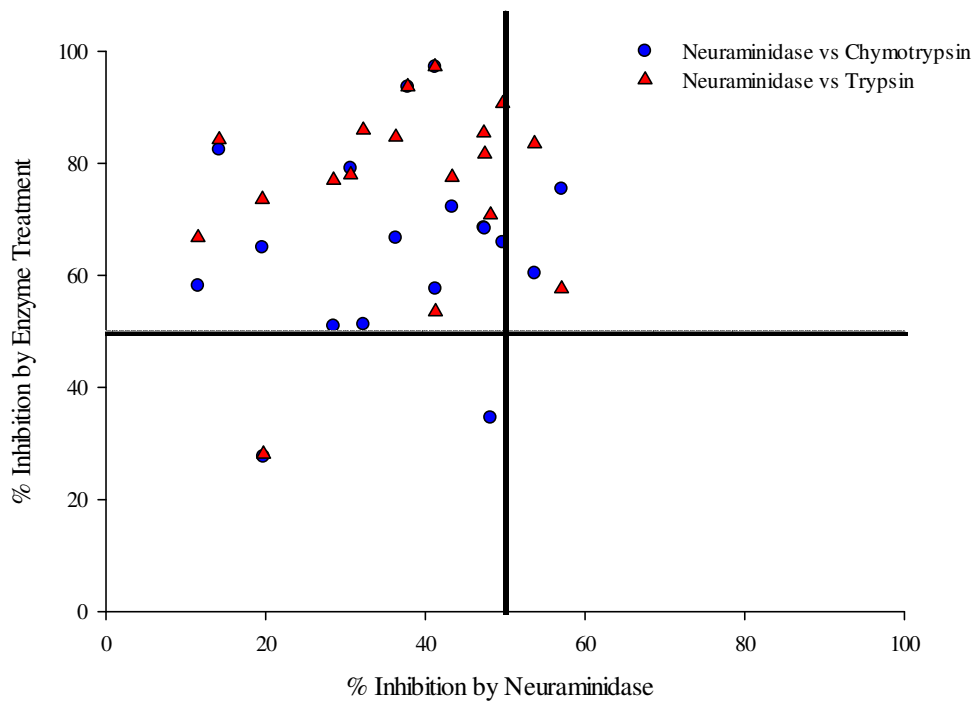


Figure 4. 9. Comparison of invasion phenotypes of 18 Ghanaian *P. falciparum* Field Isolates.

Inhibition of invasion due to chymotrypsin (blue circles) and trypsin (red triangles) were compared to inhibition due to neuraminidase (*x-axis*). Lines represent 50% inhibition.

4.5. Dependence of Ghanaian Clinical Isolates on CR1 for Invasion

To further confirm the use of CR1 as a receptor for invasion in Ghanaian field isolates, soluble CR1 (sCR1) and polyclonal chicken antibodies raised against sCR1 were used to inhibit CR1 mediated invasion in untreated and neuraminidase-treated cells.

4.5.1. Kintampo Parasite Isolates

All 8 samples tested from Kintampo were significantly inhibited by anti-CR1 antibodies in neuraminidase-treated cells compared to the control IgY antibody ($p < 0.05$, Student's t-test) (Figure 4.10.). These results confirm the NrCsTs invasion profile obtained from the enzymatic treatments, which is synonymous with the use of CR1. Invasion efficiencies in IgY antibodies were not significantly different from invasion efficiencies in media only (Control) in both untreated and neuraminidase-treated erythrocytes (Figure 4.10.) ($p > 0.05$, Student's t-test).). In addition, invasion efficiency in the presence of anti-CR1 in untreated erythrocytes did not differ from invasion efficiency in the Control and in IgY antibodies for all Kintampo isolates tested. EIMK011, EIMK020 and EIMK077 were additionally tested using soluble CR1 (sCR1), which reduced invasion efficiency significantly in both untreated and neuraminidase-treated erythrocytes compared to parasite invasion efficiencies in media only (Control) or with fetuin (Figure 4.10.).

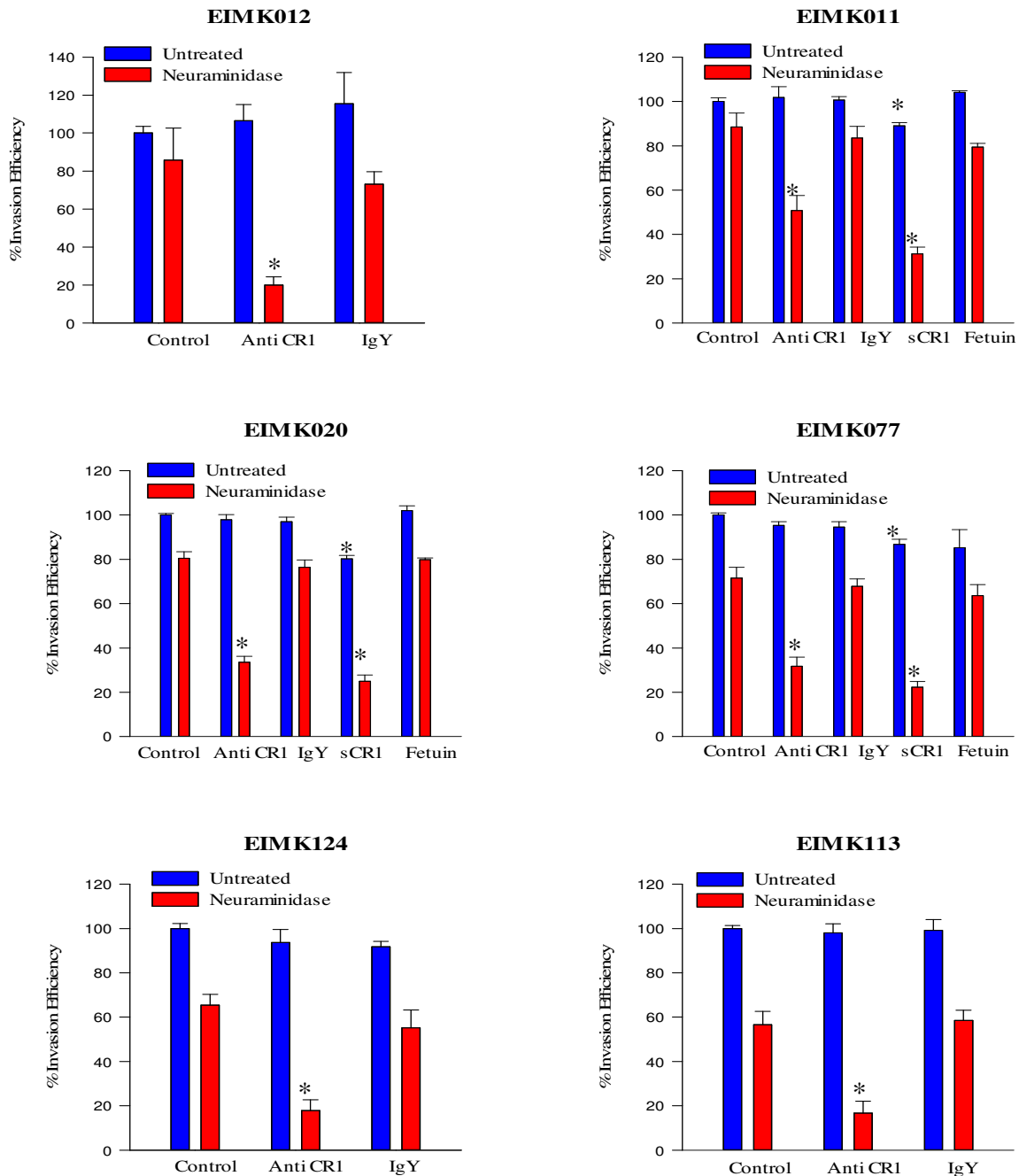


Figure 4.10. CR1-dependent invasion of *P. falciparum* field isolates from Kintampo.

CR1-mediated invasion was inhibited with either 50 $\mu\text{g}/\text{mL}$ of sCR1 or 12 $\mu\text{g}/\text{mL}$ of polyclonal chicken antibodies raised against sCR1 (anti-CR1). IgY and Fetuin were used as protein controls respectively. Control wells were treated with media only. Error bars represent SEMs. * Invasion inhibition by anti-CR1 or sCR1 was statistically significant relative to IgY and Fetuin respectively ($p < 0.05$, Student's t-test).

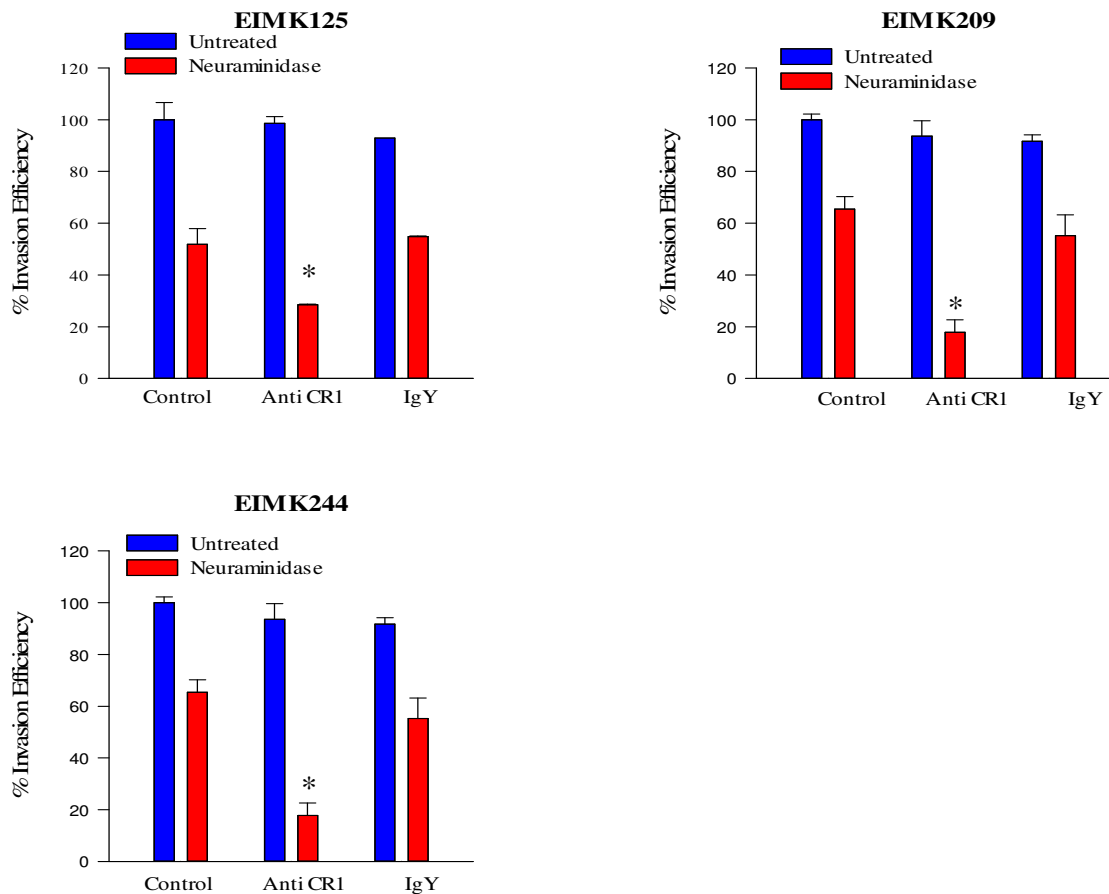


Figure 4.10. (Continued). CR1-dependent Invasion of *P. falciparum* field isolates from Kintampo.

CR1-mediated invasion was inhibited with either 50 $\mu\text{g}/\text{mL}$ of sCR1 or 12 $\mu\text{g}/\text{mL}$ of polyclonal chicken antibodies raised against sCR1 (anti-CR1). IgY and Fetuin were used as protein controls respectively. Control wells were treated with media only. Error bars represent SEMs. * Invasion inhibition by anti-CR1 or sCR1 was statistically significant relative to IgY and Fetuin respectively ($p < 0.05$, Student's t-test).

4.5.2. Accra Parasite Isolates

EIMA005 and EIMA008 were both significantly inhibited by anti-CR1 antibodies in neuraminidase-treated cells compared to media only (Control) and the control IgY antibody ($p < 0.05$, Student's t-test) (Figure 4.11.). This is also consistent with the NrCsTs invasion profile identified based on enzyme sensitivities, which is synonymous with the use of CR1. Invasion efficiencies in IgY antibodies were not significantly different from invasion efficiencies in media only (Control) in both untreated and neuraminidase-treated erythrocytes (Figure 4.11.) ($p > 0.05$, Student's t-test). In addition, invasion efficiency in the presence of anti-CR1 in untreated erythrocytes did not differ from invasion efficiency in the Control and in IgY antibodies for both Accra parasite isolates tested.

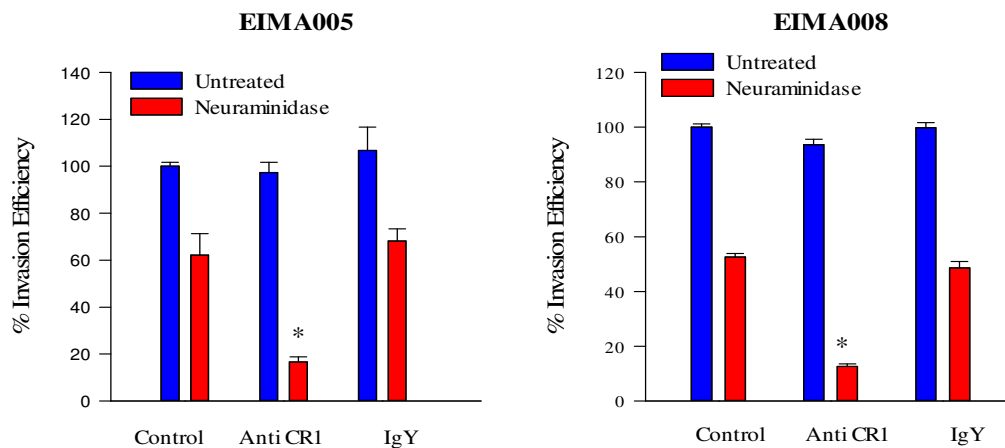


Figure 4.11. CR1-dependent invasion of *P. falciparum* field isolates from Accra.

CR1-mediated invasion was inhibited with 12 μ g/mL of polyclonal chicken antibodies raised against sCR1 (anti-CR1). IgY was used as a protein control. Control wells were treated with media only. Error bars represent SEMs. * Invasion inhibition by anti-CR1 was statistically significant relative to IgY ($p < 0.05$, Student's t-test).

m4.5.3. Navrongo Parasite Isolates

The ability of antibodies against CR1 to inhibit invasion was tested in 6 out of the 7 strains obtained from Navrongo; EIMN031, EIMN062, EIMN063, EIMN096, EIMN110, and EIMN196. In all the strains tested from Navrongo, antibodies against CR1 were able to significantly inhibit erythrocyte invasion in neuraminidase-treated erythrocytes compared to invasion efficiencies in media only (Control) and in the presence of IgY antibodies ($p < 0.05$, Student's t-test) (Figure 4.12.). Though antibodies against CR1 in neuraminidase-treated erythrocytes also inhibited EIMN031 significantly, the parasites were still able to invade erythrocytes with almost 60% invasion efficiency (Figure 4.12.), suggesting the use of a pathway not mediated by sialoglycoproteins (GYP A, B, or C), or CR1. Furthermore, invasion efficiency in the presence of anti-CR1 in untreated erythrocytes did not differ from invasion efficiency in the Control and in IgY antibodies for EIMN031, EIMN062, EIMN063, and EIMN096. Anti-CR1 antibodies significantly inhibited invasion of untreated erythrocytes by EIMN110 and EIMN196, compared to the Control and the IgY antibody ($p < 0.05$, Student's t-test).

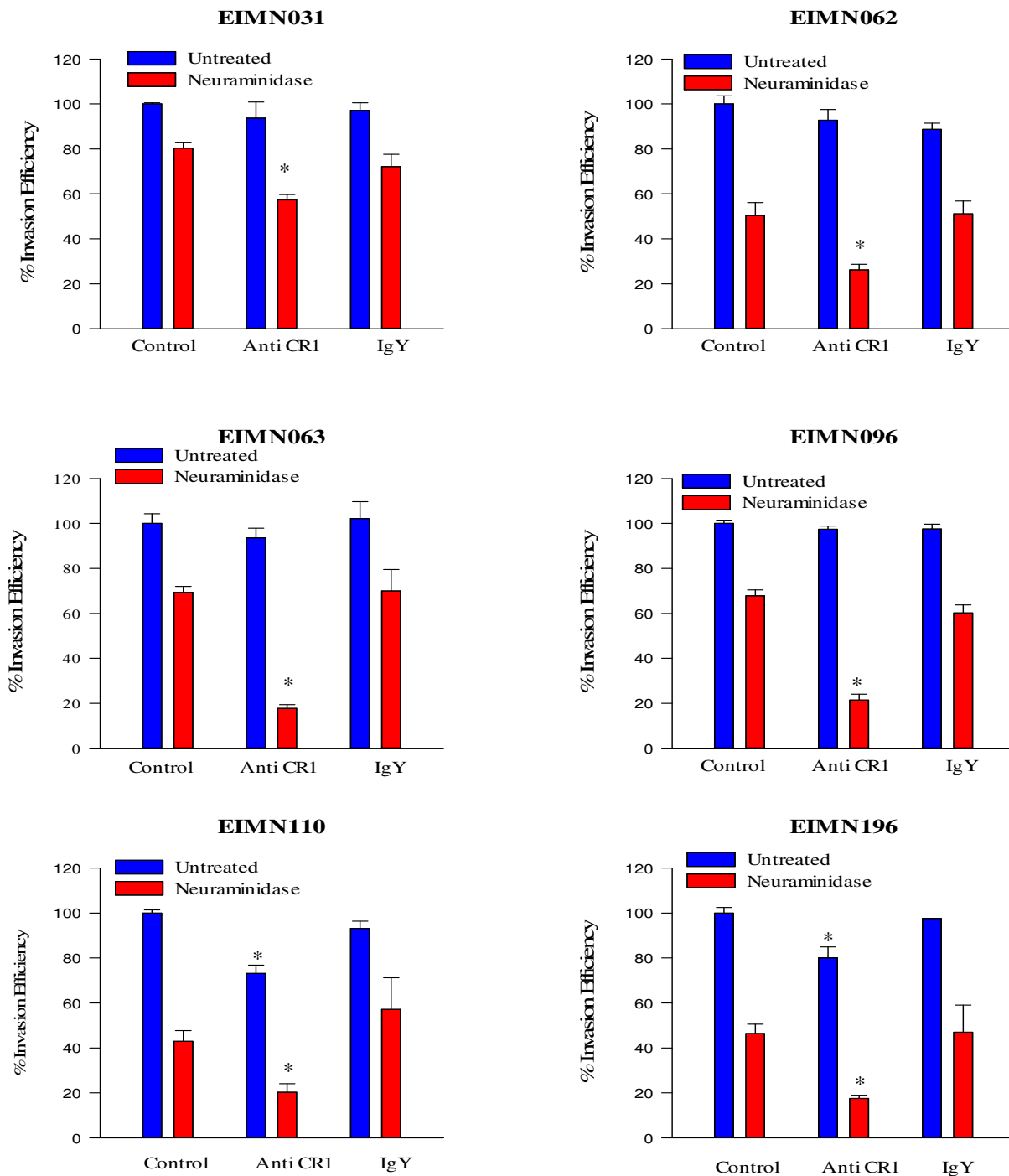


Figure 4.12. CR1-dependent invasion of *P. falciparum* field isolates from Navrongo.

CR1-mediated invasion was inhibited with 12 $\mu\text{g}/\text{mL}$ of polyclonal chicken antibodies raised against sCR1 (anti-CR1). IgY was used as a protein control. Control wells were treated with media only. Error bars represent SEMs. * Invasion inhibition by anti-CR1 was statistically significant relative to IgY ($p < 0.05$, Student's t-test).

4.6. Dependence of Ghanaian Clinical Isolates on BSG for Invasion

Recent studies indicate that basigin may be a major erythrocyte receptor used by both laboratory strains and field isolates for invasion (Crosnier *et al.*, 2011, Lopez-Perez *et al.*, 2012). To determine the dependence of Ghanaian field isolates on basigin as a receptor for erythrocyte invasion, monoclonal mouse antibodies raised against human BSG (anti-BSG) were used to inhibit invasion in untreated and neuraminidase-treated erythrocytes. Five isolates were tested using anti-BSG antibodies: EIMK011, EIMK020, EIMK077, EIMN062 and EIMA008.

Antibodies against BSG were able to reduce invasion significantly in both untreated and neuraminidase-treated erythrocytes compared to invasion efficiencies in media only (Control) and in the presence of the isotype control antibody IgG ($p < 0.05$, Student's-test) in all isolates tested (Figure 4.13.). The invasion efficiency of parasites in untreated erythrocytes was reduced to 20-30% by anti-BSG antibodies in EIMK011, EIMK020, EIMK077, and EIMN062. However, anti-BSG antibodies were able to inhibit only 50% of invasion by EIMA008 parasites in untreated erythrocytes relative to inhibition by IgG.

Anti-BSG antibodies reduced invasion efficiency in neuraminidase-treated erythrocytes to 10-20% compared with invasion in the media only Control and in the IgG isotype control in all isolates tested (Figure 4.13.).

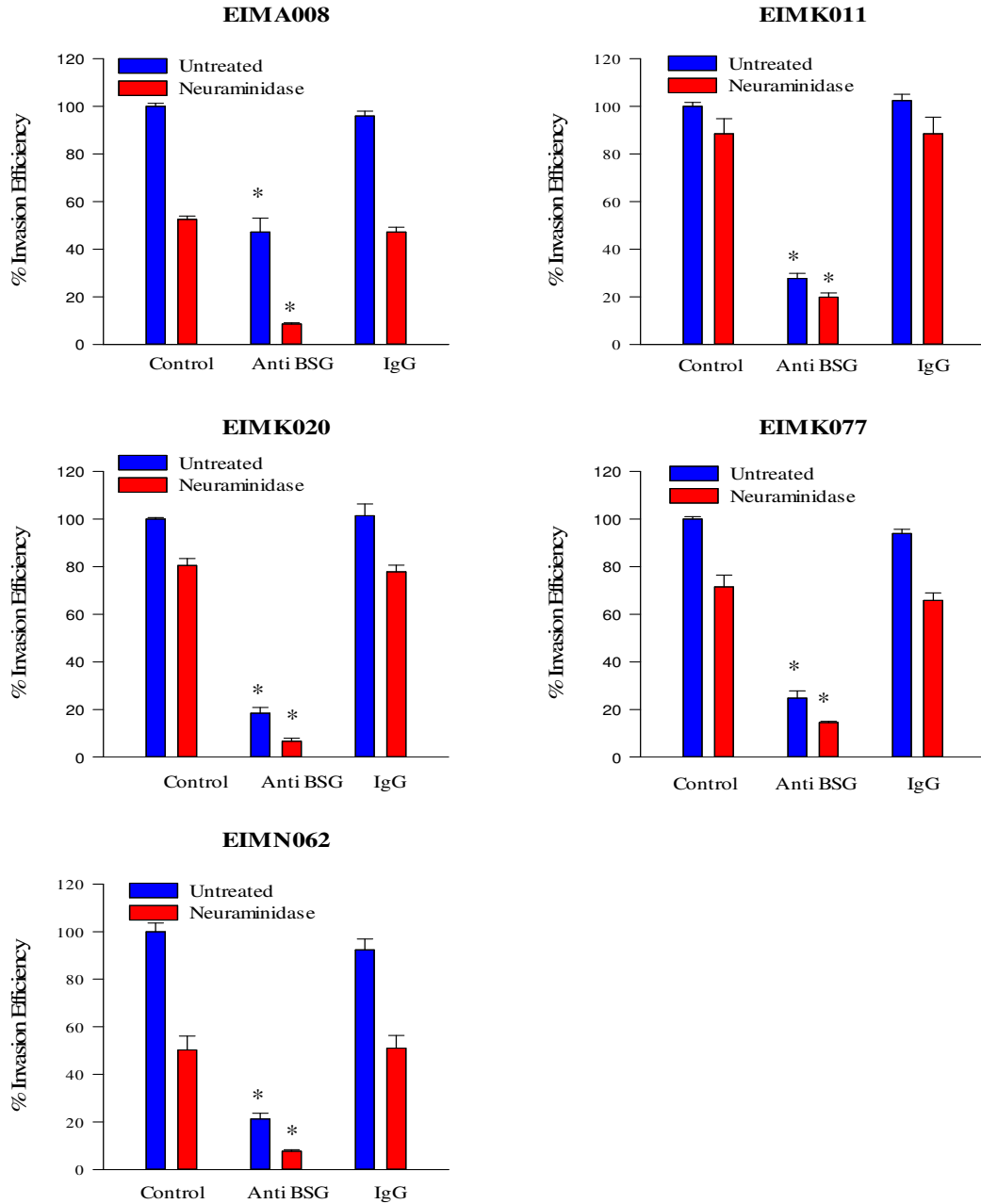


Figure 4.13. BSG-dependent invasion of Ghanaian *P. falciparum* field isolates.

BSG-mediated invasion was inhibited by 10 μ g/mL of monoclonal mouse antibodies raised against BSG. IgG was used as a protein control. Control wells were treated with media only. Error bars represent SEMs. * Invasion inhibition by anti-BSG was statistically significant relative to IgG ($p < 0.05$, Student's t-test).

CHAPTER FIVE

5.0. DISCUSSION AND CONCLUSION

5.1. DISCUSSION

In this study, we aimed at determining the invasion profiles of *P. falciparum* field isolates in Ghana, by testing the ability of these isolates to invade erythrocytes treated with neuraminidase, chymotrypsin, and trypsin that selectively remove specific receptors on the erythrocyte surface. Parasites that invaded enzyme-treated erythrocytes with a mean invasion efficiency of 50% or more relative to untreated erythrocytes were classified as resistant to the enzyme treatment. Based on invasion assays performed using these enzymes, four different invasion profiles emerged among Ghanaian clinical isolates tested.

These were:

1. Neuraminidase-resistant, chymotrypsin- and trypsin-sensitive (NrCsTs)
2. Neuraminidase-, chymotrypsin- and trypsin-sensitive (NsCsTs)
3. Neuraminidase-, chymotrypsin- and trypsin-resistant (NrCrTr) and
4. Neuraminidase-resistant, chymotrypsin-resistant and trypsin-sensitive (NrCrTs)

Neuraminidase-resistant phenotypes, also as known SA-independent phenotypes, were the most prevalent among Ghanaian field isolates, as 92% of isolates tested showed the ability to invade neuraminidase-treated erythrocytes with more than 50% invasion efficiency relative to untreated erythrocytes.

Studies in sub-Saharan Africa show that there is diversity in invasion mechanisms among isolates from different parts of the sub-region. Recent findings in Kenya have also shown the dominance of SA-independent strains within the population (Deans *et al.*, 2007,

Awandare *et al.*, 2011), which is consistent with results presented in this study. Conversely, erythrocyte invasion mechanisms of Ghanaian clinical isolates differ from Gambian clinical isolates that have been shown to be largely dependent on neuraminidase-sensitive receptors for invasion (Baum *et al.*, 2003, Gomez-Escobar *et al.*, 2010). Furthermore, high levels of antibodies against SA-dependent pathways have been reported in people living in Africa, where malaria is endemic (Persson *et al.*, 2008), suggesting the *P. falciparum* field isolates in endemic areas may have developed the ability to use alternative pathways to invade erythrocytes to ensure survival. The findings stated in this report may suggest that *P. falciparum* isolates in Ghana may have the ability to switch to SA-independent phenotypes in order to evade the host immune system.

The majority of field isolates that were resistant to neuraminidase were sensitive to both trypsin and chymotrypsin, thus appearing to use a receptor that is neuraminidase-resistant, trypsin- and chymotrypsin-sensitive (NrCsTs). This invasion phenotype is characteristic of the pathway used by parasite ligand PfRh4 and its erythrocyte receptor CR1 (Spadafora *et al.*, 2010, Tham *et al.*, 2010).

Comparison of invasion phenotypes across geographic areas revealed some interesting patterns. For example, all the samples tested from Kintampo had a similar invasion profile of NrCsTs with the exception of EIMK125, which had a NrCrTs phenotype. This may be because Kintampo is a holoendemic area, with almost year round transmission of malaria. Therefore, children with malaria may have already developed antibodies against SA-dependent pathways, and parasites are therefore likely to use SA-independent pathways.

The receptor-ligand interaction that is resistant to both neuraminidase and chymotrypsin has not yet been characterized. A similar phenotype was found in Peruvian and Brazilian isolates and quantitative PCR determined that EBA-181 was expressed most in those strains (Lopez-Perez *et al.*, 2012), suggesting that EBA-181 may mediate invasion in strains with this phenotype. Not surprisingly, samples from Kintampo also seemed generally more resistant to neuraminidase than samples from Navrongo, and more sensitive to trypsin and chymotrypsin, further indicating that CR1 may be a major receptor for erythrocyte invasion in Kintampo parasite isolates.

Samples from Navrongo showed greater variation in invasion phenotypes than those from Kintampo. Three different phenotypes were recorded among the seven samples from Navrongo: NrCsTs, NsCsTs, and NrCrTr. Four isolates showed a phenotype similar to isolates from Kintampo (NrCsTs), indicating the use of CR1. EIMN031 showed an atypical invasion phenotype that was resistant to all three enzymes (NrCrTr), whilst, EIMN110 and EIMN196 were sensitive to all three enzymes (NsCsTs). The phenotype NrCrTr has been linked with the use of basigin, a recently discovered receptor. South American clinical isolates which showed this phenotype expressed high levels of PfRh5 and PfRh2a (Lopez-Perez *et al.*, 2012), indicating that these isolates could be using basigin, which interacts with PfRh5 (Crosnier *et al.*, 2011). This particular field isolate, however, failed to adapt to culture conditions, and further investigations could not be conducted.

The phenotype exhibited by EIMN110 and EIMN196, which is sensitive to all enzymes, may indicate that this parasite strain is using a receptor that has not yet been characterized. The same phenotype was identified in field isolates in South America and a significant

correlation was established between this phenotype and strains with polymorphic PfRh4, which contains a modified amino acid repeat sequence (DEVE) (Lopez-Perez *et al.*, 2012). This may imply that PfRh4 may mediate both SA-dependent and independent pathways. Other studies were able to demonstrate that PfRh2 mediated multiple pathways i.e. both SA-dependent and independent pathways (Sahar *et al.*, 2011). Interestingly, Gunalan *et al.* (2011) also suggested that all Rh proteins may be processed in a similar fashion since proteolytic cleavage sites in PfRh2a were similar to those in PfRh1. Taken together, this information may suggest that PfRh4 may also be proteolytically processed leading to fragments with different binding specificities, suggesting that the phenotype NsCsTs may be attributed to PfRh4 binding to an unknown receptor in a SA-dependent manner.

Since the majority of isolates tested showed SA-independent phenotypes, further investigations were conducted using antibodies against known SA-independent receptors, i.e. CR1 and BSG, to determine the extent of dependence on either receptor. Antibodies against CR1 were used to inhibit invasion in untreated and neuraminidase-treated erythrocytes. Antibodies against CR1 reduced invasion efficiency in neuraminidase-treated erythrocytes from about 50-80% in erythrocytes treated with media alone or IgY, to about 10-60%, representing a 25-90% inhibition of SA-independent invasion. However, anti-CR1 antibodies were unable to inhibit invasion significantly in untreated erythrocytes compared to the controls of media only and IgY. Three isolates from Kintampo were tested additionally with sCR1 which significantly inhibited invasion in both untreated and neuraminidase-treated erythrocytes relative to the controls of media only and IgY. These results are also similar to findings reported by Awandare *et al.* (2011) in Kenyan isolates suggesting that field isolates in Ghana may also use CR1 as a major receptor for SA-

independent invasion of erythrocytes. More interestingly, the extent of inhibition by anti-CR1 antibodies in neuraminidase-treated erythrocytes for EIMK011 and EIMN031 was less than that of other isolates tested. Both isolates invaded with efficiencies greater than 50% in neuraminidase-treated erythrocytes in the presence of anti-CR1 antibodies. Furthermore, EIMK011 showed an invasion phenotype consistent with the enzyme sensitivity of CR1, but still showed the ability to invade erythrocytes when SA- and CR1-dependent pathways were removed. This may indicate that EIMK011 may use a receptor that shows the same sensitivity to neuraminidase, trypsin and chymotrypsin as CR1. Taken together, the results demonstrate that EIMK011 and EIMN031 isolates may be using receptors other than CR1 or SA receptors for invasion, further demonstrating the functional redundancy exhibited by *P. falciparum* parasites.

The Ok blood group antigen, BSG, has been recently identified to be critical for invasion. Therefore, antibodies against BSG were used to inhibit invasion in both untreated and neuraminidase-treated erythrocytes to determine the dependence of the parasite isolates on BSG in the presence and absence of SA. Anti-BSG reduced invasion efficiency below 25% in both untreated and neuraminidase-treated erythrocytes in four of the isolates tested (EIMK011, EIMK077, EIMK020, and EIMN062). These results are similar to what has been demonstrated in laboratory strains and in some field isolates from Senegal (Crosnier *et al.*, 2011). This indicates that the interaction between BSG and its parasite ligand, PfRh5, is essential for parasite growth in Ghanaian field isolates. This interaction appears to be upstream of glycophorin interactions since it appears to be independent of the presence or absence of SA. This appears to be consistent with suggestions made by

Galinski *et al.*, (1992), and again by Galinski and Barnwell (1996) that PfRh proteins act prior to interactions involving EBL proteins.

Antibodies against full length recombinant PfRh5 have been shown to have a similar ability to inhibit parasite growth and invasion in untreated erythrocytes (Bustamante *et al.*, 2013). In addition, antibodies against an EGF-like protein, PfRipr could also inhibit parasite growth and invasion to a similar extent as anti-PfRh5 antibodies (Chen *et al.*, 2011). Chen *et al.* (2011) established that PfRipr complexes with PfRh5, which binds to basigin (Crosnier *et al.*, 2011). PfRh5, PfRipr and BSG are thought to form a complex similar to the complex formed by AMA-1 and RON proteins complex which is essential in tight junction formation, suggesting that the interaction between PfRh5, PfRipr and BSG may also be involved in tight junction formation.

The data presented in this study shows that antibodies against BSG were able to reduce parasite growth and invasion to below 30% in both untreated and neuraminidase-treated erythrocytes in 4 out of the 5 isolates tested, whereas antibodies against CR1 showed similar ability in only neuraminidas- treated erythrocytes. This indicates that the majority of field isolates in Ghana may invade erythrocytes in a BSG-dependent manner regardless of the presence or absence of SA, but are only dependent on CR1 in the absence of SA. Curiously, one isolate, EIMA008 did not appear to invade in a BSG-dependent manner in the presence of SA. In this strain, parasites were able to invade untreated erythrocytes with more than 50% efficiency in the presence of anti-BSG antibodies, relative to the controls of media only and the isotype control antibody, IgG. Nevertheless, EIMA008 was dependent on BSG in the absence of SA, showing an invasion efficiency below 30% in neuraminidase-treated erythrocytes in the presence of anti-BSG antibodies. Since BSG is

thought to be involved in tight junction formation, these results may indicate that there may be other parasite ligands and erythrocyte receptors that may be involved in tight junction formation.

In summary, four invasion phenotypes were identified among *P. falciparum* clinical isolates in Ghana. Nearly all parasites tested appeared to invade erythrocytes in a SA-independent manner. The most common receptor used among the isolates was NrCsTs, which implicates CR1 as a major receptor for invasion. However, three other phenotypes were observed: NsCsTs, which may be mediated by variant PfRh4 and a novel receptor; NrCrTs, perhaps involving the use of EBA-181; and NrCrTr, which is synonymous with the use of BSG as a major receptor for invasion. Interestingly, two of these atypical phenotypes were observed in Navrongo where the malaria transmission is high and seasonal. Invasion efficiency of all the parasites tested was inhibited by the presence of antibodies against CR1 in neuraminidase-treated erythrocytes, confirming that Ghanaian isolates may be highly dependent on CR1 as a receptor for invasion. Antibodies against BSG were able to inhibit >50% invasion in both untreated and neuraminidase-treated erythrocytes in nearly all strains, suggesting that parasites in Ghana invade in a BSG-dependent manner. These results also suggest that basigin, along with other proteins may be involved in events upstream of CR1 and sialoglycoproteins.

5.2. CONCLUSION

The most prevalent erythrocyte invasion mechanism identified among Ghanaian clinical isolates suggests the use of CR1 and its parasite ligand PfRh4. All the isolates tested in this

study invaded erythrocytes in a CR1-dependent manner in erythrocytes depleted of SA, suggesting the use of CR1 as a major receptor for alternative invasion pathways. However, the parasite isolates were dependent on BSG as an erythrocyte receptor in the presence and absence of SA.

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APPENDIX**a. Reagents****Table A1.** List of reagents and their sources

Reagent	Source
Albumax II	Invitrogen
Chicken Anti-sCR1(16 mg/mL)	Gallus Immunotech Inc.
DDAO SE	Invitrogen
Gentamicin (10mg/mL)	Invitrogen
Hypoxanthine	Park Scientific Ltd
L-Glutamine	Sigma –Aldrich Co.
L-Alanine	Sigma-Aldrich Co.
Monoclonal Anti-Basigin (CD-147)	Sigma Aldrich Co.
Neuraminidase from <i>Vibrio cholerae</i>	Sigma-Aldrich Co.
Normal Human Serum (NHS)	
Percoll®	Sigma-Aldrich Co.
Phosphate Buffered Saline, pH 7.4	Sigma-Aldrich Co.
Pre-Immune Chicken IgY (14.8mg/mL)	Gallus Immunotech Inc.
RPMI 1640 Liquid (with L-Glutamine and NaHCO ₃)	Sigma-Aldrich Co.
SYBR Green I	Invitrogen
FACS flow Fluid	BD Biosciences
α-Chymotrypsin from bovine pancreas	Sigma-Aldrich Co.
Trypsin from bovine pancreas	Sigma-Aldrich Co.

b. Materials

- Flat-bottomed 96 well plate
- Latex gloves
- Alcohol swabs
- 70% Ethanol
- Permanent markers
- Sample rack
- Sterile Falcon tubes (15mL and 50mL)
- Sterile Needles
- FACS Flow Fluid
- FACS tubes

c. Preparation of Solutions**i. Albumax**

- 2 L RPMI 1640
- 100 g Albumax II
- 400 mg Hypoxanthine

ii. Parasite Wash Medium (PWM)

- 500 mL RPMI 1640
- 5 mL Gentamicin (10mg/mL)
- 50 μ L L-Glutamine (200mM)

iii. Complete Parasite Medium (CPM)

- 500 mL RPMI 1640
- 5 mL Gentamicin (10mg/mL)
- 50 μ L L-Glutamine (200mM)
- 50 mL Albumax
- 10 mL Normal Human Serum(NHS)

Table A2. List of all field isolates used in this study

Strain/Lab ID	Date thawed	Cultured	Phenotyped
EIMA005	24/01/13	Yes	Yes
EIMA008	04/07/13*	Yes	Yes
EIMK011	07/05/13	Yes	Yes
EIMK012	24/01/13	Yes	Yes
EIMK013	05/06/13	Did not grow	No
EIMK020	07/05/13	Yes	Yes
EIMK077	07/05/13	Yes	Yes
EIMK091	24/01/13	Did not survive in culture	No
EIMK113	22/03/13	Yes	Yes
EIMK124	11/04/13	Yes. Contaminated.	Yes
EIMK125	05/06/13	Yes	Yes
EIMK140	28/05/13	Did not survive in culture	No
EIMK144	28/05/13	Did not survive in culture	No
EIMK209	13/03/13	Yes	Yes
EIMK244	22/01/13	Yes	Yes
EIMN013	16/01/13	No	No
EIMN031	13/03/13	Yes. Did not survive in culture	Yes
EIMN039	24/01/13	Did not survive in culture	No
EIMN054	16/01/13	No	No
EIMN062	07/05/13	Yes	Yes
EIMN063	05/06/13	Yes	Yes
EIMN079	05/06/13	Yes	Yes
EIMN094	28/05/13	Did not grow in culture	No
EIMN096	11/04/13	Yes	Yes

*- Sample was cultured directly from the field

Table A2. (Continued). List of all field isolates used in this study.

Strain/Lab ID	Date Thawed	Cultured	Phenotyped
EIMN110	22/03/13	Yes	Yes
EIMN196	05/06/13	Yes	Yes
EIMN205	22/01/13	Did not survive in culture	No

Table A3. Invasion efficiencies of Ghanaian *P. falciparum* field isolates in neuraminidase-, chymotrypsin- and trypsin-treated erythrocytes

Parasite	% Invasion efficiency relative to untreated erythrocytes (SD)					
	Neuraminidase (0.25U/mL)	Neuraminidase (0.5U/mL)	Chymotrypsin (1mg/mL)	Chymotrypsin (4mg/mL)	Trypsin (1mg/mL)	Trypsin (10mg/mL)
A005 (n=3)	–	62.20 (22.19)	–	6.40 (4.23)	–	6.31 (2.94)
K011 (n=3)	88.45 (15.52)		41.89 (13.86)		33.23 (11.19)	
K012 (n=2)		85.82 (33.83)		33.33 (17.63)		25.00 (15.70)
K020 (n=3)	80.40 (5.98)		35.03 (16.68)		26.40 (1.25)	
K077 (n=3)	71.49 (12.03)		49.07 (12.87)		23.01 (1.29)	
K113 (n=3)		56.62 (14.56)		27.81 (15.56)		22.51 (11.90)
K124 (n=2)	67.75 (5.35)	62.10 (8.09)	48.78 (1.91)		14.07 (1.70)	
K209 (n=2)		58.73 (12.94)		42.41 (2.73)		46.47 (9.30)
K244 (n=3)		65.42 (11.77)		10.06 (11.33)		11.50 (13.95)
N031 (n=1)		80.27 (3.40)		72.38 (1.94)		71.87 (7.03)

“n” denotes the number of experiments. All experiments were set up in duplicates. Data are presented as means (SD) of one to three separate experiments for each parasite strain.

Table A3. (Continued). Invasion efficiencies of Ghanaian *P. falciparum* field isolates in neuraminidase-, chymotrypsin- and trypsin-treated erythrocytes

Parasite	% Invasion efficiency relative to untreated erythrocytes					
	Neuraminidase (0.25U/mL)	Neuraminidase (0.5U/mL)	Chymotrypsin (1mg/mL)	Chymotrypsin (4mg/mL)	Trypsin (1mg/mL)	Trypsin (10mg/mL)
N062 (n=2)	50.31 (11.60)	–	34.40 (8.37)	–	9.40 (2.47)	–
N096 (n=2)	67.75 (5.35)	–	48.78 (1.91)	–	14.07 (1.70)	–
N110 (n=2)	–	42.93 (9.44)	–	24.60 (5.13)	–	42.38 (6.51)
A008 (n=2)	52.54 (2.68)	–	31.65 (3.07)	–	18.37 (7.30)	–
K125 (n=1)	51.82 (8.60)	–	65.45 (0.34)	–	29.20 (2.06)	–
N063 (n=1)	69.35 (3.80)	–	20.97 (5.32)	–	22.04 (0.76)	–
N079 (n=1)	52.66 (0)	–	31.50 (3.77)	–	14.58 (1.11)	–
N196 (n=1)	46.32 (5.95)	–	39.65 (6.45)	–	16.49 (4.67)	–

“n” denotes the number of experiments. All experiments were set up in duplicates. Data are presented as means (SD) of one to three separate experiments for each parasite strain.

Table A4. CR1-dependent invasion of Ghanaian *P. falciparum* field isolates in untreated erythrocytes.

Parasite	% Invasion efficiency relative to untreated erythrocytes (SD)				
	Control	Anti CR1	IgY	sCR1	Fetuin
A005 (n=3)	100	100.49 (12.42)	117.07 (23.88)	–	–
K011 (n=3)	100	101.73 (12.20)	100.66 (3.68)	88.93 (6.06)*	104.04 (3.24)
K012 (n=2)	100	106.57 (16.99)	115.54 (32.83)	–	–
K020 (n=3)	100	97.84 (4.57)	96.93 (4.15)	80.24 (3.06)*	101.90 (4.33)
K077 (n=3)	100	95.34 (3.89)	94.49 (5.94)	86.75 (4.59)*	85.18 (16.52)
K113 (n=3)	100	98.02 (10.00)	99.10 (12.25)	–	–
K124 (n=2)	100	9.97 (7.24)	94.76 (3.48)	–	–
K209 (n=2)	100	92.68 (16.20)	78.30 (24.74)	–	–
K244 (n=3)	100	93.60 (14.69)	91.70 (6.05)	–	–
N031 (n=1)	100	93.65 (10.19)	97.08 (4.85)	–	–

“n” denotes the number of experiments. Data are presented as means (SD) of one to three separate experiments for each parasite strain. * Denotes that invasion inhibition by anti-CR1 or sCR1 was statistically significant compared to the effects of IgY or fetuin, respectively ($P < 0.05$ vs. respective control proteins, paired Student’s t-test)

Table A4. (Continued). CR1-dependent invasion of *P. falciparum* field isolates in Ghana in untreated erythrocytes.

Parasite	% Invasion efficiency relative to untreated erythrocytes (SD)				
	Control	Anti CR1	IgY	sCR1	Fetuin
N062 (n=3)	100	92.77 (9.51)	88.67 (5.41)	–	–
N096 (n=2)	100	97.38 (2.80)	97.58 (4.24)	–	–
A008 (n=2)	100	93.56 (4.04)	99.73 (3.78)	–	–
K125 (n=1)	100	98.54 (3.79)	84.18 (12.38)	–	–
N063 (n=1)	100	93.54 (6.08)	102.15 (10.64)	–	–
N079 (n=1)	100	94.20 (8.64)	84.95 (0.00)	–	–
N196 (n=1)	100	80.00 (6.94)*	97.54 (0.00)	–	–

“n” denotes the number of experiments. Data are presented as means (SD) of one to three separate experiments for each parasite strain. All experiments were set up in duplicates. * Denotes that invasion inhibition by anti-CR1 or sCR1 was statistically significant compared to the effects of IgY or fetuin, respectively ($P < 0.05$ vs. respective control proteins, paired Student’s t-test)

Table A5. CR1-dependent invasion of Ghanaian *P. falciparum* field isolates in neuraminidase-treated erythrocytes.

Parasite	% Invasion efficiency relative to untreated erythrocytes (SD)				
	Control	Anti CR1	IgY	sCR1	Fetuin
A005 (n=3)	62.20 (22.19)	16.64 (5.16)*	68.15 (12.78)	–	–
K011 (n=3)	88.45 (15.52)	50.77 (16.35)*	83.53 (12.82)	31.20 (7.72)*	79.46 (8.73)
K012 (n=2)	85.82 (33.82)	19.95 (8.67)*	73.16 (12.91)	–	–
K020 (n=3)	80.40 (5.98)	33.54 (5.28)*	76.33 (6.71)	25.01 (5.66)*	79.84 (1.43)
K077 (n=3)	71.49 (12.02)	31.77 (9.81)*	67.75 (8.36)	22.19 (5.11)*	63.61 (9.89)
K113 (n=3)	56.62 (14.56)	16.66(13.20)*	58.54 (11.16)	–	–
K124 (n=2)	63.70 (3.00)	21.41 (4.68)*	61.27 (3.39)	–	–
K209 (n=2)	58.73 (12.94)	41.13 (4.89)*	65.02 (7.50)	–	–
K244 (n=3)	65.41 (11.77)	17.77 (11.94)*	55.17 (19.61)	–	–
N031 (n=1)	80.27 (3.40)	57.29 (3.40)*	72.04 (7.76)	–	–

“n” denotes the number of experiments. Data are presented as means (SD) of one to three separate experiments for each parasite strain. All experiments were set up in duplicates. * Denotes that invasion inhibition by anti-CR1 (12µg/mL) or sCR1 (50µg/mL) was statistically significant compared to the effects of IgY (12µg/mL) or fetuin (50µg/mL), respectively (P < 0.05 vs. respective control proteins, paired Student’s t-test)

Table A5 (Continued). CR1-dependent invasion of Ghanaian *P. falciparum* field isolates in neuraminidase-treated erythrocytes.

Parasite	% Invasion efficiency relative to untreated erythrocytes (SD)				
	Control	Anti CR1	IgY	sCR1	Fetuin
N062 (n=2)	50.31 (11.60)	26.09 (5.06)*	51.07 (11.50)	–	–
N096 (n=2)	67.75 (5.35)	21.37 (5.28)*	60.20 (6.97)	–	–
N110 (n=2)	42.93 (9.44)	20.21 (7.68)*	57.17 (28.10)	–	–
A008 (n=2)	52.54 (2.68)	12.57 (1.98)*	48.62 (4.52)	–	–
K125 (n=1)	51.82 (8.60)	28.47 (0.34)*	54.74 (0.34)	–	–
N063 (n=1)	69.35 (3.80)	17.14 (2.28)*	69.89 (13.69)	–	–
N079 (n=1)	52.66 (0.00)	10.34 (0.89)*	40.43(6.64)	–	–
N196 (n=1)	46.31 (5.95)	17.54 (9.20)*	47.02 (16.87)	–	–

“n” denotes the number of experiments. Data are presented as means (SD) of one to three separate experiments for each parasite strain. All experiments were set up in duplicates. * Denotes that invasion inhibition by anti-CR1 (12µg/mL) or sCR1 (50µg/mL) was statistically significant compared to the effects of IgY (12µg/mL) or fetuin (50µg/mL), respectively (P < 0.05 vs. respective control proteins, paired Student’s t-test)

Table A6. BSG-dependent invasion of *P. falciparum* field isolates in Ghana in neuraminidase-treated erythrocytes.

Parasite	% Invasion efficiency relative to untreated erythrocytes (SD)		
	Control	Anti BSG	IgG
Invasion in untreated erythrocytes			
K011 (n=3)	100	27.66 (5.51)*	102.37 (6.49)
K020(n=2)	100	18.40 (4.81)*	101.32 (9.90)
K077(n=3)	100	24.73 (7.37)*	93.83 (4.39)
N062 (n=2)	100	21.29 (4.67)*	92.37 (9.15)
A008 (n=2)	100	47.22 (11.78)*	95.95 (3.97)
Invasion in neuraminidase-treated erythrocytes			
K011 (n=3)	80.40 (5.98)	19.82 (4.30)*	88.46 (17.14)
K020 (n=2)	88.45 (15.52)	6.57 (2.74)*	77.82 (5.46)
K077 (n=3)	71.49 (12.02)	14.46 (1.27)*	65.80 (7.81)
N062 (n=2)	50.31 (11.60)	7.73 (1.40)*	51.07 (10.63)
A008 (n=2)	52.54 (6.78)	8.60 (0.92)*	47.18 (4.00)

“n” denotes the number of experiments. Data are presented as means (SD) of one to three separate experiments for each parasite strain. All experiments were set up in duplicates. * Denotes that invasion inhibition by anti-BSG (10µg/mL) was statistically significant compared to the effects of IgG (10µg/mL) (P < 0.05 vs. respective control antibodies, paired Student’s t-test)