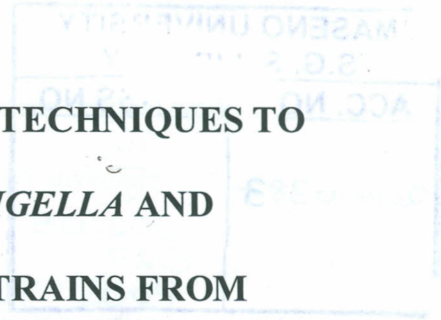

USE OF CONVENTIONAL AND MOLECULAR TECHNIQUES TO DETERMINE THE PREVALENCE OF *SHIGELLA* AND ENTEROINVASIVE *ESCHERICHIA COLI* STRAINS FROM DIARRHOEA PATIENTS IN SELECTED HEALTH FACILITIES IN RURAL WESTERN KENYA

BY

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A Research Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biomedical Science and Technology

DEPARTMENT OF BIOMEDICAL SCIENCES AND TECHNOLOGY

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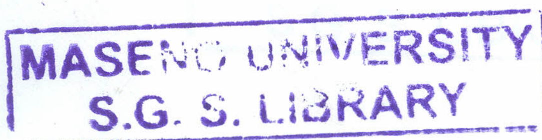

ABSTRACT

Diarrhoea is a major cause of infant morbidity and mortality worldwide. It accounts for 1.6 – 2.5 million deaths annually and each child in the developing world experiences an average of three episodes of diarrhoea per year. *Shigella* spp and enteroinvassive *Escherichia coli* (EIEC) are common etiologic agents of bacillary dysentery. Currently, conventional culture techniques for identifying *Shigella* spp from stool has low sensitivity since the diagnosis is often obscured due to the presence of low number of causative organisms, competition from commensals and inappropriate sample collection. The aim of this study was to determine the prevalence of *Shigella* and EIEC using conventional culture method and molecular technique targeting the *ipaH* gene, common to both *Shigella spp* and EIEC. Stool specimens from 440 patients of all ages presenting to nine health facilities with diarrhoea were investigated for *Shigella* species and EIEC by conventional culture and a subset of the specimens evaluated by molecular technique. Of the 440 specimens cultured, 48 (10.9%) yielded *Shigella* species; *S. flexneri* (56%), *S. dysenteriae* non-type 1 (25%), and *S. boydii* and *S. sonnei* (8.3% each). No EIEC strains were isolated. Of the 421 specimens evaluated by PCR, 78 (18.5%) tested positive for *ipaH* gene, 331 (78.6%) tested negative, and 12 (2.9%) were weakly positive. All the *Shigella* species (100%) isolated by culture and an additional 33 (7.8 %) cases not identified by culture, were detected by *ipaH* PCR. Bloody specimens were more likely to yield a *Shigella* by culture (56%) and PCR (35%) than other types of diarrhoea ($P<0.05$). The findings of this study demonstrate that the magnitude of shigellosis in rural western Kenya is much higher than previously thought. Information from this study will help in evaluating the need to strengthen the basic preventing measures and other control measures against these pathogens.

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CHAPTER ONE

1.0 INTRODUCTION

The genus *Shigella* is divided into four species (subgroups) namely: *Shigella dysenteriae, Shigella flexneri, Shigella boydii* and *Shigella sonnei* representing subgroups A, B, C and D, respectively (Murray *et al.*, 1995). The subgroups are further divided into 41 serotypes based on O antigen variations: 15 serotypes in *S. dysenteriae,* 19 in *S. boydii*, 6 in *S. flexneri* and 1 in *S. sonnei* (Ewing, 1986; and Strockbine and Maurelli, 2005). Shigellosis is an acute infectious disease caused by all the four species of *Shigella*, with severity of signs and symptoms of infection ranging from most to least serious in the same order as the serogroup (serovar) designations A to D (Greenwood, *et al.,* 2003). *Shigella flexneri* is the predominant species of *Shigella* in endemic areas accounting for over 50% of culture positive cases and exhibits heterogenicity in their serotype distribution in resource poor countries (Seidlein *et al.*, 2006).

Shigella species are pathogens of humans and other primates. The pathogenesis of infection with these bacteria and Enteroinvasive *Escherichia coli* (EIEC) are similar. *Shigella* spp and EIEC are the principle etiologic agents of bacillary dysentery, a common diarrhoea disease of humans in developing countries, and may also cause outbreaks in developed countries (Kotloff *et al.*, 1999). Transmission of the disease is primarily by person-to-person contact through contaminated hands. When associated with outbreaks the microorganisms are usually transmitted through contaminated food and/or drinks (Greenwood *et al.*, 2003). The bacteria causes disease by invading and multiplying within the colonic epithelium following the ingestion of as few as 10 viable bacteria.

Clinical manifestations of classical bacillary dysentery include fever, vomiting, abdominal pain, tenesmus (painful straining to pass stools), bloody stool and mucus resulting from invasion of the intestinal mucosa by the pathogen (Nataro and Kaper, 1998).

Pathogenesis in Shigellae and EIEC depends on both chromosomal and plasmid genes. The process of penetration and destruction of colonic epithelium is mediated in part by a large (120 – 140 MDa) plasmid that codes for the genetic determinants for epithelial cell invasion. The invasion plasmid antigen (*ipa*) genes *ipaA*, *ipaB*, *ipaC* and *ipaD* (Hale, 1991; Buysse *et al*., 1987; Sansonetti *et al*., 1982) are located on the large plasmid of all virulent *Shigella* and EIEC organisms. Apart from the *ipaABCD* genes, a new gene *ipaH* has been identified (Yang *et al*., 2005; Venkatesan *et al*., 2001). The gene is present in multiple copies on the plasmid and the chromosome of *Shigella* and EIEC bacteria (Buysse *et al*., 1995; Venkatesan *et al*., 1989; Venkatesan *et al*., 2001), with the number of copies varying between 4 and 10. Five complete alleles of *ipaH* namely *ipaH1.4*, *ipaH2.5 ipaH4.5 ipaH7.8*, and *ipaH9.8* have been shown to exist on the virulence plasmid of *S. flexneri* (Venkatesan *et al*., 2001). However, the function of these alleles is not clear yet. The *ipaH* is a 60kd antigen-coding gene and is not an indicator of invasive phenotype but is specific for *Shigella spp* and EIEC. Because of its presence in the chromosome and plasmid in multiple copies, a DNA probe for *ipaH* is less compromised by plasmid loss or selective deletion events of the invasion plasmid and is more sensitive as a diagnostic tool for *Shigella* and EIEC than the *ipaABCD* gene (Venkatesan *et al*., 1989).

Currently, conventional techniques for identifying *Shigella* spp from stool relies on culture methods. However, these methods are less sensitive since the diagnosis is often obscured due to the presence of low numbers of causative organism, competition from other commensal organisms and inappropriate sample collection. As a result, the magnitude of the disease has been underestimated. It has been shown that the sensitivity of *ipa*H polymerase chain reaction (PCR) technique for detection of *Shigella* from stool is 10^4 fold more sensitive than the conventional culture method and 100% specificity for *Shigella* spp and EIEC. Direct culture requires $2x10^6$ cfu/ml of *S. flexneri* in a specimen, to detect the pathogen (Dutta *et al.*, 2001; Thong *et al.,* 2005). Previous surveillance studies have shown that *Shigella* spp contributes to 16% (54% from *S. flexneri*) of diarrhoea cases and is the most prevalent etiologic agent of diarrhoea in rural western Kenya as determined by conventional culture method (Brooks *et al.,* 2003; Brooks *et al.,* 2006). However, culture method detects only a small fraction of the shigellosis cases and identification of EIEC is almost impossible. As a result, little data is available on the contribution of EIEC as an etiologic agent of diarrhoea in western Kenya. Moreover, there is no information on detection of *Shigella* spp or EIEC, from patients presenting with diarrhoea, using molecular methods such as PCR in an epidemiologic setup in western Kenya.

1.1 PROBLEM STATEMENT

Diarrhoea is one of the principle causes of morbidity and mortality among children under five years old in the developing world. It is estimated that diarrhoea accounts for 1.6 –

2.5 million deaths annually, and a child in the developing world experiences an average of 3 episodes of diarrhoea per year (Kotloff *et al.*, 1999). In a rural western Kenyan community and similarly in Asia, *Campylobacter* species and diarrhoeagenic *E. coli* predominates among children less than 5 years old and are progressively replaced by *Shigella* spp with increase in age (Shapiro *et al.*, 2001; Seidlein *et al.*, 2006; Brooks *et al.*, 2006). The under five mortality rate in Nyanza province was estimated at 206 deaths per 1000 live births (MOH, 2004), and in Siaya and Bondo Districts, diarrhoea reportedly is the third most common cause of morbidity among children accounting for 5% of sicknesses. In western Kenya, the standard procedure used for identification of enteric pathogens is by stool culture, a method with several practical limitations. Selection of the pathogenic bacterial colonies from normal colonic flora on culture media is difficult if the colonies of the pathogen are few. In addition, EIEC which causes diarrhoea in a manner similar to that of *Shigella* spp can not be identified by the routine microbiologic methods and requires special assays.

1.2 JUSTIFICATION

Currently, conventional techniques for identifying *Shigella* spp from stool relies on culture methods. However, these methods are less sensitive since the diagnosis is often obscured due to the presence of low numbers of the causative organisms, competition from other commensal organisms and inappropriate sample collection. It has been estimated that about 23% of outpatient diarrhoea cases and 13.2% of inpatient cases worldwide are of unknown aetiology (Lanata *et al.*, 2002). Because an etiologic agent is not detected for such a large portion of patients with diarrhoea, a possibility exists that a

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portion of the undiagnosed illnesses may be attributed to *Shigella* or EIEC undetectable by conventional (culture) technique. In this study, a molecular technique targeting the *ipaH* gene, common to both *Shigella spp* and EIEC, was used to identify the pathogens. The PCR technique was found to be more sensitive and provided more information on the magnitude of diarrhoea caused by EIEC and *Shigella* spp. In addition, it also provided information on the frequency of Shigellosis unidentifiable by culture.

The results obtained from this study have shown the proportion of diarrhoea caused by *Shigella* spp and EIEC and the various serotypes of *Shigella* isolates associated with different types of diarrhoea. This information is useful in evaluating the need for a vaccine and other control measures against these pathogens.

1.3 GENERAL OBJECTIVE

The aim of this study was to determine the prevalence of *Shigella* species and Enteroinvasive *Escherichia coli* using conventional culture method and molecular technique so as to provide data for future intervention such as vaccine development.

1.3.1 SPECIFIC OBJECTIVES

a. To determine the prevalence of *Shigella* and EIEC from stool of patients presenting with diarrhoea at selected health facilities.

b. To compare the recovery of *Shigella* from direct plating and enrichment broth.

c. To identify serotypes of *Shigella* species associated with different types of diarrhoea.

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d. To determine the number of cases with shigellosis, by PCR technique, which are undetectable by culture method

e. To detect *Shigella* species and EIEC using *ipa*H PCR technique

1.3.2 NULL HYPOTHESES

a. Enteroinvasive *Escherichia coli* (EIEC) is a common etiologic agent of diarrhoea in rural western Kenya.

b. MacConkey broth does not increase the recovery of *Shigella* spp on culture.

c. There is no association between the *Shigella* serotypes and types of diarrhoea.

d. *ipa*H PCR technique does not increase the overall detection of *Shigella* spp in stool samples obtained from diarrhoea patients.

1.4 LIMITATIONS

In this study, patients or the caretakers were asked to provide information on symptoms of the disease and the stool appearance. The study relied on the information provided by the patient or the caretaker, especially when only a rectal swab was obtained. This information might not be accurate. Polymerase chain reaction (PCR) is a molecular technique, which could be inhibited by several factors leading to false negative results. In this study, PCR templates were obtained from stool specimen pre-inoculated in an enrichment broth that could contain underlying factors that could have possibly inhibited PCR.

CHAPTER TWO

2.0 LITERATURE REVIEW

Diarrhoea is one of the major causes of infant morbidity and mortality worldwide (Byass and Ghebreyesus, 2005). The normal intestinal tract regulates the absorption and secretion of electrolytes and water to meet physiological needs of the body. More than 98 percent of the 10 litres per day of fluid entering the adult intestines are reabsorbed (Keusch, 2001). The remaining stool water, related primarily to the indigestible fibre content, determines the consistency of normal faeces from dry, hard pellets to mushy, bulky stool, varying from person to person, day to day, and stool to stool (Keusch *et al.*, 2006). This variation complicates the definition of diarrhoea, which by convention is present when three or more loose stools are passed within 24 hours. According to World Health Organization (WHO), diarrhoea accounts for 1.6 – 2.5 million deaths annually and each child in the developing world experiences an average of three episodes of diarrhoea per year (Kotloff *et al.*, 1999; Parashar *et al.*, 2003; Kosek *et al.*, 2003). Diarrhoea contributes to 3.8% of deaths globally and is fourth among the leading causes of deaths in Africa (Murray *et al.*, 2001). In Kenya, National Health Sector Strategic Plan (NHSSP) reports that diarrhoea contributes to 12.8% of deaths among children under five and that the under five mortality rate is 26 percent points higher in rural than in urban areas (117 and 93 deaths per 1000 live births). The under five mortality rate in Nyanza province was estimated at 206 deaths per 1000 live births (MOH, 2004), and in Siaya and Bondo districts, diarrhoea reportedly is the third most common cause of morbidity among children accounting for 5% of sicknesses (Phillips-Howard *et. al.*, 2003b)

Diarrhoea can be of an invasive type, which is characterized by passage of blood and mucus along with stools. Pus cells and red blood cells (RBCs) are often seen by microscopic examination. On the other hand, non-invasive diarrhoea is watery and profuse with no pus cells or RBCs seen by direct microscopy (Bhatia and Ichhpujani, 1999). Most diarrhoea illnesses are acute, usually lasting for not more than 3-5 days and are secondary to infectious causes namely bacterial, viral or parasitic. The most common microorganisms responsible for diarrhoea worldwide include rotavirus, diarrhoeagenic *Escherichia coli*, *Shigella*, *Campylobacter*, *Vibrio* and non-typhoidal *Salmonella* (Carlos and Saniel, 1990; Guerrant *et al.*, 1990). Bacterial diarrhoea accounts for 41% of outpatient cases and 44.1% of inpatient cases globally (Lanata and Black, 2002).

Early childhood diarrhoea during periods of critical postnatal development may have long-term effects on linear growth and on physical and cognitive functions. Dysentery has the most deleterious consequences on both ponderal and linear growth in children. There is a loss of about half a kilo in annual weight gain, and one and a quarter centimetres in annual height gain associated with dysentery in children less than 5 years old (Alam *et al.*, 2000). *Shigella* and Enteroinvasive *E. coli* are enteric pathogens capable of causing dysentery, a severe gastroenteritis and life-threatening diarrhoea often characterized by blood in stool. The annual incidence rates of diarrhoea in six Asian countries is 4.0/1000 and 25.4/1000 for persons of all ages and children under 5 years, respectively, with an annual incidence rate of shigellosis in children under 5 years old estimated at 13.2/1000 (Seidlein *et.al.*, 2006). Globally, the annual number of *Shigella* cases is estimated to be 164.7 million of which 163.2 million occur in developing

countries. Sixty nine percent (69%) of all cases and 61% of all deaths attributable to shigellosis involve children under 5 years old (Kotloff *et al.*, 1999).

Taxonomically, the genus *Shigella* is a typical member of *Enterobacteriaceae,* existing as Gram negative facultative intracellular enteric pathogen closely related to the genus *Escherichia*, specifically, the enteroinvasive serogroups of *E. coli* (Greenwood *et al.*, 2003; Venkatesan *et al.*, 1988). Their DNA relatedness is very high; the two are often difficult to differentiate biochemically (Murray *et al.*, 1995; Lan *et al.*, 2004). The current classification scheme recognizes four species or serovars within the genus *Shigella*, namely *S. dysenteriae, S. flexneri, S. boydii* and *S. sonnei* representing serogroups A, B, C and D respectively (Murray *et al.*, 1995). The serogroups are further divided into 41 serotypes based on O antigen variation. There are 15 *S. dysenteriae* serotypes, 6 *S. flexneri* serotypes (12 sub-serotypes), 19 *S. boydii* serotypes, and 1 *S. sonnei* serotype (Ewing, 1986; Strockbine and Maurelli, 2005). The severity of symptoms of infection ranges from most to least severe in the same order as the serogroup (serovar) designations A to D (Greenwood *et al.*, 2003). *Shigella flexneri* is the predominant species of *Shigella* in endemic areas accounting for over 50% of culture positive cases and exhibits heterogenicity in serotype distribution in resource poor countries (Seidlein *et al.*, 2006; Brooks *et al.*, 2003; Brooks *et al.*, 2006; Shapiro *et al.*, 2001). However, little data is available on the type of diarrhoea caused by the various *Shigella* serotypes.

Like *Shigella*, *E. coli* are members of the family Enterobacteriaceae. Most of the *E. coli* are motile and Gram negative bacilli. Although *E. coli* strains are part of the healthy

bowel faecal flora of both human and lower animals, pathogenic strains can cause an impressive variety of diseases and each strain is associated with different conditions. The versatility of *E. coli* strains is due to the variable acquisition of virulence genes. Some of the virulence factors resemble those found in other pathogenic species while others seem to be unique (Salyers and Whitt, 2002).

Several strains of *E. coli* that can cause severe and life threatening diarrhoea have been recognized (Nataro and Kaper, 1998). *E. coli* is an underestimated diarrhoeal pathogen in developed and developing countries. This organism may also cause gastroenteritis by varying mechanisms, and on the basis of this, diarrhoeagenic *E. coli* has been subdivided into six major groups (or virotypes) namely: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC), diffuse adherent *E. coli* (DAEC), and enteroinvasive *E. coli* (EIEC) (Greenwood *et al.*, 2003; Murray *et al.*, 1995; Nataro and Kaper, 1998; Salyers and Whitt, 2002). ETEC produces cholera toxin-like enterotoxins that elicit profuse watery diarrhoea. EPEC causes infantile diarrhoea. EHEC is a defined subset of shiga-like (vero) toxin producing *E. coli* that may lead to haemorrhagic uremic syndrome (HUS) in severe infections. EAEC is the most recently described diarrhoeagenic *E. coli* causing chronic diarrhoea in all age groups. EIEC strains invade intestinal epithelium and produce dysentery similar to that caused by *Shigella spp* (Greenwood *et al.*, 2003; Murray *et al.*, 1995) but not HUS. EIEC strains are biochemically, genetically and pathogenetically closely related to *Shigella spp*. Like *Shigella spp*, EIEC strains are lysine decarboxylase,

motility and lactose negative and are often misdiagnosed (Murray *et al.*, 1995; Nataro and Kaper, 1998; Cheasty and Rowe, 1983; Lan *et al.*, 2001).

Like *Shigella spp* but unlike most *E. coli* strains associated with enteritis, EIEC invades the colonic mucosa, multiplies within mucosal epithelial cells, disrupts epithelial cells and spreads laterally to adjacent cells. EIEC infection may cause enteritis characterized by fever, abdominal cramp, and watery diarrhoea, or typical bacillary dysentery with leucocytes, blood and mucus (Greenwood *et al.*, 2003; Seidleine *et al.*, 2006; Hale, 1991). Most cases of shigellosis and EIEC infections are individual cases and are due to person-to-person transmission. When associated with outbreaks, the microorganisms are usually transmitted orally through contaminated food and/or drinks. However, *Shigella* species have a uniquely low infective dose (ID_{50}), between 10 – 100 microorganisms, and the bacteria seem relatively unaffected by gastric acid or bile (Gorden and Small, 1993; Small *et al.*, 1994; Greenwood *et al.*, 2003).

Following ingestion, the Shigellae multiply in the small intestine and are mechanically carried to the large intestine. The primary step in the disease process is the invasion into the cells of the colonic epithelium by transversing both the mucus and glycocalyx layers which coat the epithelium (Brogden *et al.*, 2000). On coming into contact with colonic intestinal epithelium, the bacteria are engulfed by an invagination of the cell membrane, and are internalized in a vacuole within the cell. Lysis of the endocytic vacuole precedes multiplication and spread of the bacteria throughout the cytoplasm. The movement of intracellular bacteria generates the formation of cell protrusions that contain a bacterium

at their tips, which are subsequently internalized by neighbouring cells (Parsot, 2005). Upon uptake in this manner, the bacteria are found enclosed within double membranes. Lysis of the two cells membranes that surround the bacteria in internalized protrusions completes the process of intercellular dissemination, which allows the bacteria to spread from cell to cell without being exposed to the outside medium (Philpott *et al.*, 2000; Geyter *et al.*, 2002). The result of this process is the formation of ulcers through which blood and inflammatory cells reach the lumen of the intestine. Inflammation, together with sloughing of the epithelial cells, results in ulcerative lesions. The incubation period varies from 1 – 3 days (DuPont, 1995), after which the patient experiences a sudden onset of symptoms of abdominal cramps, fever and diarrhoea with stool frequently containing blood and mucus (Seidlein *et al.*, 2006; Brooks *et al.*, 2003; Nataro and Kaper, 1998; Hale, 1991). The cellular response is mainly by polymorphonuclear (PMN) leucocytes, which can be seen readily on microscopic examination of the stool together with red blood cells (RBCs) and sloughed epithelial cells (Greenwood *et al.*, 2003). Symptoms persist for 2 – 7 days and they can cause severe dehydration, kwashiorkor, vitamin B12 deficiency, fluid loss, electrolyte imbalance, and affect growth (Checkley *et al.*, 2003).

The pathogenicity of bacillary dysentery requires coordinated expression of a number of components that control the epithelial cell invasion, and intracellular replication and spreading phenotype characteristic of *Shigella* and EIEC (Hale, 1991). Many plasmid associated virulence determinants are common to both *Shigella* and EIEC (Lan *et al.* 2001). All the bacterial genes necessary for entry into epithelial cells are clustered within a 30kb region of a large 180 – 220 kb virulence plasmid (Hale, 1991). This region

encodes invasion plasmid antigen (*ipa*) genes *ipaA*, *ipaB*, *ipaC* and *ipaD*, and *mxi-spa* loci necessary for full pathogenesis (Buysse *et al.*, 1987; Sansonetti *et al.*, 1982).

The *ipa* gene cluster resides near a region of the plasmid that is a hot spot for spontaneous mutation (deletions) which can be lost thus leading to loss of virulence. These genes are present in all virulent *Shigella* and EIEC strains (Hale, 1991; Pal *et al.*, 1989; Sansonetti *et al.*, 1982). The Ipa proteins are secreted proteins, of which IpaB (57kDa), IpaC (43kDa) and IpaD (39kDa) are effectors of the invasion phenotype (Buysse *et al.*, 1987; Venkatesan *et al.*, 1988; Hartman *et al.*, 1990; Yang *et al.*, 2005). IpaB is thought to function in the lysis of the phagocytic vacuole and induction of apoptosis in macrophages by binding to and activating caspase1, thus triggering cell death (apoptosis) and facilitating the release of the bacteria into the surrounding tissue. IpaB also stimulates the release of interleukin-1-β, which leads to an acute inflammatory response in the intestinal mucosa (Sansonetti *et al.*, 1995). IpaC has been shown to promote uptake of *Shigella* spp into the eukaryotic cell whereas actin depolymerisation is a function of IpaA. The uptake is mediated by secretion of the invasins, IpaA and ipaB-C complex (Hale, 1991; Yang *et al.*, 2005). The virulence plasmid also encodes a surface protein icsA. The i*csA* locus express a 120-kDa outer membrane protein, which plays an important role in the interactions established between host cell microfilaments and the bacterial surface (actin-based motility), thus leading to intracellular spread (Bernardini *et al.*, 1989).

Recently, an additional plasmid antigen designated *ipaH* has been identified. The 60kDa ipaH protein is encoded by a recognized multicopy antigen gene that is unique to the genus *Shigella* and EIEC (Buysse *et al.*, 1987; Hartman *et al.*, 1990; Yang *et al.*, 2005; Venkatesan *et al.*, 2001; Thong *et al.*, 2005). In contrast to the single copy representation of *ipaBCDA* and *icsA* antigen genes, the *ipaH* gene is represented in multiple copies on both the chromosome and the plasmid of *Shigella* spp and EIEC (Buysse *et al.*, 1995; Venkatesan *et al.*, 1989; Venkatesan *et al.*, 2001). Five copies of *ipaH* gene have been mapped on the virulence plasmid of *S. flexneri* and were designated *ipaH9.8, ipaH7.8, ipaH4.5, ipaH2.5,* and *ipaH1.4* (Venkatesan *et al.*, 2001). Thus, this genetic marker is not lost after serial passages of *Shigella* organisms in the laboratory.

Shigella serotypes vary geographically and temporarily (Seidlein *et al.*, 2006). Such shifts pose a double challenge for vaccine developers, who must choose the most relevant serotype for inclusion in a multivalent vaccine. Previous studies showed that an initial episode of *Shigella* diarrhoea did not diminish overall risk of subsequent shigellosis but did confer 72% protection against illness due to the homologous serotype (Ferreccio *et al.*, 1991). This response has been shown to correlate with the presence of antibodies specific for Shigella somatic lipopolysaccharide antigen (Coster *et al.*, 1999). An example is shigellosis due to *S. sonnei* which is rare among people growing up and living in developing countries due to exposure of the population to *Plesiomonas shigelloides* found in surface water and a serotype (serotype 17) of which possesses a cell wall lipopolysaccharide identical to that of *S. sonnei* (Sack *et al.*, 1994). Serotype diversity due to variations in the somatic (O) antigen likely facilitates evasion of the host humoral

immune response. This supports the existence of asymptomatic carriers and why the disease is endemic in this region. Therefore, to have a major epidemiological impact, a shigellosis vaccine may need to contain antigens from several *Shigella* species and/or serotypes. Alternatively, an antigen present in the most common serotypes or in most representative *Shigella* serotypes causing endemic or epidemic infections would be ideal hence the importance of determining the *Shigella* serotype distribution in rural western Kenya where shigellosis is endemic.

The current method of diagnosing bacterial diarrhoea in public health facilities is mainly by conventional culture techniques, which are less sensitive than molecular methods (Dutta *et al.*, 2001; Toma *et al.*, 2003; Thiem *et al.*, 2004). Therefore, the multicopy nature of the *ipaH* gene and its specificity for *Shigella* spp and EIEC strains makes the *ipaH* gene probe a more attractive diagnostic tool for clinical and epidemiologic investigations. It has been estimated that about 23% of outpatient diarrhoea cases and 13.2% of inpatient cases worldwide are of unknown aetiology (Lanata *et al.*, 2002). Because an etiologic agent is not detected for such a large portion of patients with diarrhoea, there exists the possibility that a proportion of the undiagnosed illnesses may be attributed to *Shigell*a or EIEC undetectable by conventional (culture) technique. The aim of this study was to determine the prevalence of *Shigella* spp and EIEC using conventional culture method and molecular technique so as to provide data for future vaccine development and other control strategies.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 STUDY SITE

This study was incorporated within an on-going diarrhoea surveillance study in Asembo in Rarieda (formally in Bondo) District and Gem in Siaya District in Nyanza province, western Kenya (appendix A). This is a rural community bordering Lake Victoria and homogeneously populated by the Luo ethnic community. Fishing, raising cattle, poultry and subsistence farming are main occupations. Rainfall is biannually seasonal occurring generally from March to May and from October to December. Endemic diseases in this region include malaria, schistosomiasis and a high rate of HIV transmission. Malnutrition is also common (Kwena *et al.*, 2003; Phillips-Howard *et al.*, 2003b). For this study, three health centres (Abidha, Lwak and Ongielo) were selected from Asembo and four (Akala, Aluor, Njenjra and Yala) from Gem. Also included were the two district hospitals located in Bondo and Siaya towns (see the map in Appendix A).

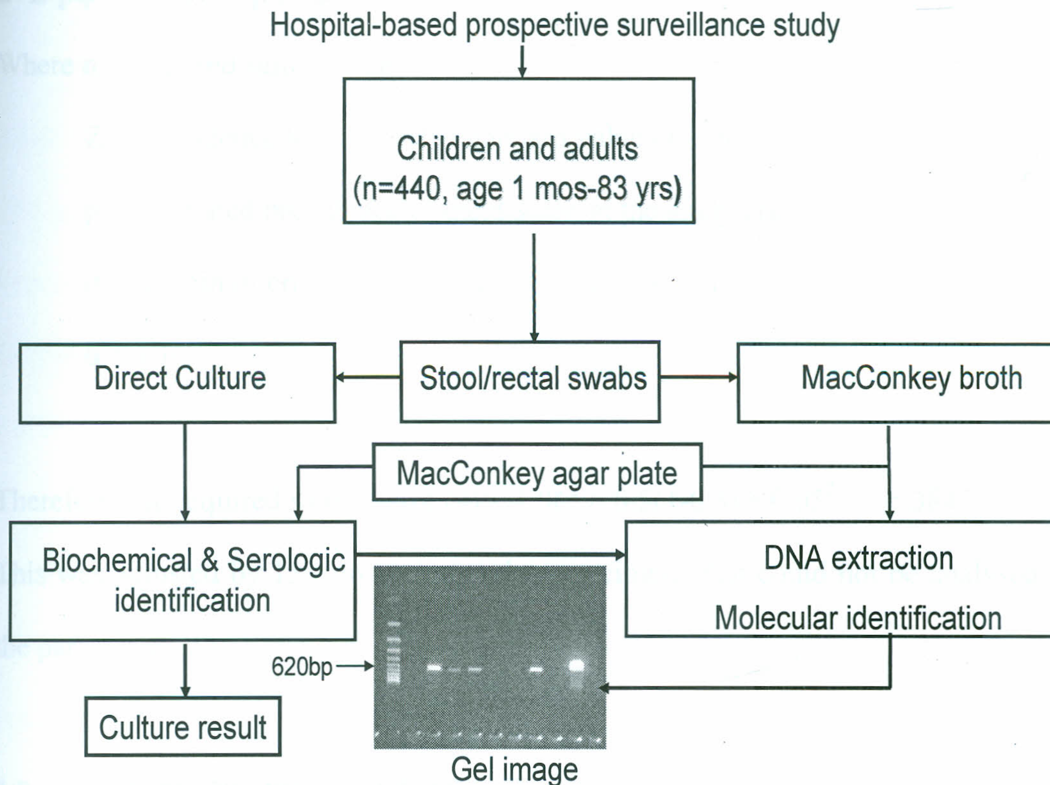
3.2 STUDY POPULATION

Asembo and Gem together have an estimated population of 125,000 people within an area of approximately 500Km^2 (Phillips-Howard *et al.*, 2003a). During the two months (April – May 2007) of this study, 440 patients of all ages who presented with diarrhoea (three or more loose stools within the last 24 hours) were enrolled.

3.3 STUDY DESIGN

This was a surveillance study that prospectively enrolled all consenting patients presenting with diarrhoea at the outpatient health facilities, and all children aged ≤ 5 years admitted to the two district hospitals. Clinical information was collected on all enrolled patients using a standard questionnaire (Appendix B). In the prospective analytical part of the study, the recovery or detection of *Shigella* spp using direct culture, MacConkey broth, and *ipaH* PCR assay from MacConkey broth were compared. Frequencies of detecting the pathogens from different types of stool were also calculated. The study design is summarized below in figure 1

Figure 1 Summary of the study design



3.4 SAMPLE SIZE

A study by Brooks *et al* estimated that 16% of diarrhoea illnesses are caused by *Shigella* spp (Brooks *et al.*, 2006), when tested by conventional culture methods. However, for this study, 50% prevalence was used to calculate the sample size since no previous study has reported the use of molecular diagnostic methods to determine the prevalence of *Shigella* and EIEC in this region. The sample size was calculated using the following

formula for sample size calculation for prevalence studies (Daniel, 1999; Cornfield, 1951).

Formula:

$$\mathbf{n=Z^2pq/d^2 \quad = Z^2 \times p\,(1\text{-}p) \div d^2}$$

Where **n** = required sample size

Z = confidence level at 95% (standard value of 1.96)

p = estimated prevalence of the disease in the study area

d = margin of error at 5% (standard value of 0.05)

q = 1-p

Therefore, the required sample size (n) = $1.96^2 \times 0.5(1-0.5) \div 0.05^2 = 384$

This was adjusted by 15% to accommodate specimens that could not be analysed for all the parameters.

3.5 SAMPLING PROCEDURE

3.5.1 Recruitment

In this study, patients of all ages presenting at any of the six clinics with diarrhoea (defined as ≥ 3 loose stools in 24 hours period within the preceding 5 days) were identified, consent obtained and enrolled by a trained health facility recorder (HFR) assigned in every health facility. Enrolled patients were assigned a patient identification number, which consisted of the first three letters of the health facility name and a four-digit number. The patient's age, sample appearance, and disease symptoms were obtained

by the HFR through questionnaires on sample collection form 1 (Appendix B). Thereafter, the patient was requested to provide a stool specimen. Patients or caretakers who did not give informed consent were excluded from the study. Diarrhoea in this study was defined as bloody if blood was visible in the specimen or if the patient (or their caretaker) reported seeing blood in their stool.

3.5.2 Sample Collection

The patients were provided with a wide mouthed stool cup, two plastic paper bags and tongue depressors or applicator sticks and given instructions to collect the specimens by themselves. Rectal swabs were taken by trained health facility recorders (HFR) from patients who were unable to provide a whole stool specimen. Two swabs were made from every whole stool specimen and placed by the HFR, immediately, in Cary Blair transport medium. Both the swab and the whole stool in cups were kept cool (in a cool box containing cold ice packs) for same day's transport to CDC/KEMRI microbiology laboratory situated near the provincial headquarter, Kisumu City (Appendix A). All the specimens were accompanied to the laboratory by the sample collection forms and were processed within 24 hours after collection.

3.6 SPECIMEN PROCESSING

3.6.1 Stool Culture

The laboratory analysis included direct culture and broth inoculation of the faecal samples collected. A swab from each stool sample was inoculated on MacConkey agar (MAC) and Xylose lysine desoxycholate (XLD) agar plates (direct plating). The second

swab was inoculated to 10mL volume of single strength MacConkey broth in accordance with manufacturer's instruction. The inoculated broth media were then sub-cultured, after 18-24 hours aerobic incubation, on MAC plates. The plates were incubated at 35 – 37°C aerobically for 18 – 24 hours. Colonies suspicious of *Shigella* spp or EIEC, obtained on MAC and XLD plates, were selected for further identification using standard laboratory procedures (Perilla *et al.*, 2003). *Shigella* spp were confirmed serologically using the *Shigella* polyvalent "O" group antisera then serotyped using the monovalent antisera (Denka Seiken, Tokyo-Japan). Isolates which failed to react with the *Shigella* antisera were tested biochemically for mucate fermentation and acetate utilization to identify EIEC strains (Farmer *et al.*, 1985; Ewing, 1986; Wayant *et al.*, 1995). Biotyping with Analytical profile index (API 20E) was also employed where serological tests were negative. Isolates identified as *E. coli* or as *Shigella* spp biochemically were tested by PCR to detect the presence *ipaH* gene. The results obtained were recorded on worksheets and transferred into a laboratory record book and computer spreadsheet.

3.6.2 *ipaH* PCR for Detection of *Shigella* and EIEC Strains

3.6.2.1 *Preparation of the template*

The *ipa*H PCR was performed from the 18 – 24 hour old MacConkey broth previously inoculated with the stool specimen and incubated at 35 – 37°C aerobically. A volume of 300µL of the broth was transferred into a 0.65mL tube Eppendorf tube and boiled at 95°C for 10 minutes to denature the bacteria and release the DNA. The specimens were then centrifuged at 10000rpm for 10 minutes. About 200µL of the supernatant was transferred

to a sterile 1.5mL Eppendorf tube and kept frozen at -86^{0}C. The DNA extracts were used as the template (sample) for testing the presence of the *ipaH* gene by PCR (Appendix C).

3.6.2.2 *Preparation of the master mix*

Primers for amplification of the *ipaH* gene, 620bp amplicon, were obtained from CDC, Biotechnology core facility in Atlanta, Georgia-USA (see primer sequence in appendix C). The primers used were IPAH15B (forward primer) and IPAH8B (Reverse primer) (Youssef *et al.*, 2000). Briefly, a typical 25µL reaction mixture consisted of 1µL primer mixture (0.5 µ L of forward primer and 0.5µL of reverse primer) o f 100µM primer concentration, 1µL of sample DNA template and the final volume adjusted to 25µL with sterile molecular grade water in a tube containing a Ready-To-Go PCR bead (GE Healthcare UK Limited, Little Chalfont UK). When each bead is rehydrated, the mixture contains $MgCl_2$, enzyme (PuReTaq DNA polymerase), dNTPs, stabilizers, buffers and BSA (Amersham Biosciences, UK Limited, 2004).

3.6.2.3 *PCR and gel documentation*

Amplification was carried out using Eppendorf Mastercycler (Eppendorf AG. 22331 Version 2.30.33-09, Hamburg - Germany). The cycling conditions included an initial pre-incubation at 95°C for 75 sec, template denaturation at 95°C for 15 sec, annealing at 65°C for 45 sec, and extension at 72°C for 45 sec for a total of 35 cycles. Final extension occured at 72°C for 10 min. The DNA fragments were separated in 1.8% agarose gel prepared as a mixed 1.1% Metaphor agarose (Cambrex BioScience, Rockland, ME USA) and 0.7% SeaKem GTG agarose (Cambrex BioScience, Rockland, ME USA) in 1X TBE.

Gels were stained with 1μg/μl ethidium bromide solution, visualized and image captured using a gel documentation system (UVP BioImaging Systems, Cambridge-United Kingdom) (appendix C).

3.7 DATA COLLECTION AND MANAGEMENT

3.7.1 Data Entry and Storage

Patients identified at each health facility were interviewed by a trained health facility recorder (HFR). The patients or their caretakers were requested to provide information on patients' names, age, date when diarrhoea started, and stool appearance. The responses were recorded on a scannable questionnaire form (Appendix B). This form accompanied the sample to the laboratory. The forms were scanned using a commercial software package (Cardiff TELEform Software, 1991-2003). Scanned forms were reviewed and corrected where necessary using the TELE form software. An Excel spreadsheet for laboratory test results was prepared in the laboratory and exported to SAS for analysis. The soft copy of the data was protected using a password while the hard copies were stored in data storage room only accessible to the investigator.

3.7.2 Reporting Results

Culture results were compiled and reported back to the clinicians as soon as they became available in any case not later than seven days after sample collection. However, the PCR results were not available immediately and could not have been used for daily patient management.

3.7.3 Data Analysis

Data were analyzed using SAS computer software (version 9.1, SAS Institute Inc., Cary, NC, USA). Chi-square statistics (Cochran-Mantel-Haenszel Statistics) was used to measure associations between diarrhoea types and culture results, and also diarrhoea types and PCR results. A *p*-value (two tailed) of less than 0.05 was considered significant at 95% confidence interval. Prevalence of the different serogroups and serotype were determined using descriptive statistics.

3.8 ETHICAL CONSIDERATIONS

3.8.1 Risks

There was no risk associated with obtaining a stool specimen using the procedure described for this study. However, obtaining a rectal swab may have caused some pain but the pain was minimal. Providing a stool sample could have been embarrassing, to some clients, but posed no medical risk.

3.8.2 Benefits

The culture and sensitivity results obtained from the specimen were sent back to the respective clinics to help clinicians manage treatment of diarrhoea patients better.

3.8.3 Informed Consent

Informed consent was obtained from all enrolled patients for the diarrhoea surveillance study. A consent form was explained to the client, if an adult, and translated into a language they understood. The patient or the caretaker was asked to sign on the

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appropriate consent form (Appendix D, E or F) if he/she agreed to participate in the study. In the case of minors, their parents or guardians gave consent on their behalf prior to participation in the study. Children who could assent were requested to do so before enrolment. The consent forms were forwarded to the laboratory accompanied with the faecal specimen.

3.8.4 Approval.

This study was done under the KEMRI approval of epidemiologic and laboratory surveillance for diarrhoeal diseases in western Kenya – CDC study protocol number 2616 and by the School of Graduate Studies (SGS), Maseno University.

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CHAPTER FOUR

4.0 RESULTS

4.1 SPECIMEN CHARACTERIZATION

During the two months of surveillance, April and May 2007, a total of 440 stool specimens were collected from patients, who presented with diarrhoea at any of the nine health facilities. The median participant age was 3 years with an average age of 15 years (range 1 month to 83 years). Children aged ≤ 5 years old constituted 54.1% of the patients. The mean duration of diarrhoea before the patients presented to a clinic for treatment was 3.5 days (range 1 – 23). Diarrhoea days of ≤ 3 days fell within the median quartile and ≤ 4 within the third quartile. On the other hand, an average number of stools produced was 5.1 stools (range 3 – 20) within 24 hours and a frequency of ≤ 4 stools per day fell within the median quartile (Table 1).

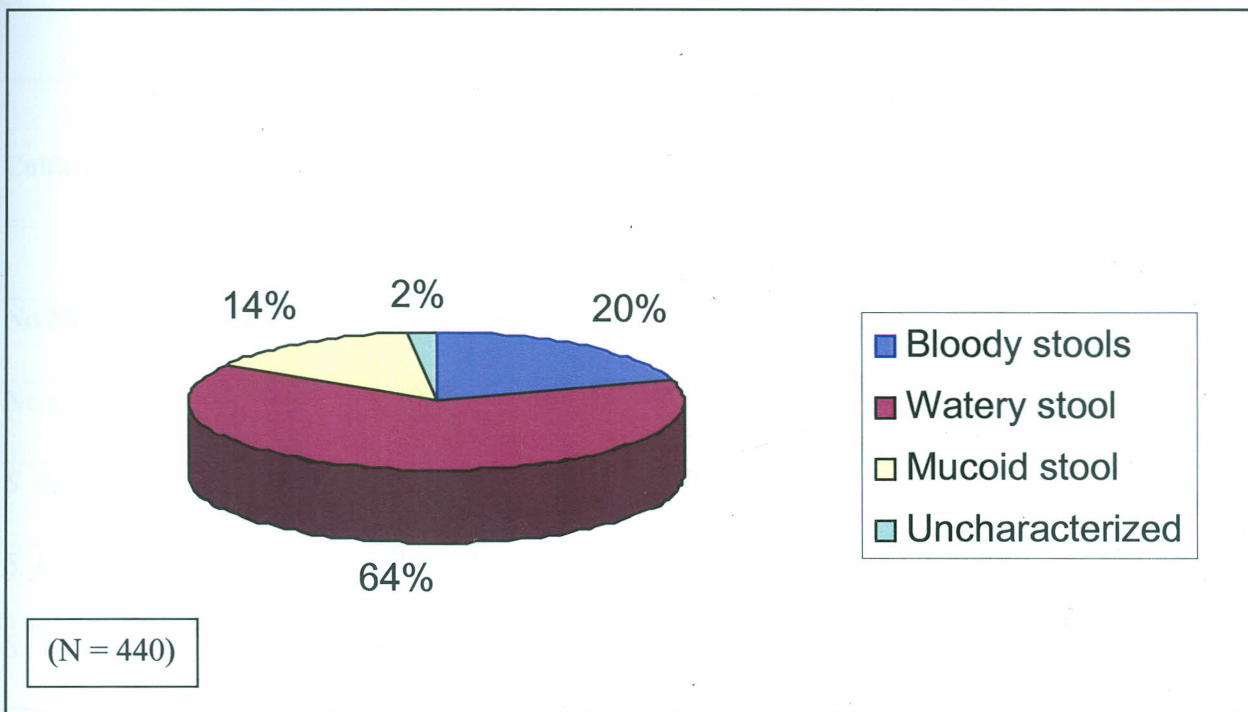
Table 1 Characteristics of diarrhoea patients enrolled from selected health facilities in Asembo and Gem community from April to May 2007

Characteristics		Number (%)
Age	< 5	238 (54.1)
	≥ 5	202 (45.9)
	Mean age	15 years
Diarrhoea	Mean frequency of diarrhoea[a]	5.1
	Mean duration of diarrhoea[b]	3.5

Key: [a] number of stools passed in a 24 hour period
[b] number of diarrhoea days before visiting the health facility for enrolment.

Of these specimens, 87 (20%) were from patients with bloody diarrhoeal illnesses, 283 (64%) from persons with watery diarrhoeal illnesses, 62 (14%) from persons with mucoid diarrhoeal illnesses, and 8 (2%) from persons with uncharacterized diarrhoeal stools (Figure 2).

Figure 2: Appearances of stool specimens from patients presenting with diarrhea



4.2 CULTURE RESULTS

From the bacteriological culture, 48 (10.9%) of the 440 directly plated specimens yielded *Shigella* spp (Table 2). However, the isolation and identification rate from the MAC broth was low [9 (2%)]. Of the 48 *Shigella* isolates, 12 (25%) were *S. dysenteriae* non-type 1, 27 (56%) were *S. flexneri*, 4 (8%) were *S. boydii,* 4 (8%) were *S. sonnei,* and 1

(2%) ungroupable *Shigella spp*. The recovery rate of *S. dysenteriae* non-type 1 and S. *flexneri* from stool specimens was significantly higher by direct plating compared to MacConkey broth (p-value = 0.019 and p-value <0.001 respectively). However, there was no EIEC isolated from the specimens using both methods.

Table 2: Isolates obtained by direct plating and broth culture methods from patients with diarrhoea from selected health facilities in Asembo and Gem community from April to May 2007

Culture Result	**Direct plating, N = 440**	**MacConkey broth, N = 440**	***p* – value**
	Frequency, n (%)	**Frequency, n (%)**	
No *Shigella*, No EIEC	390 (88.64)	429 (97.50)	<0.001
No growth	2 (0.45)	2 (0.45)	1.0
S. dysenteriae non-type 1	12 (2.73)	3 (0.68)	0.019
S. flexneri	27 (6.14)	5 (1.14)	<0.001
S. boydii	4 (0.91)	0 (0.00)	0.06
S. sonnei	4 (0.91)	1 (0.23)	0.186
Shigella spp	1 (0.23)	0 (0.00)	0.5
EIEC	0 (0.00)	0 (0.00)	

Various *Shigella* serotypes were isolated and identified indicating heterogenecity in the in their prevalence. *S. dysenteriae* serotype 2, and *S. flexneri* serotytypes 2a, 2b and 6 were the most commonly isolated pathogens (Table 3).

Table 3: Serogroups and serotypes of Shigella species identified by culture from patients with diarrhoea from selected health facilities in Asembo and Gem community

Serogroups (%) n=48	Serotype	Frequency	Percent (n=48)
S. dysenteriae (25.0)	Type 2	9	18.75
	Type 3	1	2.08
	Type 4	2	4.17
S. flexneri (56.3)	1a	3	6.25
	1b	2	4.17
	2a	5	10.42
	2b	7	14.58
	3a	1	2.08
	4a	2	4.17
	6	7	14.58
S, boydii (8.4)	C	3	6.25
	C3	1	2.08
S. sonnei (8.3)	Phase I	1	2.08
	Phase II	3	6.25
Untypable (2.1)		1	2.08

Stool appearances and serotypes of *Shigella* isolated from each specimen was also analysed. *S. dysenteriae* type 2 was the most common serotype isolated from bloody

diarrhoea specimens cultured [7 (8%) out of 87] (Table 4). There was no isolate from the uncharacterized stool.

Table 4: Serotypes of *Shigella* spp isolated from faecal specimens of different appearance from patients with diarrhoea from selected health facilities in Asembo and Gem community from April to May 2007

Bacteria Serotypes, n=48	Number (%) of stool characteristics			Total
	Bloody n = 87	**Mucoid** n = 62	**Watery** n = 283	
S.dysenteriae type 2	7 (8.05)	1 (1.61)	1 (0.35)	9
S.dysenteriae type 3	1 (1.15)	0	0	1
S.dysenteriae type 4	1 (1.15)	1 (1.61)	0	2
S. flexneri 1a	2 (2.30)	0	1 (0.35)	3
S. flexneri 1b	1 (1.15)	0	1 (0.35)	2
S. flexneri 2a	3 (3.45)	1 (1.61)	1 (0.35)	5
S. flexneri 2b	2 (2.30)	2 (3.23)	3 (1.06)	7
S. flexneri 3a	1 (1.15)	0	0	1
S. flexneri 4a	2 (2.30)	0	0	2
S. flexneri 6	3 (3.45)	2 (3.23)	2 (0.71)	7
S. boydii C	1 (1.15)	0	2 (0.71)	3
S. boydii C3	1 (1.15)	0	0	1
S. sonnei Phase I	0	0	1 (0.35)	1
S. sonnei Phase II	2 (2.30)	0	1 (0.35)	3
Untypable	0	0	1 (0.35)	1

4.3 DETECTION OF SHIGELLA SPECIES AND EIEC BY *ipaH* PCR

One of the objectives of this study was to detect *Shigella* spp and EIEC using PCR technique. The extracted DNA of the bacteria, from faecal specimens pre-inoculated in MAC broth, was subjected to amplification for the *Shigella* and EIEC specific *ipaH* gene. Of the 421 specimens tested in this study, 78 (18.5%) specimens were positive for the *ipaH* gene, 12 (2.9%) were weakly positive and 331 (78.6%) were negative for the target gene. PCR detected all (100%) the *Shigella* species isolated and identified by standard culture method. In addition, 30 out of 421 (7.1%) more cases, which were not found by culture, were detected by PCR (table 5).

Table 5: Comparison between conventional culture results and *ipaH* PCR results obtained from diarrhoea patients

Technique	Results	
	Positive, n (%)	**Negative, n (%)**
Direct culture	48 (10.9)	392 (89.1)
ipaH PCR	78 (18.5)	331 (78.6)
	OR, 1.92; 95% CI, 1.28-2.89; *P*<0.001	

Of the 421 specimens tested, 85 (20.19%) were bloody, 60 (14.25%) were mucoid, 268 (63.66) were watery, and 8 (1.9%) were uncharacterized faecal specimens (Table 6).

Table 6: Stool appearance and *ipaH* PCR results for patients with diarrhoea from selected health facilities in Asembo and Gem community from April to may 2007

Specimen Appearance	Number (%) of Specimens with PCR Results n=421			Total
	Positive	Weakly positive	Negative	
Bloody	30 (35.3%)	5 (5.9)	50 (58.8)	85
Mucoid	14 (23.3)	0	46 (76.7)	60
Watery	33 (12.3)	7 (2.6)	228 (85.1)	268
Uncharacterized	1 (12.5)	0	7 (87.5)	8

The rate of detection by *ipaH* PCR was significantly higher for bloody faecal specimens (Odd ratio, 3.5; $P<0.0001$) and significantly less for watery diarrhoea (Odds ratio, 0.3; $P<0.0001$). The detection of *ipaH* was not statistically significant for the other types of diarrhoea. Of the 85 bloody specimens, 30 (35.29%) were positive and 5 (5.9%) were weekly positive by PCR compared to 33 (12.3%) and 14 (23.3%) positive from watery and mucoid diarrhoea specimens, respectively.

CHAPTER FIVE

5.0 DISCUSSION

Diarrhoea remains one of the most common illnesses in children and one of the major causes of infant and childhood mortality in developing countries. Considering the usual scanty resources available in the developing countries, a reduction of diarrhoea-related mortality may be possible by identifying the most common causative agents and high-risk subjects and targeting them for intensive intervention.

In Kenya, diarrhoea is the fourth leading cause of mortality among children under five years and accounts for 40% of outpatient cases. This study was clinic-based and had more children aged ≤ 5 years (54.09%) than the other population which likely reflects the greater burden of diarrhoeal illness in young children. In this rural community, *Shigella* species, particularly *S. flexneri,* predominated as the cause of diarrhoeal illnesses varying from watery to bloody diarrhoea. Previous studies also showed that organism remains the major cause of diarrhoea in patients of all ages (Brooks *et al.*, 2006).

This study showed that the isolation and identification rate from the MAC broth was low [9 (2%)] compared to direct culture method [48 (10.9%) p-value < 0.001)]. This could be due to overgrowth of enteric flora which could have masked the growth *Shigella* spp and EIEC and made typical colony appearances difficult to recognize and select for subsequent identification procedures. There was also heterogenecity in the *Shigella* serotypes causing diarrhoea in rural western Kenya region with *S. flexneri* serotypes 2a, 2b and 6 being dominant which is consistent with previous studies (Brooks *et al* 2006).

Although *S. dysenteriae* type 1 is the major cause of bloody diarrhoea in outbreak situations, this study was carried out during low reporting diarrhoea season and most of the bacteria identified are not usually associated with outbreaks but are endemic etiologic agents of diarrhoea. The results presented here show that *S. dysenteriae* type 2 (18.75%) was the most prevalent serotype of *S. dysenteriae* and a major cause of diarrhoea in this region (Table 4). Bloody specimens were more likely to yield a *Shigella* spp (56.25%, Odds ratio, 7.1; $P<0.0001$) by culture compared to other types of stool and a similar trend was also observed by *ipaH* PCR (Odds ratio, 3.5; $P<0.0001$) which is consistent with studies done in Vietnam (Thiem *et al.*, 2004). Clinical shigellosis is serotype specific and, presumably, associated with immune responses to the bacterial lipopolysaccharide, a somatic antigen (O-antigen), and confers protection against the homologous serotype but the duration of the protective effect is thought to last for at least 5 to 7 months after infection (Lerman *et al.*, 1994). Serotype diversity due to the variations in O-antigen likely facilitates evasion of the host humoral immune response. This supports the existence of asymptomatic carriers and why the disease is endemic in this region. A case control study would be necessary to determine the prevalent rates in the non-diarrhoea persons and serum level antibodies between episodes. Though controls were not enrolled in this study, the finding suggests that a shigellosis vaccine may need to be a cocktail of antigens from several *Shigella* species and/or serotypes in order to have a major epidemiological impact.

MacConkey broth supports the growth of both *Shigella* spp and other coliform bacteria. The coliform bacteria (normal flora) are often found in large numbers compared to

Shigella spp (pathogens) in faecal specimens. The detection of bacterial genes also depends on the quantity and quality of the genetic material amplified. In a study conducted among patients with diarrhoea in Vietnam, the number of PCR cycles required to detect a PCR product, which is inversely related to the DNA load in the specimen, was highest (over 36 cycles) for patients with culture-negative, non-bloody diarrhoea and lowest (25 cycles) for those with culture-positive, bloody diarrhoea (Thiem, V.D. *et al.*, 2004). Another study conducted in India also showed that detection of *Shigella* species by *ipaH* PCR decreased in the presence of a high level of background flora and in the presence of natural inhibitors found in stool samples when DNA was prepared directly from the faecal specimen (Dutta *et al.*, 2001). In the current study, MacConkey broth was used to cultivate the bacteria in order to increase their population in the medium and therefore increase the chances of detection by molecular testing while reducing detection of non-viable bacteria. The broth was also used to reduce the concentration of the natural PCR inhibitors present in the stool specimen. However, the broth did not increase the chances of identifying the bacteria when sub-cultured possibly attributable to an overgrowth of competing coliform bacteria. This may explain why the isolation and identification rate by direct culture of faecal specimens was higher (10.9%) compared to indirect culture (2%) from the subculture of the broth.

EIEC causes diarrhoea in a similar manner to *Shigella* species. However, it has been reported to be less prevalent (less than 1.5%) compared to other enteropathogens in shigellosis endemic areas (Youssef *et al.*, 2000; Gaudio *et al.*, 1997; Dutta *et al.*, 2001). A previous study reported low prevalence of EIEC (0.7%) compared to other

diarrhoeagenic *E. coli* strains identified by convention methods from diarrhoea patients in rural western Kenya. From this study no EIEC strain was identified by conventional culture method which is in consistent with previous reports that showed the low prevalence of EIEC strains as a cause of diarrhea compared to other enteropathogens (Brooks *et al.,* 2006; Vargas *et al.*, 2004). However, a longer surveillance study using molecular methods would provide valuable information on the EIEC epidemiological patterns.

Studies of the epidemiology of shigellosis have generally relied on standard culture methods of diagnosis. In this study, a PCR system based on the *ipaH* gene was used and compared with standard bacteriologic methods to determine the prevalence of shigellosis and EIEC among patients presenting with diarrhoea. The distinctive property of the *ipaH* gene is that it is the first recognized multicopy antigen gene of *Shigella* species that is unique to the *Shigella* genus and enteroinvasive *E. coli* (Venkatesan *et al.*, 1989). The potential advantage that this PCR system offers over standard culture methods is the improved sensitivity. The assay was extremely reliable; being able to detect 48 out of 48 (100%) culture confirmed Shigella infections. This PCR assay also identified and confirmed a *Shigella* species which was only identified biochemically and could not be confirmed serologically. This untypable strain would have remained undiagnosed had this assay not been used. Furthermore, it also detected 30 more cases which could not be identified by culture, thus detecting the presence of *Shigella* spp and/or EIEC in 18.5% (78 of 421) of diarrhoea cases tested. This finding also concur with a previous study India which reported 15.3% prevalence from diarrhoea cases even though Luria broth was used

as an enrichment broth instead of MacConkey broth (Dutta et al., 2001). Out of 440 specimens, the frequencies of *S higella* dysentery cases were 6.1%, watery diarrhoea 3.2%, and mucoid diarrhoea 1.6% by direct culture method; but when *ipaH* PCR was employed, the frequencies were found to be 7.1%, 7.8% and 3.3% for bloody, watery and mucoid diarrhoea, respectively. Patients who have the most severe form of shigellosis or EIEC infection also shed the most organisms in stool. A direct relationship exists between bacterial load, detection by culture and number of PCR cycles required to detect the PCR product. The data presented in this report shows that 2.9% cases exhibited weak bands after amplification indicating low quantity of the PCR product which could be a result of low quantity of DNA in the template and reflects low bacterial load in the specimen.

The data presented in this study show the prevalence of bacterial diarrhoea of 10.9% and 18.5% by culture and *ipaH* PCR, respectively. The molecular technique almost doubled the rate of detection (OR = 1.92, $p < 0.0001$) when compared by conventional culture method. In a study done in Thailand (Gaudio *et al.*, 1997), the *ipaH* PCR was shown to increase the rate of detection of *Shigella* species or EIEC by 37% over standard culture techniques. However, the later study used PCR directly on the faecal specimens. In this report, data was collected during the low diarrhoea season (Phillips-Howard *et al.*, 2003b) and the faecal specimen was diluted to reduce the concentration of non-viable cells. The detection rates could be higher if the specimens were collected during the dry seasons which mark the higher peaks of diarrhoea incidences.

Most people who are infected with *Shigella* develop diarrhoea, fever, and abdominal cramps. Severity of the disease ranges from mild to very severe diarrhea. The incubation period varies from 1 – 3 days (DuPont, 1995; MMWR Surveillance Summaries, 1996), after which the patient experiences a sudden onset of symptoms of the disease. In this study, the data collected indicates that about a half of patients presented at the health facility for treatment in ≤ 3 days after the onset of diarrhoea and an average of 5 stools in 24 hours period. This confirms the acuteness and severity of shigellosis which is a major cause of diarrhoea in developing countries. However, there was no significant statistical correlation between duration of diarrhoea or frequency of diarrhoea and *Shigella* serogroups (p-value > 0.05). A similar trend was observed when comparing the duration of diarrhoea and detection of *ipa*H gene by PCR. Nevertheless, there existed a significant correlation between the PCR result and the frequency of diarrhoea within an episode ($r = 0.76$, p-value <0.05). This could be attributed to the fact that the higher the infection, the more the frequency of diarrhoea and the more the chance that the PCR technique would detect the target gene. This could not be strongly observed from culture technique probably because of the antimicrobials taken before stool specimen was collected and overgrowth of normal flora.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The results of this study indicate that *ipaH* PCR technique is a powerful molecular diagnostic tool for detecting *Shigella* species and EIEC in epidemiologic investigations in areas where the two are endemic. It is likely that when the organisms were detected by PCR but not by culture, other factors were involved such as low inoculum, overgrowth of commensal bacteria, or adverse culture conditions that contribute to the variation in detection. Such sensitive molecular detection is especially important for Shigellae, since they can produce disease with as few as 10 to 100 organisms. Assuming that the majority of the patients with PCR-positive or weakly positive but culture-negative diarrhoea are infected with *Shigella* spp or EIEC, infections with these organisms may be responsible for over 20% of the diarrhoea episodes in rural western Kenya. This is much more than was previously thought on the basis of the conventional culture methods alone. Further, the PCR technique was able to detect a *Shigella* species which could not be identified serologically. This strain might represent a new serotype of *Shigella* which could have been missed if the PCR system had not been used. Thus, the *ipaH* PCR may be judged as superior for its sensitivity and specificity.

Bloody specimens, compared to other types of stool, were more likely to yield a *Shigella* spp by culture and *ipaH* PCR. In this study, the information on sample appearance was provided by the patients or their caretakers hence the information might not be accurate. In addition, stool samples from comparable control population were not screened to

assess the extent of asymptomatic carriage of the bacteria. Lastly, the concentration of natural PCR inhibitor and unviable bacterial cells present in stool specimen were not determined to estimate their effects on the PCR results.

6.2 RECOMMENDATIONS

a) From this study, introduction of *ipaH* PCR technique is highly recommended in order to augment the conventional culture techniques and thus to improve the quality of detection and clinical diagnosis of the bacterial pathogens in health institutions and during field surveillance. The *ipaH* assay system, which amplifies sequence present on both the invasion plasmid and the chromosome, has been shown to detect *Shigella* and EIEC organisms that have lost the invasion-essential plasmid and are therefore considered non-invasive. Hence this assay is likely to identify false-negative strains as determined by the invasion plasmid-dependent assay system.

b) A molecular diagnostic study targeting the invasion plasmid of *Shigella* and EIEC should be carried out in western Kenya to determine the proportion of the invasive strains and changes in the epidemiology of shigellosis in the community. In endemic areas, acquired immunity may attenuate symptoms leading to high asymptomatic infection rates. Since humans are the only known host of the *Shigella* spp, asymptomatic cases should be investigated to determine the carriage rate of the bacteria in the population.

c) Efforts to prevent diarrhoeal illnesses through basic measures should be strengthened by developing and introducing a *Shigella* vaccine to reduce the high infection rates.

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