ANALYSIS OF REACTIVITY AND FUNCTIONALITY OF SERUM ANTIBODIES FROM MONOVALENT AND MULTIVALENT *Shigella* BIOCONJUGATE VACCINATED RABBITS AGAINST *Shigella* ISOLATES FROM KENYA

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DECLARATION

This thesis is my original work and has not been submitted for a degree in any other University or institution.

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ABSTRACT

Shigella species is a major cause of bacterial diarrheal mortalities and morbidities worldwide and second in Kenya, especially in children under five years. Antibiotics are needed in treatment of shigellosis however, increasing resistance necessitates other control measures such as *Shigella* vaccine but none has widely been approved. An immunogenic novel monovalent and multivalent bioconjugate vaccine (linking *Shigella* O-antigen (LPS) to Exoprotein A of *Pseudomonas aeruginosa*) against the four major *Shigella* serotypes responsible for 80% global morbidity; *S. flexneri* (Sf) 2a, 3a, 6 and *S. sonnei* is in development. The monovalent and quadrivalent bioconjugate vaccine constructs have been evaluated for immunogenicity in rabbit models and is planned for clinical trial in Kenya. Therefore, it is necessary to further understand the antibody response (reactivity and functionality (BA)) from the monovalent and quadrivalent (adjuvanted 4V-adj) and without an adjuvant (4V) *Shigella* bioconjugate vaccinated rabbit serum against *Shigella* isolates from Kenya. This retrospective laboratory-based study, nested within an ongoing parent diarrheal surveillance protocol in Kenya since 2009, was conducted to test the immunized rabbit serum against 129 archived *Shigella* strains from Kenyan. Specifically, this study assesses the specific reactivity of serum antibodies against the four major *Shigella* serotypes targeted; cross reactivity of the serum antibodies against *Shigella* serotypes (Sf 1b, 2b, 4a, 4b, *Shigella* spp, *S. dysenteriae* and *S. boydii)* not targeted by the formulation and the BA of the serum antibodies against a subset of reactive *Shigella* isolates. The 129 isolates were serotyped and screened for retention of the virulence factor O-antigen LPS by plating on Congo red (CR) culture plate. The specific and cross reactivity of the immunized rabbit serum to the virulent *Shigella* isolates was determined by colony blot assay. The BA of the serum antibodies against reactive *Shigella* isolates was assessed by a serum bactericidal assay (SBA) and analyzed using Nist Integrated Colony Enumerator and Opsititre software. Of the 129 *Shigella* isolates, 109 (85%) retained their virulence; 67 serotypes were targeted by the vaccine while 42 were not-targeted serotypes. From the virulent isolates, a subset of 22 isolates targeted by vaccine [Sf 2a (4), 3a (10), 6 (4), *S. sonnei* form I (4)] and 19 isolates not targeted by the vaccine [Sf 1b (5), 2b (5), 4a (2), 4b (3), *S boydii* (2) and *S. dysenteriae* (3)] were assessed for reactivity. Specific reactivity Serum from rabbits immunized with the 4V-Adj and the respective monovalent vaccines against the 22 target serotypes had 100% specific reactivity. The serum from 4V-Adj vaccinated rabbits cross-reacted with Sf 2b (75%), Sf 4a (100%), one Sf 1b (25%) and Sf 4b (33%). Serum from rabbits immunized with the Sf 2a monovalent vaccine cross-reacted with *Shigella* serotypes; one Sf 3a and three Sf 2b, while the Sf 3a monovalent serum cross-reacted with one Sf 2a. The surviving colonies were enumerated using excel based Nist Integrated Colony Counter and analyzed using the Opsititre software and presented in tables. There was ≥4-fold increase in BA between the pre and post vaccine rabbit sera with all the *Shigella* serotypes tested except one cross-reactive Sf 3a indicative that, the rabbit's serum antibodies had functional activity. Collectively, these results demonstrate that the monovalent/ quadrivalent bioconjugate *Shigella* vaccine may be more broadly protective than designed offering a promising solution to reduced morbidity and mortalities associated with *Shigella* spp. These results strengthen the vaccine development strategies and lay a solid foundational basis for *Shigella* bioconjugate vaccine human challenge studies here in Kenya.

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LIST OF ABBREVIATIONS, ACRONYMS

4V	Quadrivalent without adjuvant
4V-Adj	Quadrivalent with an adjuvant
Ab	Antibodies
AI	Associate Investigator
BA	Bactericidal Assay
BRC	Baby Rabbit Complement
CFU	Colony Forming Units
CHIM	Controlled Human Challenge Models
CR	Congo Red
DoD	Department of Defense
ELISA	Enzyme Linked Immunosorbent Assay
GI	Gastrointestinal
HUS	Haemolytic Uremic Syndrome
HI	Heat Inactivated
FHP	Force Health Protection
LB	Luria -Bertani
LBA	Luria-Bertani agar
LMTB	LimmaTech Biologics
LPS	Lipopolysaccharide
MHK	Microbiology Hub Kericho
NICE	National Institute of Standards and Technology Integrated Colony Enumerator

PBS	Phosphate Buffered Saline
PI	Principal Investigator
SBA	Serum Bactericidal Antibody assay
SERU	Scientific and Ethics Research Unit
SEVI	Subunit Enteric Vaccines and Immunology
S. spp	*Shigella* species
SF	*Shigella flexneri*
SS	*Shigella sonnei*
T3SS	Type III Secretion System
USAMRD-A/K	US Army Medical Research Directorate- Africa/Kenya
WRAIR	Walter Reed Army Institute of Research

DEFINITION OF TERMS

Functionality	Ability to cause bacterial killing also referred to as bactericidal activity in this study
Reactivity	Antibodies binding to *Shigella* isolates
Monovalent	Vaccine construct composed of anti-LPS against O-Antigen of one specific target serotype
Multivalent	Vaccine composed of anti-LPS against O-Antigen of the 4 target serotypes. Also referred to as quadrivalent in this study
*Shigella*4V	The LMTB tetravalent bioconjugate *Shigella* vaccine under development/ clinical trial studies

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CHAPTER ONE

INTRODUCTION

1.1. Background Information

The genus *Shigella* are Gram-negative bacteria from the family *Enterobacteriaceae* (Christabel, 2012). *Shigella* spp are a major cause of bacterial diarrhea worldwide, accounting for approximately 165-190 million cases and 1.1 million deaths per year, mainly in developing countries and disproportionately in children (Azmi *et al*., 2014; Nüesch-Inderbinen *et al*., 2016; C. Yang *et al*., 2016). There are four species and several subtypes of *Shigella*: *S. sonnei, S. boydii, S. flexneri* and *S. dysenteriae* with over 50 serotypes except for *S. sonnei* that has only one serotype(Ghosh *et al*., 2014; Livio *et al*., 2014; Torraca *et al*., 2020). The species differ in geographic distribution and antimicrobial susceptibility (Ghosh *et al*., 2014). *Shigella sonnei* is encountered mostly in industrialized countries and *S. flexneri* in developing countries (Azmi *et al*., 2014; Jeong *et al*., 2011). The most predominant isolate in Kenya is *S. flexneri* followed by *S. sonnei* (Patricia B. Pavlinac *et al*., 2014; Swierczewski *et al*., 2013). Diarrheal surveillance studies in Kenya show that *Shigella* spp were the second leading bacterial cause of diarrhea in both children and adults at healthcare facilities in Kericho, Kisii, Kisumu, Homa Bay and Migori counties (Kotloff *et al*., 2012; P. B. Pavlinac *et al*., 2016; Swierczewski *et al*., 2013). The serotypes of *S.flexneri* previously identified one of the sites from the GEMs study in Kenya include, S. *flexneri* 1b, 2a, 3a, 4a, 6 and 7a (Livio *et al*., 2014).

The clinical manifestation of the infection is Shigellosis which is characterized by profuse diarrhea with or without blood and gastrointestinal disease. Shigellosis leads to morbidity and mortality in both children and adults (Shimanovich *et al*., 2017). Shigellosis is not easily diagnosed by conventional culture methods due to the fastidious nature of the *Shigella* isolate.

Therefore, empirical treatment with effective antibiotics is recommended to reduce the duration of illness, risk of complications and transmission of the disease (Humphries & Linscott, 2015; Nüesch-Inderbinen *et al*., 2016). However, selective pressure created by use and misuse of antibiotics has been identified as a major factor contributing to increased antimicrobial resistance in bacteria which is a significant public health problem worldwide (Aggarwal, 2016). In Kenya, as most diarrheal infections are treated empirically, there is increased usage of β-lactams and quinolones in the treatment of shigellosis (Sang *et al*., 2012), resulting in increased resistance. Moreover, antibiotic susceptibility of *Shigella* spp. is reported to be dynamic and varies geographically with time and the resistance antibiotics are not recommended as empirical treatment for shigellosis (Kotloff *et al*., 2012). In Kenya, 80-100 % of *Shigella* isolates were found to be resistant to tetracycline, ampicillin, trimethoprime/sulfamethoxazole and there emerging resistance to quinolones such as ciprofloxacin (Kotloff *et al*., 2012; P. B. Pavlinac *et al*., 2016; Swierczewski *et al*., 2013; WHO, 2020). The increasing resistance of *Shigella* spp. to various antibiotics used in its treatment exacerbates management and control emphasizing the need for more effective preventive measures to morbidity and mortalities associated with shigellosis infections (Kazi *et al*., 2015; Leski *et al*., 2013).

Shigella spp are highly invasive gastrointestinal (GI) pathogens with a low infective dose of 10-100 bacilli (Hale & Keusch, 1996; Kazi *et al*., 2015). The pathogenesis of *Shigella* spp involves a combination of plasmid and, in some serotypes, chromosomally encoded virulence factors, secreted proteins and bacterial LPS leading to *Shigella* infection as well as serotype-specific immunity. Additionally, immune evasion mechanisms due to bacterial cell secreted effectors such as VirG protein facilitate spread of the bacteria from cell to cell leading to profuse diarrhea and establishment of the infection (Mattock & Blocker, 2017; Schroeder & Hilbi, 2008). The

chromosomal virulence factors located on a Pathogenicity Island encoding proteins such as SHI-1 aids in the production of enterotoxin and intestinal colonization, SHI-2 and -3 involved in iron-siderophore complexes and host inflammatory response, SHI-O in modification of O-antigen and serotype conversion and Stx-phage P27 that produces shiga toxin also play a role in *Shigella* pathogenesis (Mattock & Blocker, 2017). Plasmid-encoded virulence factors including type III secretion system (T_3SS) apparatus encoded within the macromolecule needle-like structure (IpaB, IpaC and IpaD) form part of the *Shigella* antigenic structures that lead to colonization and disease (Ashida *et al*., 2015). The LPS, is a major cell surface component that causes non-specific pathophysiological reaction and acute inflammatory effects observed during Shigellosis (Cruz *et al*., 2014; The *et al*., 2016).

The O-antigen of the LPS aids the *Shigella* bacterium to evade destruction by the host response and in complement-mediated lysis resulting in bacterial cell death (Kim *et al*., 2016). The *Shigella* LPS plays a vital role in host resistance to non-specific defense encountered during tissue invasion leading to multiplication and escape from phagocytosis of the cells by tissue macrophages (Cruz *et al*., 2014; Killackey *et al*., 2016). Additionally, the antigenic variability of the O-antigen on the LPS is the basis for grouping and sub-typing of *Shigella* spp (Livio *et al*., 2014; Noriega *et al*., 1999). *Shigella* overcomes the innate immune response leading to activation of the adaptive antibody and cell mediated immune responses. The memory B and T cell responses provide short lived serotype-specific immunity against *Shigella* re-infections (Kim *et al*., 2016; Koestler *et al*., 2019). The serotype-specific antibody immune responses are therefore the foundational basis for serotype specific monovalent and multivalent *Shigella* vaccine formulations in development. The key roles of the Ipa and LPS virulence factors in Shigella pathogenesis combined with being exposed on the bacterial surface makes them

attractive vaccine targets (Mani *et al*., 2016; Mattock & Blocker, 2017). However, the potential for a globally effective vaccine depends on the ongoing characterization and understanding of the specificity or cross reactivity of the vaccine induced antibodies against *Shigella* spp serotypes (WHO, 2020). To determine the utility of these vaccines in LMICs, such as Kenya, there is need to further investigate anti O-antigen LPS serum antibodies against a range of *Shigella* sub-types from Kenya.

Although no vaccine has been widely approved or licensed, milestones have been achieved in the current *Shigella* conjugate vaccine development strategies(Levine *et al*., 2007; Mattock & Blocker, 2017; Passwell *et al*., 2010; Turbyfill *et al*., 2018). Some *Shigella* vaccines with successful pre-clinical studies include the artificial Invaplex (containing IpaB, C, LPS and other proteins) in phase 1 trial, the live attenuated WRSs2 mutant (VirG deletion) advanced to clinical development and a live *Shigella* vaccine (deletion of *guaBA*, *sen* and *set* genes) in a phase 1 study (Turbyfill *et al*., 2018; WHO, 2020). Building on previous successes achieved with *Shigella* conjugate vaccines, a polysaccharide-bioconjugate approach such as with Flexyn2a developed by LimmaTech Biologics (LMTB) has been shown to be safe, cost effective, highly immunogenic and tolerable (even among infants) vaccine (Riddle *et al*., 2016). The bioconjugate vaccine formulation consist of *Shigella* serotype-specific O-antigens (of LPS) bio-conjugated to a protein carrier, *Pseudomonas aeruginosa* Exoprotein A (Chen & Kotloff, 2016; Riddle *et al*., 2016). Transitioning the vaccine formulation from a single valency to multi-valency is required to induce immunity against the four major *Shigella* serotypes responsible for approximately 89% of global morbidity (Chen & Kotloff, 2016; Riddle *et al*., 2016; Turbyfill *et al*., 2018; WHO, 2020), specifically *S. flexneri* 2a, 3a, 6 and *S. sonnei* and possibly other serotypes as recommended by WHO (WHO, 2020).

Finding suitable shigellosis models for use in evaluating various *Shigella* vaccines remains a major challenge. Animal models currently being used include rabbits, guinea pigs, mice, non-human primates' such as rhesus macaques, chicks, and piglets. Non-human primates' rhesus macaques models are expensive and have very stringent regulatory and ethical requirements for pre-clinical vaccine trials (Oaks *et al*., 1996). Rabbits and guinea pigs are commonly used for vaccine testing as they have less ethical constraints and induce reactivity close to that expected in humans (Marteyn, 2016). Recently, a series of experiments have been conducted in Switzerland using guinea pigs and rabbits to evaluate the immunogenicity of *Shigella* O-antigen based bioconjugate vaccines, comparing monovalent and multivalent/ quadrivalent (4V) formulations, administered intramuscularly alone, or in combination with an adjuvant. The quadrivalent vaccine (*Shigella*4V) by LMTB is currently in a phase 1/2a safety and dose finding clinical trial in Kenya. What is still unclear is the breadth of the reactivity of the serum collected after immunization of rabbits with *Shigella* serotypes (targeted and not targeted by the vaccine formulation) from Kenya. To assess this gap, a series of ELISA colony spot blot experiments against, a representative subset of confirmed *Shigella* isolates from Kenya targeted by the vaccine as well as serotypes not targeted by the vaccine formulation was accomplished.

Additionally, much needed information on the functionality (bactericidal activity) of the post LMTB quadrivalent bioconjugate vaccine serum by activation of the classical complement pathway resulting in bacterial death is yet to be available. However, serum from subjects immunized with Flexyn2a did have bactericidal activity against *S. flexneri* 2a, 2457T (Riddle *et al*., 2016). *In-vitro* experimental studies done in 2016 by Lin *et al.,* and Kim *et al.,* showed that successful Serum Bactericidal Assay (SBA) was capable of demonstrating functional capabilities of the antibodies to confer bactericidal activities within detectable protective levels (Kim *et al*.,

2016). The automated enumeration method NIST's integrated colony enumeration (NICE, Mathworks, Natick, MA) has proven to achieve a higher throughput compared to manual/ conventional colony counting systems (Kim *et al*., 2016). The SBA was used for testing the bactericidal activity of the rabbit serum with the subset of reactive *Shigella* serotypes. NICE software was used for enumeration of surviving bacterial colonies (Azmi *et al*., 2014a) for the determination of the bactericidal activities of immunized rabbit sera against control strains and clinical *Shigella* isolates from Kenya.

1.2. Statement of the Problem

Shigella has been listed as a category B agent of bioterrorism by the Centers for Disease Control and Prevention. *Shigella* spp have been reported as the second leading bacteria causing diarrheal morbidities and mortalities in both children and adults in Kenya. Although shigellosis results in major life-long complications including, stunted physical and cognitive growth in children, toxic megacolon, haemolytic uremic syndrome, arthritis as well as irritable bowel syndrome/ disease, these symptoms are often not associated with the infection. The increasing antimicrobial resistance together with inadequate public health preventive and control measures in Kenya further exacerbate the management of the disease.

The severity of shigellosis and acquired immunological response specific to *Shigella* infection varies with the serotype and geographical region which complicated the vaccine development strategies. The O-antigen of the LPS has been key for seroepidemiologic data and is a candidate for *Shigella* vaccine development with measurable immunologic response and protection against *Shigella* infection in animal models (rabbits & guinea pigs) and human volunteers. In the recent past, a novel *Shigella* bioconjugate vaccine has been developed in monovalent and multivalent constructs. Impressive immunogenic response has been shown from phase1/ 2a and 2b human

vaccine studies with the bioconjugate monovalent *S.flexneri* 2a (Flexyn2a) vaccine and provided a basis for the development of a quadrivalent construct targeting the four important *Shigella* serotypes. However, no study has investigated the specificity/ cross reactivity of vaccine induced serum and the associated functionality against *Shigella* strains from Kenya a target population for the safety and immunogenicity testing. Additionally and following success with the safety and dose finding, the possible next steps in the vaccine trial will be efficacy testing which forms the basis for the functionality test in this study for both targeted and not targeted by the vaccine formulation.

1.3 General Objective

To investigate the reactivity and functionality of serum antibodies from monovalent and quadrivalent (4V and 4V-Adj) *Shigella* bioconjugate vaccinated rabbit against *Shigella* isolates from Kenya.

1.3.1 Specific Objectives

i. To assess the specific and cross reactivity of monovalent and quadrivalent (4V and 4V-Adj) *Shigella* bioconjugate vaccinated rabbit serum antibodies against the Sf 2a, 3a, 6, *S. sonnei* and other (Sf 1a & b, 2b, 4a & b, 5a, 3b, *Shigella* spp, *S. dysenteriae* & *S. boydii*) serotypes from Kenya.

ii. To assess the bactericidal activity of the monovalent and quadrivalent (4V and 4V-Adj) *Shigella* bioconjugate vaccine serum antibodies against a subset of the targeted and non-targeted reactive *Shigella* isolates.

1.3.2 Research Questions

i. How do the serum antibodies from *Shigella* bioconjugate vaccinated rabbits react specifically and broadly with the *Shigella* strains tested?

ii. What is the breath of the bactericidal activity of the serum antibodies from monovalent and 4V and 4V-Adj bioconjugate vaccinated rabbits against the reactive *Shigella* strains tested?

1.4 Significance of the study

This study results demonstrates the following;

i. The ability of induced antibodies from *Shigella* bioconjugate vaccinated rabbits to bind to *Shigella* serotype bacterial cells (specific and cross reactivity by colony blot assay). This implies that the vaccine under development can be able form an specific antigen antibody complex thus preventing bacteria from further invasion of the host leading to shortened pathogenesis.

ii. The functionality capabilities of the antibodies (Bactericidal activity by SBA) by *in vitro* activation of complement system leading to bacterial death. This activity mimics the in vivo complement activity indicative that the vaccine construct would therefore be able to not only bind but cause bacterial killing leading to elimination of the *Shigella* bacteria, preventing disease progression and reducing mortality/ morbidity.

- Collectively, these results add more knowledge therefore strengthen the vaccine development strategies and also forms a foundational basis for prospective future bioconjugate candidate vaccine challenge studies with the Kenyan population.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Shigella* Biology

Shigellosis is caused by water-borne Gram negative bacteria of the *Enterobacteriaceae* family the genus *Shigella* (S) that, invade and colonize the human GI system leading to watery or bloody diarrhea depending on the species implicated (Azmi et al., 2014; H. N. Njuguna et al., 2013). The four species of *Shigella* and their serovars are grouped into, *S. dysenteriae* (Group A), *S.flexneri* (Group B), *S. boydii* (Group C), and *S. sonnei* (Group D). These are further characterized by O-antigen or biochemically to approximately 50 serotypes (Azmi *et al*., 2014). Identification of *Shigella* spp is mainly via stool culture with serotyping of the isolates to subtype level using antisera and molecular methods though this is limited to genus and species level only (WHO, 2020). Diarrheal infection due to *Shigella* spp presents with loose stools with or without blood depending on the causative species, severe abdominal pain, nausea, vomiting, fever, headache, loss of appetite and long term complications such as, toxic megacolon, arthritis, irritable bowel syndrome/ disease and stunted growth in children (Mattock & Blocker, 2017).

2.2 Transmission and Epidemiology of *Shigella* Infection

Shigella infection can be transmitted through ingestion of as low as 10-100 bacteria in water and food contaminated with feces mostly in developing countries as well as directly from person to person in more developed countries such as United States of America (USA) (Torraca *et al*., 2020). Human beings are the main reservoirs of *Shigella* spp and the transmission routes that lead to infection include, oral fecal route, sexually transmitted diseases, bacteremia but especially shigellosis and surgical procedures along the GI. Infections due to *Shigella* vary with seasons (though not fixed) and between regions worldwide with more endemic disease in

developing countries while occurring more as outbreaks in developed countries (Taneja & Mewara, 2016).

This highly invasive bacterium causes millions of deaths worldwide. Studies (Christabel, 2012; Kotloff *et al*., 2012) suggest that the majority of those affected are children especially in developing countries in Africa such as Kenya and Asia as well as in adults across the globe. Although rare, some studies have reported *Shigella* infections outside the gastrointestinal tract as a causative agent of blood stream infection, meningitis (Endris *et al*., 2013), abscess and sexually transmitted infection in men having sex with men (WHO, 2020). The most common species causing shigellosis globally and in Kenya are *S. flexneri* and *S. sonnei* (Figure 2.1) with a few *S. dysenteriae* and *S. boydii* (Livio *et al*., 2014; Torraca *et al*., 2020).

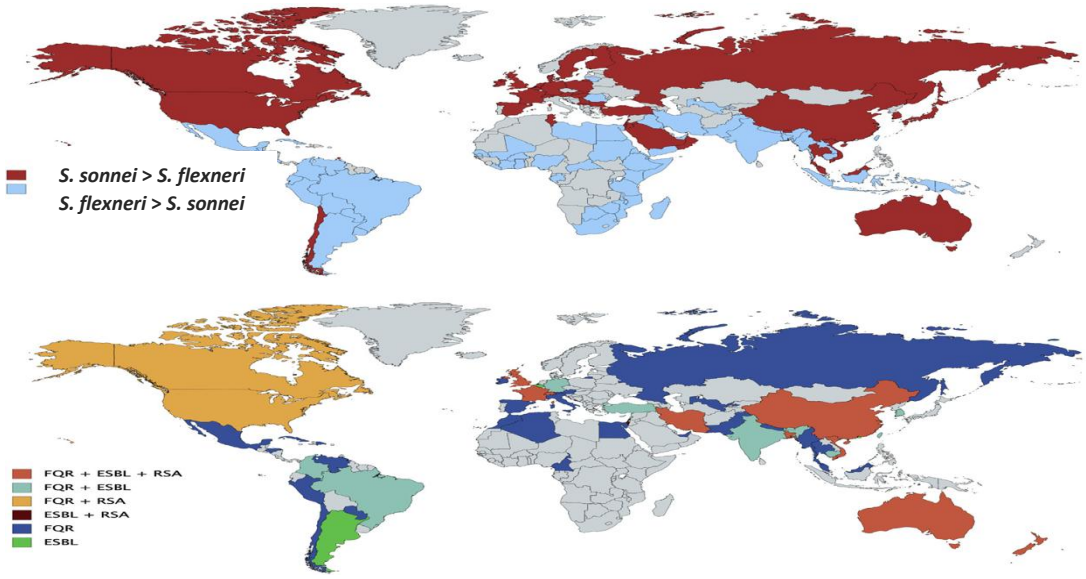

Figure 2.1.: Global epidemiology of most prevalent *Shigella* species and their antimicrobial resistance (Torraca et al., 2020)

A review of shigellosis and serotypes in Africa indicate that the serotypes differ across countries with *Shigella flexneri* isolated in most sub-Saharan Africa, Ethiopia, Kenya and Ghana. In Kenya, the incidence of shigellosis is largely under estimated due to syndromic treatment and

management (Pavlinac *et al*., 2014). However, *Shigella* has been reported as the first or second leading cause of diarrheal in Nairobi, and western regions (Kisii, Kericho, Kisumu) respectively accounting for 4-12% shigellosis and has certain seasonal variation (C. Njuguna *et al*., 2016; P. B. Pavlinac *et al*., 2016; Shah *et al*., 2016). The most common *Shigella* spp identified in Kericho, Kisumu and Kisii are *S. flexneri* and *S. sonnei* with a few *S. dysenteriae* and *S. boydii* (Livio *et al*., 2014; P. B. Pavlinac *et al*., 2016; Swierczewski *et al*., 2013). The reported associated mortalities and morbidities to shigellosis was mostly with other *Shigella* species other than *S. dysenteriae* (Tickell *et al*., 2017). Unlike in other countries, in Kenya, *Shigella* infection has only been reported from fecal, water, soil and raw vegetables samples (Christabel, 2012; Kotloff *et al*., 2012).

2.3 Treatment for Shigellosis and Antibiotic Resistance

Although shigellosis can be a self-limiting infection, antibiotics are necessary for treatment and management of the severe infection. Treatment of shigellosis depends on the species being queried or isolated as causative agent of the diarrheal disease and is largely influenced by the innate and inter-species distribution of resistant genes (Torraca *et al*., 2020; WHO, 2020). Most often, shigellosis cases are managed by empherical treated (P. B. Pavlinac *et al*., 2016).

Increase in resistance to commonly used antibiotics such as ampicillin, trimethoprime-sulfamethoxazole, tetracycline, (Swierczewski *et al*., 2013) nalidixic acid and azithromycin (Nüesch-Inderbinen *et al*., 2016) raises major concern on the available but limited treatment options (WHO, 2020). The increased resistant bacteria may be due to selective pressure of antibiotics associated with over prescription from empirical treatment, over the counter self-medication or use of some of the drugs as prophylaxis in Kenya and many parts of the world (Aggarwal *et al*., 2016; Torraca *et al*., 2020). Although there are reported resistance on

ciprofloxacin, it still remains the current recommendation for shigellosis treatment by the WHO and the Kenya ministry of health (Rhee *et al*., 2019; WHO, 2020).

2.4 Mechanisms of Virulence and Immunological Response by *Shigella* spp

The pathogenesis of the highly invasive *Shigella* spp leading to severe disease is attributed to the virulence factors which are either chromosomally- or plasmid-encoded. The virulence invasive plasmid antigens or Ipa proteins (IpaB, IpaC and IpaD) are located at the tip of T3SS protein complex and facilitate bacterial entry into the host cells (F. Yang *et al*., 2005a). Individual *Shigella* isolates may harbor more than one detectable virulence genes and or toxins including, IpaH present as multiple copies e.g. *Shigella* enterotoxin (ShET) 1 and 2, Shiga toxin 1 and 2 expressed in *S. flexneri* 2a and *S. dysenteriae* respectively (Farshad *et al*., 2006). Although Ipa virulence genes were identified in 100% of *Shigella* isolates from pediatric diarrheal stool in Brazil and Iraq they were missing or deleted in environmental samples from Bangladesh (Cruz *et al*., 2014; Farshad *et al*., 2006). These virulence factors result in secretion of cytokines by the host cells which leads to inflammation in the colonic epithelium, enterotoxic effects to the colon, establishment of a replicative niche leading to destruction of the epithelial cells. Eventually, effectors for down-regulation of inflammation and production of innate immune response are elicited (Mattock & Blocker, 2017).

The plasmid encoded virulence protein VirG (IcsA) is present in all virulent *Shigella* isolates and facilitates the intercellular spread of *Shigella* into the host epithelial cells enhancing further invasion and cell to cell spread by actin-based motility (Mattock & Blocker, 2017). Another virulent factor, LPS, is the serotype determinant, based on the structure of the O-polysaccharide and therefore differs with each serotype. LPS has also been implicated in differentiating between virulent or avirulent strains within a serotype. For example, the *S. sonnei* phase/form I

and II differ in that *S. sonnei* I are able to retain the pINV gene (T_3SS and O-antigen) making it the more virulent prototype while *S. sonnei* II have lost the pINV gene and are avirulent (Kim *et al*., 2016; Torraca *et al*., 2020).

In shigellosis, host re-infection may occur leading to sero-conversion and production of anti-LPS antibodies (IgG & IgA) against serotype specific *Shigella* strains thus activating a short lived adaptive immune response (Chen & Kotloff, 2016). A study done among Israeli soldiers (Shimanovich *et al*., 2017) indicate that these immunoglobulin's have been found in both symptomatic and asymptomatic subjects but at higher level in asymptomatic individuals during outbreaks. The antibodies produced may also facilitate phagocytosis by the macrophages(Faruque *et al*., 2002) however, studies of this nature have not been performed in Kenya to assess the bactericidal activity of serum antibodies against *Shigella* bacteria isolated from Kenyans.

2.5 Vaccine Development Strategies

The efforts towards developing a *Shigella* vaccine have been challenging due to the diversity of *Shigella* serotypes, target genes, mode of and models for evaluation of candidates. Summarized in Table 2.1 below are some of the vaccines and the stages in their development (Marteyn, 2016; WHO, 2020). A majority of the *Shigella* vaccines are still in clinical trials and are yet to be approved. The current formulations are geared towards more immunogenic but tolerable vaccine especially in children and affordable candidates for the LMIC (WHO, 2020). As seen in Table 2.1, these vaccines have been developed in high income countries, most of which have gone through several pre-clinical trials, initial phase 1 human trial and have been approved for phase 1b or 2 and 3 clinical trials. Following the pre-clinical and initial human trials, some of the vaccine constructs such as *Shigella*4V are currently being tested or planned for testing in Kenya

which is one of the LMIC in sub-Saharan Africa where shigellosis is endemic. The population in Kenya region would benefit from the vaccine once approved.

Table 2.1.: Vaccine pipelines and stages in development

Candidate name/identifier platform	Developer	Pre-clinical	Phase I	Phase II	Phase III
Cellular candidates guaBA-based live attenuated (CVD 1208, CVD 1208S)] Mutations in the guaBA operon (genes involved in guanine biosynthesis) leading to a guanine auxotroph and decrease in virulence. Further iterations included deletions of enterotoxin genes.	CVD at the University of Maryland School of Medicine, Baltimore, Maryland USA		X		
VirG-based live attenuated (WRSS1, WRSs3, WRSf3) Primary attenuation by a 212bp deletion in the virG gene preventing intracellular spreading. Further iterations included deletions of enterotoxin genes and lipid A genes.	WRAIR, Silver Spring, Maryland USA			X	
ShigETEC Live, genetically attenuated Shigella vaccine strain that is amenable for the heterologous expression of diarrheal antigens and therefore can provide protective immunity against multiple pathogens	EveliQure Biotechnologies GmbH, Vienna, Austria	X			
Truncated *Shigella*, Mutant *Shigella* bacteria in which the gene encoding of O-antigen polymerase is disrupted	International Vaccine Institute, Seoul, Korea	X			
Ty21a typhoid vaccine expressing *Shigella* LPS *Salmonella typhi* Ty21a construct comprising a *Shigella sonnei* O-antigen biosynthetic gene region	Protein Potential LLC, Rockville, Maryland USA	X			
Inactivated trivalent *Shigella* whole cell Formalin inactivated *Shigella* whole cells	PATH, Washington DC and WRAIR, Silver Spring, Maryland USA		X		
Heat Killed Multi Serotype *Shigella* (HKMS) vaccine Heat killed preparation of 6 strains of Shigella that were subsequently combined to form an inactivated vaccine	NICED, Kolkata, India	X			
Glycoconjugate candidates Chemically prepared glycoconjugate O polysaccharide covalently linked to carrier protein	LDMI at the NICHHD, NIH, Bethesda, Maryland USA				X

Recombinant glycoconjugate O polysaccharide specific bioconjugate vaccine	Limmatech Biologics (LMTB) AG Schlieren, Switzerland			X	
Synthetic glycoconjugate use of synthetic oligosaccharides (OSs), acting as efficient functional SF2a O-SP mimics, as the haptens for a conjugate vaccine	Institute Pasteur, Paris, France	X			
Novel antigen candidates Invaplex$_{AR}$ 2nd generation macromolecular complex composed of *Shigella* LPS and the Type 3 secretions system proteins (IpaB, IpaC, and IpaD)	WRAIR, Silver Spring, Maryland USA	X			
GMMA Genetically derived outer membrane particles comprised of predicted *Shigella* outer membrane and periplasmic proteins without LPS using a novel protein vesicle technology	Sclavo Behring Vaccines Institute for Global Health S.r.l [A GSK company], Sienna, Italy		X		
OMV *Shigella* outer membrane vesicles encapsulated in nanoparticles	University of Navarra, Spain	X			
Subunit candidates DB Fusion Fusion protein of two Type III secretion system antigens, invasion plasmid antigens B (IpaB) and invasion plasmid antigen D (IpaD)	PATH, Washington, DC	X			
34 kDa OmpA Conserved and cross reactive major outer membrane protein (MOMP) of *Shigella flexneri* 2a	NICED, Kolkata, India	X			
Flexyn2a *Shigella flexneri* 2a O-Ag bioconjugate vaccine	LMTB, AG Schlieren, Switzerland		X		
***Shigella*4V** *Shigella flexneri* 2a, 3a, 6 and *S. sonnei* O-Ag bioconjugate vaccine	LMTB, AG Schlieren, Switzerland		X		

Highlighted grey is the vaccine construct under study. Data adopted from Marteyn *et al*., and WHO with slight modification (Marteyn, 2016; WHO, 2020).

2.6 Models for Evaluation of *Shigella* Vaccines

Assessment of *Shigella* vaccine candidates has been hampered by the lack of an ideal animal model for shigellosis. Several models have been utilized for the different vaccine candidates under development (Kim *et al*., 2016). Use of each model depends on the availability, ability to induce immune response that mimics the human immune response, ethical consideration, life span as well as expected reaction (Heine *et al*., 2014). Models such as non-human primate rhesus macaques, young domestic pigs, rabbits, mice/ mouse, guinea pigs and even young chicken (Marteyn, 2016) have been used. Of these models, the rhesus macaques mimics the *Shigella* human infection best however, there are very stringent ethical restriction in use of this animal model. The piglets have varied colonic destruction and guinea pigs are not efficiently infected so they are not widely used. Mice, although widely used, lack the IL-8 cytokine which is key in *Shigella* invasion. Rabbits have been widely used due to their robust immunological response following challenge with *Shigella* bioconjugate vaccine (regardless of the route of administration) though the ileum is the typical site of infection(Marteyn, 2016).

Several animal and human models have since been utilized in efforts to develop multi-serotype vaccines for appropriate interventions in endemic regions. Both infant and adult mice have been used to test several vaccines that are being developed against IpaB, IpaD, LPS and other virulence factors (Brotcke Zumsteg *et al*., 2014).

2.7 Current Vaccine Development Outcomes

The need for *Shigella* vaccine development was highlighted in the nineties when US soldiers deployed during the desert shield/ storm operations in Iraq and Chilean children developed diarrhea. When the deployed soldiers and Chilean children were studied, serotype specific anti-*Shigella* LPS and Ipa IgA and IgG antibodies were detected from 10-100% serum of *Shigella*

infected adults and children with 5.5% and 14% exhibiting IgA and IgG *Shigella* LPS seroconversion (Hyams *et al*., 1995).

Recent developments from two studies in the US (Kim *et al*., 2016; Riddle *et al*., 2016) indicate that a *Shigella* vaccine targeting the O-antigen of the four major *Shigella* serotypes (*S. flexneri* and *S. sonnei)* bio-conjugated to a protein carrier rEPA has been shown to be immunogenic. Furthermore, addition of an adjuvant increased the efficacy of the vaccine. Additionally, this bioconjugate vaccine has been tolerable among children under three years, which was a major unresolved challenge with other vaccine constructs (WHO, 2020). The phase 1/2a age descending bioconjugate vaccine trial is currently underway in Kenya. However, no vaccine has been widely approved hence the need for further investigation of the breath of host immunological response and the ability of the antibodies to confer broad protection against *Shigella* spp. serotypes (Levine *et al*., 2007; Mattock & Blocker, 2017). To this regard, it is imperative to evaluate the pre-clinical bioconjugate vaccinated rabbit serum against *Shigella* isolates from Kenya and in future the human serum from the ongoing *Shigella*4V clinical trial pending proof of concept in CHIM study.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Area

This retrospective laboratory-based study was conducted at the Microbiology Hub Kericho (MHK) located in Kericho (latitude: 0°22'2.3"S and longitude: 35°16'52.72"E), Kenya. The MHK has been conducting a study "surveillance of enteric pathogens causing diarrheal illnesses in Kenya" (protocol number SERU/SSC#1549/ WRAIR#1549) since 2009. Stool specimens from different geographical regions in Kenya are collected from humans, bacteria isolated including *Shigella* spp, identified to genus/species and stored for future testing. The *Shigella* isolates included in this study were from, Kericho, Kisumu, Kisii county and Kombewa sub-county hospitals. The LMTB and the Walter Reed Institute of Research (WRAIR), Department of Diarrheal Disease Research, bacterial diseases branch, Subunit Enteric Vaccines and Immunology (SEVI), in Maryland, USA has been conducting *Shigella* vaccine development studies using human and animal challenge models (including, rabbits, guinea pigs and mice). The most recent study evaluated the immunogenicity of the novel monovalent & 4V/4V-Adj O-antigen based bioconjugate vaccine using rabbit (New Zealand white) and guinea pig models. The four groups of *Shigella* isolates from Kenyan population archived at MHK and the rabbit serum before and after vaccination were appropriate for the reactivity and bactericidal activity assessed in this study.

3.2. Study Population: *Shigella* strains, serum samples and complement

This study used 134 archived *Shigella* strains that had been collected in a parent protocol from patients with diarrheal illnesses from different hospitals in Kenya, as described in section 3.1. The pooled serum samples from bioconjugate vaccine immunized rabbits used in the study were

obtained from LMTB, the vaccine manufacturer through WRAIR. Additionally *Shigella* positive control strains (*S. flexneri* 2a, 2457T, *S. flexneri* 3a, J17B, *S flexneri* 6, CCH060 and *S. sonnei*, Moseley) and Baby rabbit complement (BRC) (native and heat inactivated) were obtained from WRAIR. A Congo red negative *Shigella* spp isolate, K-Sspp-071 from MHK Biobank was used as negative control.

3.3. Sample Size Determination

i. *Shigella* Isolates From Stool Samples

The total number of different genus of bacterial isolates from stool samples in MHK biobank from the parent protocol was 425, out of which 134 were *Shigella* isolates. The *Shigella* samples for this study were purposively selected for this study. From the 134 *Shigella* spp selected, 5 did not grow on revival and therefore 129 were used for downstream analysis in this study. These isolates were assigned new codes containing the study, country, sequential numbers and the serotype (i.e SBA-K-Sf6-001) for this study.

ii. Serum Samples From vaccinated Rabbits

The rabbits were intra-muscularly immunized with either 1 µg monovalent or quadrivalent (combination of 1 µg each monovalent with and without adjuvant (alum)) bioconjugate vaccine on day, 14 and 28. Bleeding of the rabbits was done on day 0 (pre) before immunization and on day 42 (post III). Pools were prepared in order to obtain adequate amounts of the serum from each set of bioconjugate vaccinated rabbits then tested by ELISA and remainder stored for future testing. For this study, an aliquot of each monovalent and quadrivalent for a total of 12 Table 3.1pooled rabbit serum samples were shipped to MHK and analyzed against archived *Shigella* isolates from the aforementioned diarrheal study utilizing the colony spot blot and SBA.

Table 3.1. List of pooled pre and post III rabbit serum samples

Serum samples from Rabbits used in the study			
1	LMTB17_003_4V_pre	7	LMTB17_003_4V_Post III
2	LMTB17_003_4Vadj_pre	8	LMTB17_003_4Vadj_Post III
3	LMTB17_003_Sf3a_Pre	9	LMTB17_003_Sf3a_Post III
4	LMTB17_003_Sf2a_Pre	10	LMTB17_003_Sf2a_Post III
5	LMTB17_003_Sf6_Pre	11	LMTB17_003_Sf6-Post III
6	LMTB17_003_Ss_Pre	12	LMTB17_003_Ss_Post III

3.4. Serotyping of *Shigella* spp Isolates

A total of 129 *Shigella* isolates randomly selected from the MHK Biobank were revived by scrapping the surface of the frozen bacterial cells on MacConkey agar and incubated overnight. An isolated colony from each plate were sub-cultured on Trypticase soy blood agar (TSA) plates (Becton, Dickinson, USA), incubated overnight at 37°C and macroscopically checked for lawn culture and isolated colonies. The *Shigella* group and type verification was done by slide agglutination using commercial antisera set 2 (Denka Seiken Co. Ltd., Tokyo, Japan) as described in the manufacture's insert. Using a Pasteur pipette, a drop of normal saline was placed on the clean marked slide, a colony of the *Shigella* isolate under test picked using applicator stick, emulsified and checked for auto-agglutination, a drop of the specific antisera added and slide swirled for 1 minute. If agglutination was observed, it was marked as positive and if no agglutination was observed, negative.

3.5. Screening for *Shigella* O-antigen (LPS) Expressed on the Outer Surface of the Bacterial Cell Wall

A colony from the confirmed *Shigella* isolates were streaked on Congo Red (CR) solid media and incubated overnight for 16-18hrs at 39°C. The next day, the colonies that appeared red

(absorbed CR dye) were considered virulent from which, a subset was selected based on the serotype representation for further analysis. For the serotypes that were well represented (8 and above), every first, second, third or fifth isolate per serotype were selected. The *S. flexneri* serotypes from the non-formulation that were only 2 to 5 were all processed due to the few numbers that were available for the study.

3.6. Specific- & Cross-Reactivity of the Post-Vaccine Rabbit Serum to the Selected Virulent *Shigella* Isolates

Pre-labeled nitrocellulose membrane disks (BioRad, USA) were layered on TSA plates and inoculated with 2µl from 200µl sterile saline emulsified with CR+ *Shigella* isolate/ control strains (4-9 *Shigella* strains of same serotype and 2-5 controls per nitrocellulose disk). Following overnight incubation at 37°C, the membranes were transferred into a container with 2% casein on a mini-orbital shaker and blocked for 30 minutes at 23 ± 2°C. Each membrane was then transferred into square petri dishes (15x100mm) containing the respective quadrivalent and monovalent bioconjugate vaccinated rabbit serum (primary antibody) diluted 1:250 in 2% casein and incubated on an orbital shaker for 2 hours at room temperature (23 ± 2°C). The membranes were washed four times in Tris buffer, wash buffer x2 and Tris buffer on a shaker for 15 minutes intervals each. Then, membranes were transferred into square petri dishes with the secondary antibody, Protein A-Alkaline Phosphatase (Sigma Aldrich, USA) at 2µg/ml in 2% casein an orbital shaker for 1 hour. The membranes were, washed four times in Tris buffer, wash buffer x2 and Tris buffer on a shaker for 15 minutes intervals each and color developed in Fast Red/Naphthol AS-TR substrate (Sigma Aldrich, USA) solution in 1X Tris (Sigma Aldrich, USA)/ substrate buffer at pH 8.0 for 30 minutes. The Membranes were rinsed in deionized water and dried over night at room temperature. The spots with red color compared to the positive

control or darker than the negative control were considered positive while those with similar or pale color compared to the negative control were considered negative.

3.7. Functionality (bactericidal activity) of Serum Antibodies by Serum Bactericidal Assay (SBA)

3.7.1 Preparation of Frozen Bacterial Stocks

From the CR+ lawn culture, approximately (~)10 isolated colonies were transferred to a 15 ml polypropylene tube containing 3 ml of Luria Bertani (LB) broth incubated for 16-18 hours at 37°C, gently shaking. On the next day, the culture broth was diluted 1:100 in LB broth and incubated at 37°C, gently shaking, until the culture broth attained an OD600 of ~0.6-0.8. Using 50ml serological pipette, 12.5 ml the culture was transferred to a sterile 50 ml conical tube and centrifuged at 4,000 rpm (6,000 x g) for 10 minutes. The supernatant was discarded and bacterial pellets gently re-suspended, by quickly dragging the bottom of the tube back and forth along a microcentrifuge rack. Using a serological pipette, 12.5 ml of 35% sterile glycerol in LB broth was added to the bacteria and re-suspend, mixed well, 0.5 ml aliquoted in sterile cryovials (20 vials each isolate) and frozen at -80°C.

3.7.2 Determination of Optimal Dilution Factor, temperatures and time of the Bacterial Stocks

A frozen vial of bacteria stock was thawed at room temperature, 500 µl of assay buffer added and centrifuged at 12,000 x g for 2 minutes in an eppendorf tube. The supernatant was discarded, 1 ml assay buffer added and bacterial pallet washed in by centrifugation at 12,000 x g for 2 minutes, supernatant discarded, pellet re-suspended in 1 ml assay buffer and diluted 1:1000 in assay buffer. In well 1A of a 96 well plate, 60 µl of diluted bacteria was dispensed, 30 µl of assay buffer added to wells 1B-1H and 20 µl of assay buffer added to all wells in columns 2 and

3. A 2-fold dilution of the bacterial stock was done by pipetting 30 µl of bacteria from well 1A, mixing with well 1B, then 30 µl transferred from well 1B to 1C and so on down the plate. The final 30 µl from well 1H was discarded. Using a multichannel pipette, 10 µl was transferred from column 1 to columns 2 and 3. Using a pipette, 50 µl of HI BRC and 50 µl of native BRC added to all wells in columns 2 and 3 respectively and plate briefly mix, at 12,000 rpm for 10-15 seconds on a mini-orbital shaker. The assay plate was incubated at 37°C for 2 hours (without shaking) while LB plates (2 per 96 well assay plate) was dried in a biosafety cabinet for 30 mins to 1 hour. After the 2 hour incubation, assay plate was incubated on ice for 10-20 mins, briefly mixed by shaking at 12,000 rpm for 10-15 seconds. Using a multichannel pipette 10 µl from all wells in column 2 was transferred to the top of the LB plate, immediately tilted and the spots allowed to run for ~1.5-2 cm. This procedure was repeated for column 3, spotting these wells below the previous row on the LB plate, incubated at room temperature until absorbed into the agar and incubated upside down at 29^{o}C for *S. flexneri* 2a, 3a and 6 and 26^{o}C for *S. sonnei* for 12 to 16 hours. From well E3, 10 µl of bacterial suspension was streaked onto a Congo red plate and incubated overnight to check for retention of virulence. After 12-16 hour incubation and the dilution of bacteria from the active complement group (column 3) that yields between 80-120 CUF was determined. The individual dilutions were used for all vials in each lot of the *Shigella* serotype bacterial stock.

3.7.3 Assessment of Bactericidal Activity

The bactericidal activity of vaccinated rabbit serum against the selected *Shigella* isolates were assessed as previously described (Nahm *et al*., 2018; Weerts *et al*., 2019). Serum samples from rabbits immunized with quadrivalent (4V and 4V-Adj) and monovalent bioconjugate vaccines were diluted 1:10 in assay buffer and heat inactivated at 56°C for 30 mins. Using a pipette, 20 µl

of assay buffer was added to columns 1 through 12 of rows A through H of a 96 well round-bottom tissue culture plate. To row A of the 96-well, 30 µl of each test sample added, in duplicate i.e. test sample 1 to columns 3 and 4, followed by the rest of the samples and serially diluted 3-fold through to raw H. Target bacterial stock was thawed at room temperature, 500ul of assay buffer added, pelleted by centrifuging at 12000xg for 2 mins, supernatant discarded, pellet re-suspended in 1 ml assay buffer and diluted as per the optimal dilution. In the Hi complement control wells (column 1), 50ul of 20% heat-inactivated BRC was added and 50ul of 20% native BRC to complement control and test sample wells (column 2-12), mixed on a shaker at 700 rotations per minute (rpm) for 10-15 seconds. The assay plate was incubated at 37°C for 2 hours and reaction stopped by incubating plate on ice for 10-20 minutes. The plate was mixed on a shaker at 700rpm for 10-15 seconds and using a multichannel pipette, raw A mixed and 10ul transferred to the top of dry LB plate. The plate was tilted for the spots to run for ~1.5-2 cm and repeated for rows B to D on same plate, E to H on a second plate and incubated at room temperature for 10-15 mins. The inoculated LBA plates were incubated overnight at 26°C and 29°C. After overnight incubation, 25ml of pre-heated overlay agar was added to each plate and incubated at room temperature for 2 hours to allow surviving bacteria to develop red color. The plate was imaged and the surviving colonies enumerated using NIST's integrated colony enumerator (NICE) software and bactericidal titer calculated using average CFU/spot count of the two duplicate wells.

3.8. Data analysis

i. **Specific and Cross Reactivity (Colony spot blot)**: The qualitative data from the individual pre- and post- immunization bioconjugate vaccinated rabbit serum reactivity

(serotype specific and cross) was assessed and reported as positive (+) if red or negative (-) no color/pale/same as the negative control.

ii. **Functionality (bactericidal activity) of Serum by SBA**: The excel based NICE software was used to enumerate the colonies (Appendix 1: Sample of enumerated SBA plate) and 50% killing Index calculated using the Opsotiter software (University at Alabama at Birmingham) (Moon H. Nahm, 2018) and log transformed into interpolated titers. The fold rise in interpolated titers between pre- and post- immunized rabbit serum was calculated and Rabbit serum antibodies were considered to have bactericidal activity if they yielded ≥4-fold rise between the pre- and post- immunized sera with the *Shigella* serotypes tested.

Descriptive statistics such as percentages and fold increase were used to report the data generated and presented in tables.

3.9. Ethical Considerations

This was a retrospective laboratory-based study nested within an ongoing parent diarrheal surveillance protocol (KEMRI Scientific Steering Committees (SSC) #1549/WRAIR#1549) in Kenya since 2009. Permission to use the pooled serum samples was granted by LimmaTech Biologics AG through the WRAIR-SEVI department. This study was approved by the School of Graduate Studies (SGS) of Maseno University (Appendix 2: Approval School of Graduate Studies, Maseno University), for one year renewable by the institutional review board (IRBs) of KEMRI (SSC# 3900) and given an exemption determination by WRAIR as non-human research (Appendix 2: Approval School of Graduate Studies, Maseno University

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES

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Date: 20th September, 2018

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR ELIZABETH ODUNDO— MSC/PH/00022/2014

The above named is registered in the Master of Science Programme in the School of Public Health and Community Development, Maseno University. This is to confirm that her research proposal titled "Analysis of Reactivity and Functionality of Serum Antibodies from Multivalent Shigella Bioconjugate Vaccinated Rabbits and Guinea Pigs against Shigella Isolates from Kenya" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

20 SEP 2018

Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

Appendix 3: KEMRI/SERU/CCR/125/3900 protocol approval letter). To maintain confidentiality of the participants from whom *Shigella* isolates were obtained, the isolates were coded with unique identifiers and the investigator did not have access to participant information or demographics from the parent surveillance protocol.

The re-coding of the isolates was done by assigning new study numbers aforementioned in section 3.2 (i) and the original logs securely kept by and accessible to the quality assurance officers at MHK therefore, assuring confidentiality of participants from the parent protocol.

CHAPTER FOUR

RESULTS

4.1. Serotyping Of The *Shigella* Isolates

In order to achieve the overall objective of this project was to determine if immunization with a *Shigella* quadrivalent bioconjugate vaccine induced antibodies reactive against endemic *Shigella* isolates from Kenya, the site of a phase 1/2a *Shigella* 4V vaccine study currently underway. The first stage of the project was to identify and verify *Shigella* isolates from the biobank within the MHK laboratory with the intent to select serotypes that were contained within the quadrivalent vaccine formulation as well as *Shigella* serotypes not contained within the vaccine formulation. A total of 129 *Shigella* (S) isolates were sub-cultured on TSA plates and serotyped (Figure 4.1); *S. flexneri* (n=**70, 55%**), *S. sonnei* (n=**13, 10%**), *S. dysenteriae* (n=**14, 11%**), *S. boydii* (n=**12, 9%).** Of the isolates confirmed, 20 isolates (16%) were *S. sonnei* form II (n=**13, 65%**) and *Shigella* spp (n=**7, 35%**) not typable by slide method using the commercially available antisera.

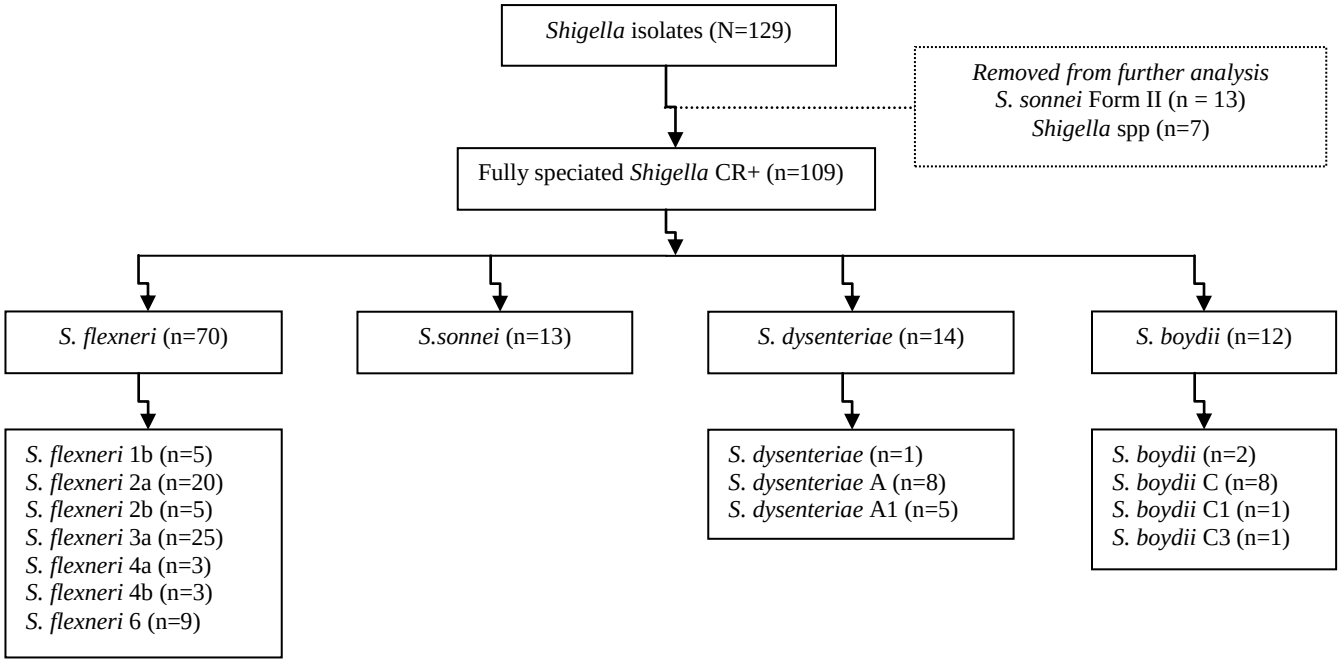


Figure 4.1. Flow chart of the distribution of *Shigella* serotypes.
Serotyping done by slide agglutination using set 2 Denka Seiken antisera containing 19 group and type antisera; 10 *S. flexneri* (group B), 3 *S.sonnei* (group D), 4 *S. boydii* (group C), 2 *S. dysenteriae* (group A).

4.2. Detection Of *Shigella* O-Antigen Expressed On The Outer Surface Of The Bacterial Cell Wall By Congo Red

Of the 129 isolates, 85% screened positive for Congo red uptake (CR+) distributed as follows; 100% *S. boydii* and *S. dysenteriae*, 96% *S. flexneri* and 42% *S. sonnei*. Most of the *S. sonnei* (58%) were form II isolates and Congo red negative indicating that they did not retain their virulence and were removed from the study (Plate 4.1).

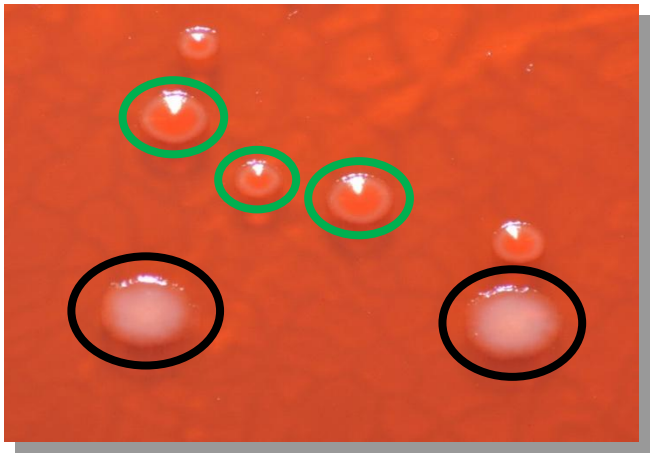


Plate 4.1: Congo Red Plate Showing CR Positive And Negative Colonies
Green circles (red colonies) positive (retained virulence) and black circles (pale colonies) Negative (did not retain virulence).

The *Shigella* serotypes selected for ensuing analysis by colony spot blots were 41 [22 (54%) and 19 (46%)] and serum bactericidal assays were 15 [10 (67%) and 5 (33%)] within formulation and non-formulation respectively (Table 4.1).

4.3. Specific and Cross Reactivity of Rabbit Serum Antibodies Against the *Shigella* Isolates

The first objective of this study was to determine the ability of antibodies, induced after immunization of rabbits with either monovalent or quadrivalent *Shigella* bioconjugate vaccines, to bind to the *Shigella* i*solates* identified and characterized as outlined above. Paired pooled rabbit serum samples collected from pre- and post-immunization were utilized in colony blot

assays to assess whether antibodies were able to bind to surface expressed antigens forming an antigen-antibody complex visualized by staining.

Table 4.1.: *Shigella* Spp Selected For Analysis By Colony Blot And Serum Bactericidal Assay.

***Shigella* serotype**		**Isolate Abbreviation**	**No. per serotype**	**CR+ No. for colony blots**	**No. for SBA**
***Shigella* serotypes contained within the vaccine formulation**	*Shigella flex* 2a	S.f2a	20	4	3
	Shigella flex 3a	S.f3a	25	10	4
	Shigella flex 6	S.f6	9	4	2
	Shigella sonnei	S.s-I	13	4	2
	Sub-Total		**67**	**22**	**11**
***Shigella* serotypes not within the vaccine formulation**	*Shigella dysenteriae*	S.d	14	2	0
	Shigella boydii	S.b	12	3	0
	Shigella flex 1b	S.f1b	5	5	0
	Shigella flex 2b	S.f2b	5	4	2
	Shigella flex 4a	S.f4a	3	2	2
	Shigella flex 4b	S.f4b	3	3	1
	Sub-Total		**42**	**19**	**5**
	Total		**109 (100%)**	**42 (37.6%)**	**16 (13.7)**
Out of the 109 CR+ *Shigella* serotypes, 38% were subjected to assess specific (22) and cross (19) reactivity by colony blot and a subset of 14% for functionality by SBA.					

All five *Shigella* control strains were incorporated in the respective colony blot runs. The positive control strains were reactive with the post III vaccination rabbit serum samples tested while the negative control was non-reactive. As expected, there was also no detectable reaction observed with all of the pre- vaccination rabbit serum (Figure 4.2).

Figure 4.2: Sample Colony Spot Blots From The Study.
The A and B are from Post III and Pre runs for Sf 2a rabbit serum; Green-Positive isolates; Red-Positive control; Black-Negative control

4.3.1 Specific And Cross Reactivity of *Shigella* Isolates Within Formulation

Serum antibodies from rabbits immunized with the quadrivalent bioconjugate vaccine formulated with alum (4V-Adj) were reactive in colony blots with the 22 *Shigella* isolates from the *Shigella* serotypes contained within the vaccine formulations. In contrast, serum antibodies after immunization with the quadrivalent vaccine delivered without alum (4V) reacted with all of the *S. flexneri* 3a and *S. sonnei* isolates, but only 50% *of S. flexneri* 2a isolates and none of *S. flexneri* 6 strains. The was observed reactivity from serum samples from monovalent bioconjugate immunized rabbits with the specific *Shigella* serotypes in line with the O-antigen used in the vaccine formulation (Sf 2a, 3a, 6, *S. sonnei*). However, in this study, there was also some cross reactivity observed between serum from monovalent bioconjugate vaccinated rabbits of *S. flexneri* 2a with one *S. flexneri* 3a isolate, of *S. flexneri* 3a with one *S. flexneri* 2a isolate and from *S. flexneri* 6 with one *S. flexneri* 2a isolate. No cross reactivity was observed *neither S. sonnei* nor the Sf 6 isolates with other monovalent rabbit serum assayed in this study (Table 4.2.).

Table 4.2.: Specific/Cross Reactivity Of Rabbit Serum With *Shigella* Isolates Contained Within Formulation

***Shigella* serotype**	**Isolate ID**	**Post immunization Rabbit serum**					
		Monovalent				**Quadrivalent**	
		***S. flexneri* 2a**	***S. flexneri* 3a**	***S. flexneri* 6**	***S. sonnei***	**4V**	**4V-Adj**
S. flexneri 2a	SBA-K-S.f2a-003	+	-	-	-	-	+
	SBA-K-S.f2a-049[p]	+	+	-	-	+	+
	SBA-K-S.f2a-100[p]	+	-	-	-	-	+
	SBA-K-S.f2a-072	+	-	+	-	+	+
S. flexneri 3a	SBA-K-S.f3a-085[p]	-	+	-	-	+	+
	SBA-K-S.f3a-089[p]	-	+	-	-	+	+
	SBA-K-S.f3a-093	-	+	-	-	+	+
	SBA-K-S.f3a-098	-	+	-	-	+	+
	SBA-K-S.f3a-047	-	+	-	-	+	+
	SBA-K-S.f3a-001[p]	+	+	-	-	+	+
	SBA-K-S.f3a-040	-	+	-	-	+	+
	SBA-K-S.f3a-044[p]	-	+	-	-	+	+
	SBA-K-S.f3a-047	-	+	-	-	+	+
	SBA-K-S.f3a-111	-	+	-	-	+	+
S. flexneri 6	SBA-K-S.f6-008	-	-	+	-	-	+
	SBA-K-S.f6-050[p]	-	-	+	-	-	+
	SBA-K-S.f6-092	-	-	+	-	-	+
	SBA-K-S.f6-124[p]	-	-	+	-	-	+
S. sonnei	SBA-K-S.s1-105[p]	-	-	-	+	+	+
	SBA-K-S.s1-107[p]	-	-	-	+	+	+
Assay Controls							
Positive	*S. flexneri* 2a,2457T	+	-	-	-	+	+
	S. flexneri 3a, J17B	-	+	-	-	+	+
	S. flexneri 6, CCH060	-	-	+	-	+	+
	S. sonnei, Moseley	-	-	-	+	+	+
Negative	K-Sspp-071	-	-	-	-	-	-

A) Key: [p] = Selected for SBA, (-) = Negative, (+) = Positive, K-Sspp-071= Negative control. B) Only serotype specific control strains were included with monovalent while all four positive controls were included with quadrivalent bioconjugate serum tested. All serotypes within formulation reacted with specific monovalent and 4V-Adj while for 4V, 50% of Sf2a and none of Sf6 reacted.

4.3.2. Cross Reactivity Of The Post III Serum With *Shigella* Isolates Not Within Formulation: Other than the four serotypes contained within the vaccine formulations, reactivity of rabbit serum after immunization with either monovalent or quadrivalent *Shigella* bioconjugate vaccines were also tested against other *Shigella* isolates. Cross reactivity was observed with seven (37%) out of the nineteen *Shigella* isolates tested. Serum from rabbits immunized with 4V-Adj reacted with three of the four (75%) *S. flexneri* 2b isolates, two (100%) *S. flexneri* 4a isolates and one isolate from both *S. flexneri* 1b (20%) and (25%) *S. flexneri* 4b. Interestingly, animals immunized with the *S. flexneri* 2a monovalent bioconjugate had antibodies that cross-reacted with three (75%) *S. flexneri* 2b in the colony blot assays. No cross reactivity was observed between serum from monovalent *S. flexneri* 3a, *S. flexneri* 6 and *S. sonnei* bioconjugate immunized rabbits and *Shigella* serotypes not contained within the vaccine formulations (Table 4.3).

The results of the colony blot reactivity were compared to the positive and negative control colors developed and reported as positive. The qualitative data from the individual pre and post III monovalent and quadrivalent bioconjugate vaccinated rabbit serum reactivity could not be reported as high, medium or low. This is because no standard was available for incorporation as control hence no comparative standard to be used for comparison of the color intensities. The comparison of the spot blot color intensities against the positive and negative controls in each run was sufficient to obtain the positive and negative colony blot results.

Table 4.3.: Cross Reactivity Of Rabbit Serum With *Shigella* Isolates Not Contained Within Formulation

***Shigella* serotype**	**Isolate ID**	**Post immunization Rabbit serum**					
		Monovalent				**Quadrivalent**	
		***S. flexneri* 2a**	***S. flexneri* 3a**	***S. flexneri* 6**	***S. sonnei***	**4V**	**4V-Adj**
S. boydii	SBA-K-S.bC3-046	-	-	-	-	-	-
	SBA-K-S.bC-065	-	-	-	-	-	-
	SBA-K-S.bC-095	-	-	-	-	-	-
S. dysenteriae	SBA-K-S.dA-012	-	-	-	-	-	-
	SBA-K-S.dA1-041	-	-	-	-	-	-
S. flexneri 2b	SBA-K-S.f2b-066	-	-	-	-	-	-
	SBA-K-S.f2b-131 [p]	+	-	-	-	-	+
	SBA-K-S.f2b-132[p]	+	-	-	-	-	+
	SBA-K-S.f2b-133[p]	+	-	-	-	-	+
S. flexneri 1b	SBA-K-S.f1b-021 [b]	-	-	-	-	+	+
	SBA-K-S.f1b -025	-	-	-	-	-	-
	SBA-K-S.f1b -042	-	-	-	-	-	-
	SBA-K-S.f1b -102	-	-	-	-	-	-
	SBA-K-S.f1b -103	-	-	-	-	-	-
S. flexneri 4a	SBA-K-S.f4a -032 [p]	-	-	-	-	-	+
	SBA-K-S.f4a-039[p]	-	-	-	-	-	+
S. flexneri 4b	SBA-K-S.f4b-068 [p]	-	-	-	-	-	+
	SBA-K-S.f4b-090	-	-	-	-	-	-
	SBA-K-S.f4b-115	-	-	-	-	-	-
Assay Controls							
Positive	*S. flexneri* 2a,2457T	+	-	-	-	+	+
	S. flexneri 3a, J17B	-	+	-	-	+	+
	S. flexneri 6, CCH060	-	-	+	-	+	+
	S. sonnei, Moseley	-	-	-	+	+	+
Negative	K-Sspp-071	-	-	-	-	-	-

A) Key: [p] = Selected for SBA, [b]= Not analyzed further. Additional trouble shooting with the isolate is still ongoing. Isolate will be included in separate study, (-) = Negative, (+) = Positive, K-Sspp-071= Negative control. B) Only serotype specific control strains were included with monovalent while all four positive controls were included with quadrivalent bioconjugate serum tested.

4.4. Functional Activity Of Serum Antibodies Induced After Immunization With Monovalent Or Quadrivalent *Shigella* Bioconjugate Vaccines

The final objective of this study was to determine if the antibodies from rabbits immunized with the *Shigella* bioconjugate vaccines that bound in the *Shigella* isolates in the reactivity (colony blot assays) possessed functional activity. To assess one parameter of functionality, rabbit serum samples reactive in the colony blots were assessed for bactericidal activity. All *Shigella* isolates tested in the SBA showed optimal growth after 16-18hr incubation at 29˚C and at 26˚C for *S. sonnei*, resulting in microcolony growth sufficient to be detected. Growth conditions were similar to historical *Shigella* strains (*S. flexneri* 2a, 2457T, *S. sonnei*, 53G and Moseley, *S. flexneri* 6, CCHO60, and *S. flexneri* 3a, J17B) as previously reported with the modification for *S. flexneri* 6, CCHO60, which was incubated at 29˚C instead of 26˚C. The 50% cutoff value established for the assays were consistent with previous results (Weerts et al., 2019).

Consistent with the colony blot results with the pre-immunization serum pools being negative, the bactericidal activity was also low to undetectable in these pooled samples from all the rabbits against all tested *Shigella* serotypes. Serum from rabbits immunized with monovalent *S. flexneri* 6 bioconjugate had bactericidal activity against *S. flexneri* 6 strains used in the study, but no cross-reactivity with other serotypes. Similar results were achieved after immunization of rabbits with monovalent *S. sonnei* bioconjugate. Interestingly, serum from rabbits immunized with monovalent *S. flexneri* 3a bioconjugate had bactericidal activity against one of the two *S. flexneri* 2a isolates, in addition to all four of the S. *flexneri* 3a strains. Serum from rabbits immunized with monovalent *S. flexneri* 2a bioconjugate was capable of killing the two *S. flexneri* 2a strains and one of the *S. flexneri* 3a isolates, albeit at much lower levels. Additionally, monovalent rabbit serum from *S. flexneri* 6 had bactericidal acidity against one *S. flexneri* 2a.

Serum from rabbits immunized with the quadrivalent *Shigella* bioconjugate (4V) had bactericidal activity against two of the *S. flexneri* 2a strains, two of the *S. sonnei* isolates and all four of the S. *flexneri* 3a isolates but none of the *S. flexneri* 6 isolates. In stark contrast, serum from rabbits immunized with 4V-Adj had bactericidal activity against all sixteen *Shigella* isolates representing the four *Shigella* serotypes contained within the vaccine formulation. The interpolated titers of the quadrivalent and monovalent anti-sera varied among the specific serotypes and between the same serotypes. The highest bactericidal titers were attained by *S. sonnei* while the lowest were by *S. flexneri* 6 monovalent sera.

Interestingly, serum from rabbits immunized with monovalent *S. flexneri* 2a monovalent bioconjugate had bactericidal activity against the two *S. flexneri* 2b strains tested comparable to *S. flexneri* 2a strain. Similarly, the serum from rabbits immunized with 4V-Adj had notable bactericidal activity with the two *S. flexneri* 2b isolates, two *S. flexneri* 4a isolates, and a *S. flexneri* 4b isolate. The interpolated (bactericidal) titers of the quadrivalent and monovalent anti-sera varied among the specific serotypes and not between the same serotypes. The bactericidal titers attained from the monovalent and 4V-Adj bioconjugate serum were identical between the two *S. flexneri* 2b tested. However, the titers from 4V-Adj bioconjugate with *S. flexneri* 4a strains varied among the two isolates tested.

Furthermore, there was observable fold increase in bactericidal titers between the pre and post III bioconjugate vaccinated rabbit serum antibodies against the *Shigella* strains tested.

Table 4.4: Bactericidal activity of rabbit serum antibodies against selected *Shigella* isolates

Shigella serotype		Isolate ID	Fold increase in antibodies induced in rabbits after immunization					
			Monovalent				Quadrivalent	
			S. flexneri 2a	*S. flexneri* 3a	*S. flexneri* 6	*S.sonnei*	4V	4V-Adj
Contained within the vaccine construct	*S. flexneri* 2a	SBA-K-S.f2a-049	70	19	—		15	12
		SBA-K-S.f2a-100	111	—			—	24
		SBA-K-S.f2a-072	100		36	—	9	9
	S. flexneri 6	SBA-K-S.f6-050	—		46	—		70
		SBA-K-S.f6-124			54			113
	S. flexneri 3a	SBA-K-S.f3a-085	—	40	—		54	20
		SBA-K-S.f3a-089		98			37	42
		SBA-K-S.f3a-044		49			34	34
		SBA-K-S.f3a-001	NR	7			4	32
	S. sonnei	SBA-K-S.s1-105	—			746	68	274
		SBA-K-S.s1-107				663	74	68
Not within the vaccine construct	*S. flexneri* 2b	SBA-K-S.f2b-131	67	—				41
		SBA-K-S.f2b-132	62					25
	S. flexneri 4a	SBA-K-S.f4a-032	—					7
		SBA-K-S.f4a-039						7
	S. flexneri 4b	SBA-K-S.f4b-068						9

Fold increase calculated by dividing the post-immunization (post III-day 42 pooled rabbit serum) bacterial titer by the pre-immunization (pre- day 0 pooled rabbit serum) bacterial titer, a fold rise of ≥4 considered as responders; < 4-fold increase = non-responder (NR); (—) = Not tested for bactericidal activity fold rise tires; tire 4 (>201), tire 3 (61-200), tire 2 (31-60) and tire 1 (4-30).

CHAPTER FIVE
DISCUSSION

5.1 Discussion

Shigella continues to be one of the leading global causes of diarrheal morbidity and mortality, a serious public health problem particularly in children within resource limited settings (Shimanovich *et al*., 2017). Depending on the *Shigella* species associated with the infections coupled with multidrug resistance, the disease can be severe resulting in long-standing sequelae such as toxic megacolon, irritable bowel syndrome, haemolytic uremic syndrome, reactive arthritis and stunted growth in children amid a myriad of other diarrheal symptoms aforementioned (MacLennan *et al*., 2019; Mattock & Blocker, 2017). It was therefore important in this study to identify the circulating serotypes that would form part of the targeted *Shigella* serotypes for vaccine development.

Of the 109 serotypes in this study, 62% were *S. flexneri* 2a, 3a,6 and *S. sonnei* serotypes similar to those from the Global Enteric Multicenter Study (GEMs, Kenya incorporated) with 63% of the *Shigella* species; *S. flexneri* 3a, 2a, 6 and *S. sonnei*, as the most predominant. These serotypes are responsible for approximately 80% of *Shigella* diarrheal infections globally, and recommended for quadrivalent *Shigella* vaccine WHO (Livio *et al*., 2014; Riddle *et al*., 2016). Other *Shigella* serotypes characterized in this study were; 11% *S. dysenteriae* implicated in epidemics/ outbreaks, 9% *S. boydii*, 7% *S. flexneri* 1b (possible for inclusion in multivalent vaccine), 7% *S. flexneri* 2b, 4% *S. flexneri* 4a these were similar to those characterized in other studies (Livio *et al*., 2014; Nyanga *et al*., 2017). Additionally, 4% *S. flexneri* 4b, were identified in this study that were not identified in the GEMs study for Kenya while *S. flexneri* 7a was identified in Kenya before (Livio *et al*., 2014), but not identified in this study. The serotypes not

identified in this study could be attributed to the limitation of the Denka Seiken *Shigella* set 2 antisera that identifies some serotypes but also possibly missed because, not all available archived *Shigella* isolates from the biobank were tested in this study. However, the isolates tested here cover a broader geographical region and age groups within Kenya as per the parent surveillance protocol as compared to GEMs, Obiero and Nyaga *et al*., studies (Livio *et al*., 2014; Nyanga *et al*., 2017; Obiero *et al*., 2017). The serotypes used in this study provide a broader distribution of *Shigella* isolates, which is considered one key factor in strengthening vaccine development strategies. This is important given the need to evaluate the efficacy of existing multivalent vaccines not only in adults but also in children in Kenya where shigellosis is endemic with diverse range of strain types and serotypes (Obiero *et al*., 2017).

The majority of *Shigella* isolates in this study retained their virulence genes with the exception of *S. sonnei* where ~58% were CR negative, supporting the findings of a study by Szujarto (Dr Valéria Szijártó, 2013). The LPS biosynthesis genes are uniquely located, in part, on the virulence plasmid of S. sonnei and therefore loss of the plasmid results in a conversion from Form I (smooth, round colonies) to Phase II (rough, large colonies) phenotype. The lack of *S. sonnei* phase II to retain virulence genes could be correlated with more self-limiting disease (Torraca *et al*., 2020) and therefore, not targeted in ongoing vaccine development strategies. This also formed the basis for not proceeding with *S. sonnei* Phase II form isolates for the ensuing assays in this study.

The specific anti *S. flexneri* 2a antibody responses observed in the phase 1 study with the monovalent bioconjugate (Chen & Kotloff, 2016; Riddle *et al*., 2016) agree with the results of all rabbit serum following serotype specific monovalent bioconjugate vaccination against the target *Shigella* strains; Sf 2a, 3a, 6 and *S. sonnei* in this study. This is indicative that, the antibodies

produced following immunization with the monovalent were specific and support the findings from desert shield/ storm operations and Kenyan adults studies on seroconversion due to exposure or infection with *Shigella* spp (Hyams *et al*., 1995; Obiero *et al*., 2017). The cross reactivity observed between some of the vaccine targeted serotypes Sf2a and Sf3a strains with Sf3a and Sf2a monovalent vaccinated rabbit serum could be due to the serogroups O-antigen factors (6, 7:8, 9) shared by the two serotypes (Hlozek *et al*., 2020). In this *in vitro* study, cross reactivity of Sf6 monovalent with one of the Sf2a isolate points to the possibility of the Sf6 monovalent could induce cross reactive antibodies. Although only one of the Sf2a isolates cross reacted, the results provides valuable information following adding to the results from initial Sf6 conjugate efficacy study by Farzam *et al*., and basis for further studies evaluating the Sf6 monovalent bioconjugate human serum with Sf2a serotypes from Kenya (Farzam *et al*., 2017).

In this study post quadrivalent bioconjugate vaccinated rabbit serum had specific reactivity with 86% (4V) and 100% (4V-Adj) with the four *Shigella* isolates targeted by the formulation. This demonstrates that the multivalent formulation was immunogenic and support the assumptions made by the GEMs, Riddle *et al* studies, and recommendation by WHO on a broadly reactive *Shigella* vaccine (Livio *et al*., 2014; WHO, 2020). Therefore, this study results show that, the post vaccinated rabbit serum from the LMTB quadrivalent bioconjugate vaccine (*Shigella*4V) construct targeting Sf 2a, 3a, 6 and *S. sonnei* which is now being evaluated in a Phase 1/2a age-descending study in Kenya confers specific protective ability against the four important *Shigella* serotypes. However, the 16% percent that include all Sf6 and 50% Sf2a that were not reactive with 4V in this study will need to be assessed.

The cross reactivity observed from serum collected from rabbits immunized with the *S. flexneri* 2a monovalent vaccine with *S. flexneri* 2b support the suggestion that could this could be due to

both chemical and conformational epitopes similar to Sf2a due to the O-factor 9 (Hlozek *et al*., 2020). Cross protection among *Shigella flexneri* serotypes has been explored previously (Noriega *et al*., 1999) in the context of animal studies and clinical evaluations. Emphasis has been placed on the ability of a vaccine to confer a significant degree of protection to most common *Shigella* serotypes especially those with shared antigenic structures (group B; 3/4, 6, 7(8)) (Noriega *et al*., 1999). No cross reactivity of serum from *S. sonnei* (group B) monovalent vaccines with other isolates tested in this study due to the structural difference not only between the two groups B and *Shigella flexneri* (group D) but also between and the other two groups of *Shigella* A and C aforementioned (Knirel *et al*., 2015).

Interestingly, immunization with the 4V-adj and 4V bioconjugate serum showed broader cross reactivity with the prevalent serotypes (*S. flexneri* 2a, 3a, 6 and *S. sonnei*) as well as with additional serotypes within group B (*S. flexneri* 1b, 2b, 4a, 4b). This is similar to results from guinea pigs immunized with a bivalent *S. flexneri* 2a/3a vaccine which significantly protecting the vaccinated guinea pigs against challenge with *S. flexneri* Y, 1b, 2b and 5b but offered nominal protection against *S. flexneri* 1a, 4b and 6 (Noriega *et al*., 1999; Van de Verg & Venkatesan, 2014). These finding support the assumption that a higher degree of cross protection can be achieved by combining *Shigella* antigenic and group factors within a vaccine (Van de Verg & Venkatesan, 2014).

An exciting outcome this study was the immune responses generated after immunizing the rabbits with the 4V-Adj (vaccine delivered with alum) that resulted in increased breadth of the immune responses to four other *Shigella* serotypes not specifically targeted by the vaccine. Although the mechanisms of action afforded by vaccine adjuvants are largely unknown, the effects of adjuvants on vaccine-induced immune responses can be multifactorial. For example,

inclusion of an adjuvant (such as alum) to a vaccine formulation can augment the magnitude of the immune response, redirect the phenotype of the immune response or expand the breath of immune response as shown with proteins (HogenEsch *et al*., 2018; Turbyfill *et al*., 2018). The addition of an alum to the *Shigella*4V bioconjugate vaccine formulation increased the breath of the immune response cross reactivity in the colony blot and SBA assays (Tables 4.2-4.4).Therefore, adjuvants may be useful in vaccine development to reduce the amount of vaccine required to reach an immunological threshold, either in terms of vaccine dose amount or the number of vaccinations (dose-sparing) or to enhance the immunogenicity of a vaccine in populations where immune responses are not as robust, such as in small children or infants (HogenEsch *et al*., 2018; WHO, 2020). Previous clinical studies with a monovalent Flexyn2a bioconjugate (Riddle *et al*., 2016) did not detect an increase in the magnitude or change in the phenotype of the immune response induced but the study did not directly measure any changes in the breadth of the immune response in terms of cross-reactivity with other *Shigella* serotypes. However, these studies were conducted in adult populations where the adjuvant effect may be limited as compared to infant populations. Archived samples from those studies could be utilized to investigate these hypotheses using a similar study structure as implemented with the rabbit serum. Nevertheless, results from this study clearly demonstrate an added advantage of alum to the quality of the immune response induced after immunization with the quadrivalent formulation and suggests the vaccine could protect against more than the four *Shigella* serotypes targeted by the vaccine.

There was ≥4-fold increase of in the bactericidal titers (Weerts et al., 2019) for all the rabbit serum with target *Shigella* serotypes indicative that there was detectable vaccine induced immunological rabbit responses. The results of functionality of the vaccinated rabbit serum with

the *Shigella* serotypes in this study correlate with antibody titers determined by ELISA conducted on the same sample set and generally align with results after immunization with the Flexyn2a vaccine (Riddle *et al*., 2016; Talaat *et al*., 2017). Furthermore, all the non-targeted cross-reactive *Shigella* serotypes had >4-fold increase in bactericidal titers with the 4V-Adj formulation therefore reinforcing the need for addition of an adjuvant to vaccine formulation as indicated by Turbyfill *et al*., 2018. The combination of binding in colony blot format and the bactericidal activity associated with immunization of rabbits with the 4V-adj formulation holds the promise of a vaccine with a broader coverage than the four *Shigella* serotypes targeted by the vaccine.

If clinical evaluations, which are underway in Kenya, also demonstrate a more broadly reactive immune response, the *Shigella* bioconjugate may offer a promising solution to the morbidity and mortality associated with *Shigella* infections. As vaccine development efforts progress, the ability of multivalent *Shigella* vaccines to elicit broadly cross-reactive immunity should be explored, especially to *Shigella* serotypes of global predominance.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

This study was undertaken to evaluate the breath of reactivity and functionality of antibodies from monovalent ad multivalent *Shigella* bioconjugate vaccinated rabbit serum against *Shigella* isolates from a low and middle income country (Kenya) where the *Shigella*4V would be usable. These results show that the monovalent and the *Shigella*4V had specific, cross reactivity and bactericidal activities indicative that, the vaccine contract cannot only bind to the antigen but also activate the complement classical pathway leading to bacterial cell death. The breath of the *Shigella*4V illustrated here lays a foundation for possible CHIM studies here in Kenya as a proof of concept for the vaccine construct following success with the ongoing phase 1/ 2a immunogenicity studies.

6.2 Conclusions

This study demonstrates that,

ii. Specificity of the quadrivalent cover the four important implicated in 80% of *Shigella* diarrheal infections globally (Sf2a, 3a, 6 and *S. sonnei*) and additional predominant *Shigella* serotypes (Sf2b, 1b, 4a, and 4b).

iii. Cross reactivity and bactericidal activities support the assumption made that a higher degree of cross protection can be achieved by combining *Shigella* antigenic and group factors within a vaccine.

- This implies that the vaccine construct could offer a broad protection than designed for and a promising and enduring solution towards prevention of shigellosis in endemic regions such as Kenya.

6.3 Recommendation and Future steps

i. Analyze additional isolates such as *S. flexneri* 1b, 5a/b, 3b that would add to the knowledge on the breath of cross reactivity with the novel quadrivalent vaccine under clinical trials.

ii. Analysis of human serum from immunization with Flexyn2a vaccine for cross reactivity with *S. flexneri* 2a, 2b and 3a isolates from Kenya as seen in this study.

iii. Pending proof of concept though controlled human challenge studies (CHIM), it would be exciting to see the results from evaluating the post *Shigella*4V tetravalent vaccine human serum from the ongoing phase 1/2a LMTB study for reactivity/ cross-reactivity and functional capabilities against recent *Shigella* isolates from Kenya.

REFERENCES

Aggarwal, P., Uppal, B., Ghosh, R., Krishna Prakash, S., Chakravarti, A., Jha, A. K., & Rajeshwari, K. (2016). Multi drug resistance and Extended Spectrum Beta Lactamases in clinical isolates of Shigella: A study from New Delhi, India. *Travel Medicine and Infectious Disease*, *14*(4), 407–413. https://doi.org/10.1016/j.tmaid.2016.05.006

Ashida, H., Mimuro, H., & Sasakawa, C. (2015). Shigella Manipulates Host Immune Responses by Delivering Effector Proteins with Specific Roles. *Frontiers in Immunology*, *6*. https://doi.org/10.3389/fimmu.2015.00219

Azmi, I. J., Khajanchi, B. K., Akter, F., Hasan, T. N., Shahnaij, M., Akter, M., Banik, A., Sultana, H., Hossain, M. A., Ahmed, M. K., Faruque, S. M., & Talukder, K. A. (2014). Fluoroquinolone Resistance Mechanisms of Shigella flexneri Isolated in Bangladesh. *PLoS ONE*, *9*(7), e102533. https://doi.org/10.1371/journal.pone.0102533

Brotcke Zumsteg, A., Goosmann, C., Brinkmann, V., Morona, R., & Zychlinsky, A. (2014). IcsA Is a Shigella flexneri Adhesin Regulated by the Type III Secretion System and Required for Pathogenesis. *Cell Host & Microbe*, *15*(4), 435–445. https://doi.org/10.1016/j.chom.2014.03.001

Chen, W. H., & Kotloff, K. L. (2016). Shigella Vaccine Development: Finding the Path of Least Resistance. *Clinical and Vaccine Immunology*, *23*(12), 904–907. https://doi.org/10.1128/CVI.00444-16

Christabel, M. (2012). Characterization of antibiotic resistance in environmental enteric pathogens from Kibera slum in Nairobi-Kenya. *Journal of Bacteriology Research*, *4*(4), 46–54. https://doi.org/10.5897/JBR12.008

Cruz, C. B. N. da, Souza, M. C. S. de, Serra, P. T., Santos, I., Balieiro, A., Pieri, F. A., Nogueira, P. A., & Orlandi, P. P. (2014). Virulence Factors Associated with Pediatric Shigellosis in Brazilian Amazon. *BioMed Research International*, *2014*, 1–9. https://doi.org/10.1155/2014/539697

Dr Valéria Szijártó. (2013). *Cross-protective potential of live Shigella mutants lacking immunodominant antigens*.

Endris, M., Mohammed, R., Takele, Y., Woldeyohannes, D., Tiruneh, M., & Diro, E. (2013). *Shigella* Bacteremia in a Patient with Visceral Leishmaniasis. *Case Reports in Critical Care*, *2013*, 1–3. https://doi.org/10.1155/2013/920729

Farshad, S., Sheikhi, R., Japoni, A., Basiri, E., & Alborzi, A. (2006). Characterization of Shigella Strains in Iran by Plasmid Profile Analysis and PCR Amplification of ipa Genes. *Journal of Clinical Microbiology*, *44*(8), 2879–2883. https://doi.org/10.1128/JCM.00310-06

Faruque, S. M., Khan, R., Kamruzzaman, M., Yamasaki, S., Ahmad, Q. S., Azim, T., Nair, G. B., Takeda, Y., & Sack, D. A. (2002). Isolation of Shigella dysenteriae Type 1 and S. flexneri Strains from Surface Waters in Bangladesh: Comparative Molecular Analysis of Environmental Shigella Isolates versus Clinical Strains. *Applied and Environmental Microbiology*, *68*(8), 3908–3913. https://doi.org/10.1128/AEM.68.8.3908-3913.2002

Farzam, N., Ramon-Saraf, R., Banet-Levi, Y., Lerner-Geva, L., Ashkenazi, S., Kubler-Kielb, J., Vinogradov, E., Robbins, J. B., & Schneerson, R. (2017). Vaccination with Shigella flexneri 2a conjugate induces type 2a and cross-reactive type 6 antibodies in humans but not in mice. *Vaccine*, *35*(37), 4990–4996. https://doi.org/10.1016/j.vaccine.2017.07.070

Ghosh, S., Pazhani, G. P., Niyogi, S. K., Nataro, J. P., & Ramamurthy, T. (2014). Genetic characterization of Shigella spp. Isolated from diarrhoeal and asymptomatic children.

Journal of Medical Microbiology, *63*(7), 903–910. https://doi.org/10.1099/jmm.0.070912-0

Hale, T. L., & Keusch, G. T. (1996). Shigella. In S. Baron (Ed.), *Medical Microbiology* (4th ed.). University of Texas Medical Branch at Galveston. http://www.ncbi.nlm.nih.gov/books/NBK8038/

Heine, S. J., Diaz-McNair, J., Andar, A. U., Drachenberg, C. B., van de Verg, L., Walker, R., Picking, W. L., & Pasetti, M. F. (2014). Intradermal Delivery of *Shigella* IpaB and IpaD Type III Secretion Proteins: Kinetics of Cell Recruitment and Antigen Uptake, Mucosal and Systemic Immunity, and Protection across Serotypes. *The Journal of Immunology*, *192*(4), 1630–1640. https://doi.org/10.4049/jimmunol.1302743

Hlozek, J., Owen, S., Ravenscroft, N., & Kuttel, M. M. (2020). Molecular Modeling of the Shigella flexneri Serogroup 3 and 5 O-Antigens and Conformational Relationships for a Vaccine Containing Serotypes 2a and 3a. *Vaccines*, *8*(4), 643. https://doi.org/10.3390/vaccines8040643

HogenEsch, H., O'Hagan, D. T., & Fox, C. B. (2018). Optimizing the utilization of aluminum adjuvants in vaccines: You might just get what you want. *Npj Vaccines*, *3*(1), 51. https://doi.org/10.1038/s41541-018-0089-x

Humphries, R. M., & Linscott, A. J. (2015). Practical Guidance for Clinical Microbiology Laboratories: Diagnosis of Bacterial Gastroenteritis. *Clinical Microbiology Reviews*, *28*(1), 3–31. https://doi.org/10.1128/CMR.00073-14

Hyams, K. C., Malone, J. D., Bourgeois, A. L., Hawkins, R., Hale, T. L., & Murphy, J. R. (1995). Serum antibody to lipopolysaccharide antigens of Shigella species among U.S. military personnel deployed to Saudi Arabia and Kuwait during Operations Desert Shield

and Desert Storm. *Clinical and Diagnostic Laboratory Immunology*, *2*(6), 700–703. https://doi.org/10.1128/CDLI.2.6.700-703.1995

Jeong, H. S., Bae, I. K., Shin, J. H., Jung, H. J., Kim, S. H., Lee, J. Y., Oh, S. H., Kim, H. R., Chang, C. L., Kho, W.-G., & Lee, J. N. (2011). Prevalence of Plasmid-mediated Quinolone Resistance and Its Association with Extended-spectrum Beta-lactamase and AmpC Beta-lactamase in Enterobacteriaceae. *Annals of Laboratory Medicine*, *31*(4), 257–264. https://doi.org/10.3343/kjlm.2011.31.4.257

Kazi, M., Drego, L., Nikam, C., Ajbani, K., Soman, R., Shetty, A., & Rodrigues, C. (2015). Molecular characterization of carbapenem-resistant Enterobacteriaceae at a tertiary care laboratory in Mumbai. *European Journal of Clinical Microbiology and Infectious Diseases*, *34*(3), 467–472. https://doi.org/10.1007/s10096-014-2249-x

Killackey, S. A., Sorbara, M. T., & Girardin, S. E. (2016). Cellular Aspects of Shigella Pathogenesis: Focus on the Manipulation of Host Cell Processes. *Frontiers in Cellular and Infection Microbiology*, *6*. https://doi.org/10.3389/fcimb.2016.00038

Kim, H. W., Kim, K. H., Kim, J. H., & Nahm, M. H. (2016). A high throughput serum bactericidal assay for antibodies to Haemophilus influenzae type b. *BMC Infectious Diseases*, *16*(1). https://doi.org/10.1186/s12879-016-1808-4

Knirel, Y. A., Sun, Q., Senchenkova, S. N., Perepelov, A. V., Shashkov, A. S., & Xu, J. (2015). O-Antigen modifications providing antigenic diversity of Shigella flexneri and underlying genetic mechanisms. *Biochemistry (Moscow)*, *80*(7), 901–914. https://doi.org/10.1134/S0006297915070093

Koestler, B. J., Ward, C. M., Fisher, C. R., Rajan, A., Maresso, A. W., & Payne, S. M. (2019). Human Intestinal Enteroids as a Model System of *Shigella* Pathogenesis. *Infection and*

Immunity, *87*(4), e00733-18, /iai/87/4/IAI.00733-18.atom. https://doi.org/10.1128/IAI.00733-18

Kotloff, K. L., Blackwelder, W. C., Nasrin, D., Nataro, J. P., Farag, T. H., van Eijk, A., Adegbola, R. A., Alonso, P. L., Breiman, R. F., Golam Faruque, A. S., Saha, D., Sow, S. O., Sur, D., Zaidi, A. K. M., Biswas, K., Panchalingam, S., Clemens, J. D., Cohen, D., Glass, R. I., … Levine, M. M. (2012). The Global Enteric Multicenter Study (GEMS) of Diarrheal Disease in Infants and Young Children in Developing Countries: Epidemiologic and Clinical Methods of the Case/Control Study. *Clinical Infectious Diseases*, *55*(suppl_4), S232–S245. https://doi.org/10.1093/cid/cis753

Leski, T. A., Vora, G. J., Barrows, B. R., Pimentel, G., House, B. L., Nicklasson, M., Wasfy, M., Abdel-Maksoud, M., & Taitt, C. R. (2013). Molecular Characterization of Multidrug Resistant Hospital Isolates Using the Antimicrobial Resistance Determinant Microarray. *PLoS ONE*, *8*(7), e69507. https://doi.org/10.1371/journal.pone.0069507

Levine, M. M., Kotloff, K. L., Barry, E. M., Pasetti, M. F., & Sztein, M. B. (2007). Clinical trials of Shigella vaccines: Two steps forward and one step back on a long, hard road. *Nature Reviews Microbiology*, *5*(7), 540–553. https://doi.org/10.1038/nrmicro1662

Livio, S., Strockbine, N. A., Panchalingam, S., Tennant, S. M., Barry, E. M., Marohn, M. E., Antonio, M., Hossain, A., Mandomando, I., Ochieng, J. B., Oundo, J. O., Qureshi, S., Ramamurthy, T., Tamboura, B., Adegbola, R. A., Hossain, M. J., Saha, D., Sen, S., Faruque, A. S. G., … Levine, M. M. (2014). Shigella Isolates From the Global Enteric Multicenter Study Inform Vaccine Development. *Clinical Infectious Diseases*, *59*(7), 933–941. https://doi.org/10.1093/cid/ciu468

MacLennan, C. A., Riddle, M. S., Chen, W. H., Talaat, K. R., Jain, V., Bourgeois, A. L., Frenck, R., Kotloff, K., & Porter, C. K. (2019). Consensus Report on Shigella Controlled Human Infection Model: Clinical Endpoints. *Clinical Infectious Diseases*, *69*(Supplement_8), S591–S595. https://doi.org/10.1093/cid/ciz891

Mani, S., Wierzba, T., & Walker, R. I. (2016). Status of vaccine research and development for Shigella. *Vaccine*, *34*(26), 2887–2894. https://doi.org/10.1016/j.vaccine.2016.02.075

Marteyn, B. S. (2016). Shigella Vaccine Development: The Model Matters. In *JSM Trop Med Res* (Vol. 1, Issue 2, p. 1011).

Mattock, E., & Blocker, A. J. (2017). How do the virulence factors of shigella work together to cause disease? *Frontiers in Cellular and Infection Microbiology*, *7*(MAR). https://doi.org/10.3389/fcimb.2017.00064

Nahm, M. H., Yu, J., Weerts, H. P., Wenzel, H., Tamilselvi, C. S., Chandrasekaran, L., Pasetti, M. F., Mani, S., & Kaminski, R. W. (2018). Development, Interlaboratory Evaluations, and Application of a Simple, High-Throughput *Shigella* Serum Bactericidal Assay. *MSphere*, *3*(3), e00146-18, /msphere/3/3/mSphere146-18.atom. https://doi.org/10.1128/mSphere.00146-18

Njuguna, C., Njeru, I., Mgamb, E., Langat, D., Makokha, A., Ongore, D., Mathenge, E., & Kariuki, S. (2016). Enteric pathogens and factors associated with acute bloody diarrhoea, Kenya. *BMC Infectious Diseases*, *16*(1), 477. https://doi.org/10.1186/s12879-016-1814-6

Njuguna, H. N., Cosmas, L., Williamson, J., Nyachieo, D., Olack, B., Ochieng, J. B., Wamola, N., Oundo, J. O., Feikin, D. R., Mintz, E. D., & Breiman, R. F. (2013). Use of Population-based Surveillance to Define the High Incidence of Shigellosis in an Urban

Slum in Nairobi, Kenya. *PLOS ONE*, *8*(3), e58437. https://doi.org/10.1371/journal.pone.0058437

Noriega, F. R., Liao, F. M., Maneval, D. R., Ren, S., Formal, S. B., & Levine, M. M. (1999). Strategy for Cross-Protection among Shigella flexneri Serotypes. *Infection and Immunity*, *67*(2), 782–788. https://doi.org/10.1128/IAI.67.2.782-788.1999

Nüesch-Inderbinen, M., Heini, N., Zurfluh, K., Althaus, D., Hächler, H., & Stephan, R. (2016). *Shigella* Antimicrobial Drug Resistance Mechanisms, 2004–2014. *Emerging Infectious Diseases*, *22*(6), 1083–1085. https://doi.org/10.3201/eid2206.152088

Nyanga, P. L., Onyuka, J., Webale, M. K., Were, T., & Budambula, V. (2017). *Escherichia coli pathotypes and Shigella sero-groups in diarrheic children in Nairobi city, Kenya*. 9.

Oaks, E. V., Picking, W. D., & Picking, W. L. (1996). Antibody response of monkeys to invasion plasmid antigen D after infection with Shigella spp. *Clinical and Diagnostic Laboratory Immunology*, *3*(2), 242–245. https://doi.org/10.1128/CDLI.3.2.242-245.1996

Obiero, C. W., Ndiaye, A. G. W., Sciré, A. S., Kaunyangi, B. M., Marchetti, E., Gone, A. M., Schütte, L. D., Riccucci, D., Auerbach, J., Saul, A., Martin, L. B., Bejon, P., Njuguna, P., & Podda, A. (2017). A phase 2a randomized study to evaluate the safety and immunogenicity of the 1790GAHB generalized modules for membrane antigen vaccine against Shigella sonnei administered intramuscularly to adults from a shigellosis-endemic country. *Frontiers in Immunology*, *8*(DEC). https://doi.org/10.3389/fimmu.2017.01884

Passwell, J. H., Ashkenazi, S., Banet-Levi, Y., Ramon-Saraf, R., Farzam, N., Lerner-Geva, L., Even-Nir, H., Yerushalmi, B., Chu, C., Shiloach, J., Robbins, J. B., & Schneerson, R. (2010). Age-related efficacy of Shigella O-specific polysaccharide conjugates in 1–4-

year-old Israeli children. *Vaccine*, *28*(10), 2231–2235. https://doi.org/10.1016/j.vaccine.2009.12.050

Pavlinac, P. B., Denno, D. M., John-Stewart, G. C., Onchiri, F. M., Naulikha, J. M., Odundo, E. A., Hulseberg, C. E., Singa, B. O., Manhart, L. E., & Walson, J. L. (2016). Failure of Syndrome-Based Diarrhea Management Guidelines to Detect *Shigella* Infections in Kenyan Children. *Journal of the Pediatric Infectious Diseases Society*, *5*(4), 366–374. https://doi.org/10.1093/jpids/piv037

Pavlinac, Patricia B., John-Stewart, G. C., Naulikha, J. M., Onchiri, F. M., Denno, D. M., Odundo, E. A., Singa, B. O., Richardson, B. A., & Walson, J. L. (2014). High-risk enteric pathogens associated with HIV infection and HIV exposure in Kenyan children with acute diarrhea. *AIDS*, *28*(15), 2287–2296. https://doi.org/10.1097/QAD.0000000000000396

Rhee, C., Aol, G., Ouma, A., Audi, A., Muema, S., Auko, J., Omore, R., Odongo, G., Wiegand, R. E., Montgomery, J. M., Widdowson, M.-A., O'Reilly, C. E., Bigogo, G., & Verani, J. R. (2019). Inappropriate use of antibiotics for childhood diarrhea case management—Kenya, 2009–2016. *BMC Public Health*, *19*(S3), 468. https://doi.org/10.1186/s12889-019-6771-8

Riddle, M. S., Kaminski, R. W., Paolo, C. D., Porter, C. K., Gutierrez, R. L., Clarkson, K. A., Weerts, H. E., Duplessis, C., Castellano, A., Alaimo, C., Paolino, K., Gormley, R., & Fonck, V. G. (2016). Safety and immunogenicity of a candidate bioconjugate vaccine against Shigella flexneri 2a administered to healthy adults: A single-blind, randomized phase i study. *Clinical and Vaccine Immunology*, *23*(12), 908–917. https://doi.org/10.1128/CVI.00224-16

Sang, W. K., Oundo, V., & Schnabel, D. (2012). Prevalence and antibiotic resistance of bacterial pathogens isolated from childhood diarrhoea in four provinces of Kenya. *The Journal of Infection in Developing Countries*, *6*(07), 572–578. https://doi.org/10.3855/jidc.2196

Schroeder, G. N., & Hilbi, H. (2008). Molecular Pathogenesis of Shigella spp.: Controlling Host Cell Signaling, Invasion, and Death by Type III Secretion. *Clinical Microbiology Reviews*, *21*(1), 134–156. https://doi.org/10.1128/CMR.00032-07

Shah, M., Kathiiko, C., Wada, A., Odoyo, E., Bundi, M., Miringu, G., Guyo, S., Karama, M., & Ichinose, Y. (2016). Prevalence, seasonal variation, and antibiotic resistance pattern of enteric bacterial pathogens among hospitalized diarrheic children in suburban regions of central Kenya. *Tropical Medicine and Health*, *44*(1), 39. https://doi.org/10.1186/s41182-016-0038-1

Shimanovich, A. A., Buskirk, A. D., Heine, S. J., Blackwelder, W. C., Wahid, R., Kotloff, K. L., & Pasetti, M. F. (2017). Functional and antigen-specific serum antibody levels as correlates of protection against shigellosis in a controlled human challenge study. *Clinical and Vaccine Immunology*, *24*(2). https://doi.org/10.1128/CVI.00412-16

Swierczewski, B. E., Odundo, E. A., Koech, M. C., Ndonye, J. N., Kirera, R. K., Odhiambo, C. P., Cheruiyot, E. K., Shaffer, D. N., Ombogo, A. N., & Oaks, E. V. (2013). Enteric pathogen surveillance in a case-control study of acute diarrhoea in the town of Kisii, Kenya. *Journal of Medical Microbiology*, *62*(11), 1774–1776. https://doi.org/10.1099/jmm.0.059139-0

Talaat, K. R., Alaimo, C., Martin, P., Bourgeois, A. L., Kaminski, R. W., Dreyer, A., Porter, C. K., Chakraborty, S., & Clarkson, K. A. (2017). *Flexyn2a, a candidate bioconjugate*

vaccine against Shigella flexneri 2a induces protective immune response in a controlled human infection model. Vaccines for Enteric Diseases, Albufeira, Portugal.

Taneja, N., & Mewara, A. (2016). Shigellosis: Epidemiology in India. *Indian Journal of Medical Research*, *143*(5), 565. https://doi.org/10.4103/0971-5916.187104

The, H. C., Thanh, D. P., Holt, K. E., Thomson, N. R., & Baker, S. (2016). The genomic signatures of Shigella evolution, adaptation and geographical spread. *Nature Reviews Microbiology*, *14*(4), 235–250. https://doi.org/10.1038/nrmicro.2016.10

Tickell, K. D., Brander, R. L., Atlas, H. E., Pernica, J. M., Walson, J. L., & Pavlinac, P. B. (2017). Identification and management of Shigella infection in children with diarrhoea: A systematic review and meta-analysis. *The Lancet Global Health*, *5*(12), e1235–e1248. https://doi.org/10.1016/S2214-109X(17)30392-3

Torraca, V., Holt, K., & Mostowy, S. (2020). Shigella sonnei. *Trends in Microbiology*, *28*(8), 696–697. https://doi.org/10.1016/j.tim.2020.02.011

Turbyfill, K. R., Clarkson, K. A., Vortherms, A. R., Oaks, E. V., & Kaminski, R. W. (2018). Assembly, Biochemical Characterization, Immunogenicity, Adjuvanticity, and Efficacy of Shigella Artificial Invaplex. *MSphere*, *3*(2), e00583-17. https://doi.org/10.1128/msphere.00583-17

Van de Verg, L. L., & Venkatesan, M. M. (2014). Editorial Commentary: A Shigella Vaccine Against Prevalent Serotypes. *Clinical Infectious Diseases*, *59*(7), 942–943. https://doi.org/10.1093/cid/ciu471

Weerts, H. P., Yu, J., Kaminski, R. W., & Nahm, M. H. (2019). A High-throughput Shigella-specific Bactericidal Assay. *Journal of Visualized Experiments*, *144*, 59164. https://doi.org/10.3791/59164

WHO. (2020). *DRAFT WHO Preferred Product Characteristics for Vaccines against Shigella*.

Yang, C., Li, P., Zhang, X., Ma, Q., Cui, X., Li, H., Liu, H., Wang, J., Xie, J., Wu, F., Sheng, C., Du, X., Qi, L., Su, W., Jia, L., Xu, X., Zhao, J., Xia, S., Zhou, N., … Song, H. (2016). Molecular characterization and analysis of high-level multidrug-resistance of Shigella flexneri serotype 4s strains from China. *Scientific Reports*, *6*(1), 29124. https://doi.org/10.1038/srep29124

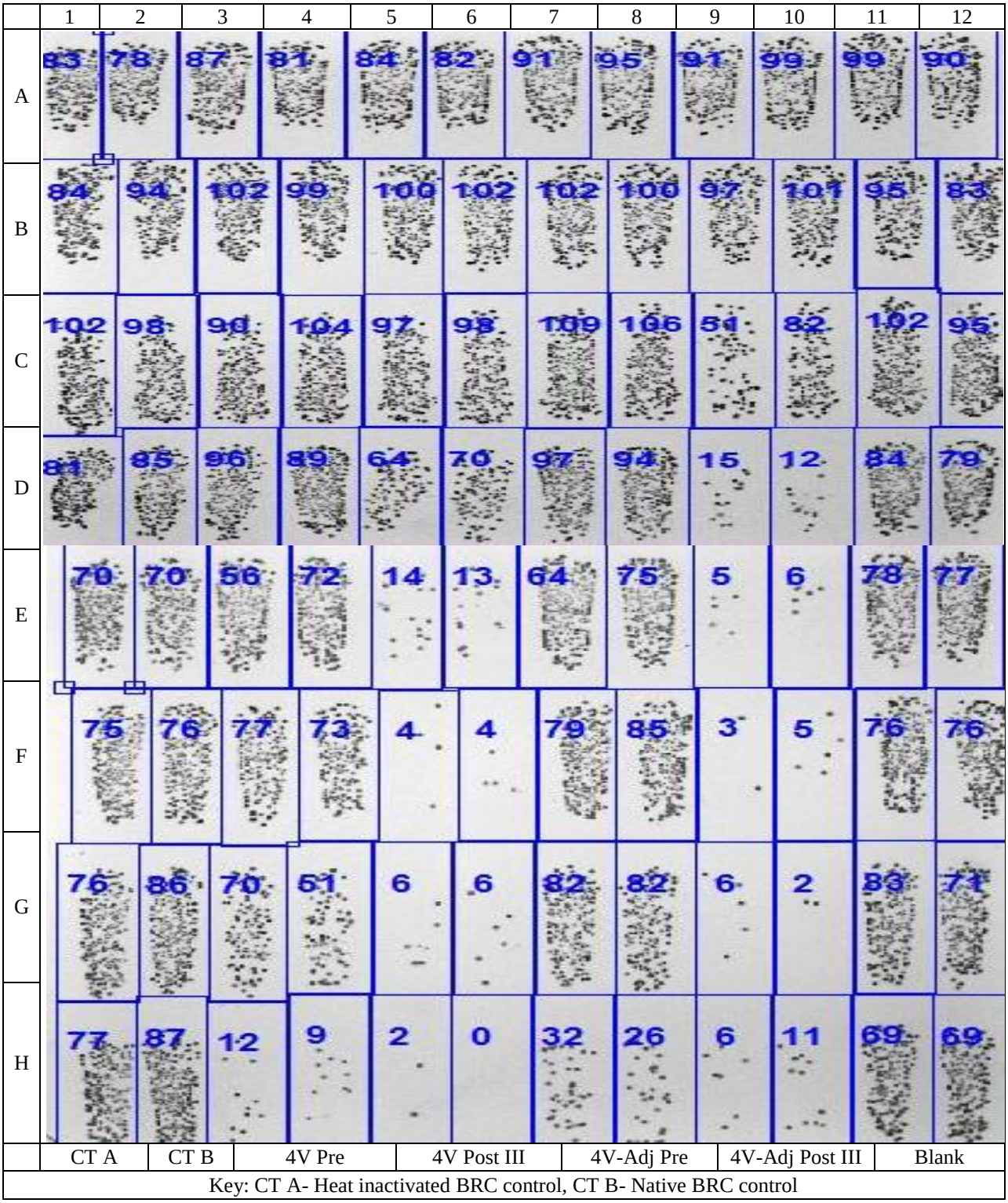
Yang, F., Yang, J., Zhang, X., Chen, L., Jiang, Y., Yan, Y., Tang, X., Wang, J., Xiong, Z., Dong, J., Xue, Y., Zhu, Y., Xu, X., Sun, L., Chen, S., Nie, H., Peng, J., Xu, J., Wang, Y., … Jin, Q. (2005a). Genome dynamics and diversity of Shigella species, the etiologic agents of bacillary dysentery. *Nucleic Acids Research*, *33*(19), 6445–6458. https://doi.org/10.1093/nar/gki954

Yang, F., Yang, J., Zhang, X., Chen, L., Jiang, Y., Yan, Y., Tang, X., Wang, J., Xiong, Z., Dong, J., Xue, Y., Zhu, Y., Xu, X., Sun, L., Chen, S., Nie, H., Peng, J., Xu, J., Wang, Y., … Jin, Q. (2005b). Genome dynamics and diversity of Shigella species, the etiologic agents of bacillary dysentery. *Nucleic Acids Research*, *33*(19), 6445–6458. https://doi.org/10.1093/nar/gki954

APPENDICES

Appendix 1: Sample of enumerated SBA plate

SBA SBA-K-S.f3a-089 Plate 1 and 2:



Key: CT A- Heat inactivated BRC control, CT B- Native BRC control

Appendix 2: Approval School of Graduate Studies, Maseno University

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref MSC/PH/00022/14

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 20th September, 2018

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR ELIZABETH ODUNDO— MSC/PH/00022/2014

The above named is registered in the Master of Science Programme in the School of Public Health and Community Development, Maseno University. This is to confirm that her research proposal titled "Analysis of Reactivity and Functionality of Serum Antibodies from Multivalent Shigella Biooconjugate Vaccinated Rabbits and Guinea Pigs against Shigella Isolates from Kenya" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

20 SEP 2018

Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

Appendix 3: KEMRI/SERU/CCR/125/3900 protocol approval letter

KENYA MEDICAL RESEARCH INSITITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
Email: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 **August 21, 2019**

TO: **ELIZABETH A. ODUNDO**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CCR**
NAIROBI

forwarded 28/Aug 2019

Dear Madam,

RE: **KEMRI/SERU/CCR/125/3900 (*RESUBMITTED INITIAL SUBMISSION*): ANALYSIS OF REACTIVITY AND FUNTIONALLY OF SERUM ANTIBODIES FROM MULTI VALENT *SHIGELLA* BIOCONJUGATE VACCINATED RABBITS AND GUINEA PIGS *SHIGELLA* ISOLATES FROM KENYA**

Reference is made to your letter dated July 29, 2019. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the following revised study documents on August 14, 2019:

1. Protocol version 1.7 dated 26 July 2019, tracked and clean
2. Letter from PI of SSC 1549 dated 18/09/2018 entitled "Permission to use archived isolates".

This is to inform you that the Committee notes that the following issues raised during the **289th Committee A** meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **July 9, 2019,** have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **August 21, 2019** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **August 20, 2020.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **July 09, 2020**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued. You may embark on the study.

Yours faithfully

ENOCK KEBENEI
THE ACTING HEAD
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT