PREVALENCE OF SELECTED BACTERIAL AND VIRAL ENTERO-PATHOGENS IN CHILDREN LESS THAN 5 YEARS OF AGE IN LIMPOPO PROVINCE, SOUTH AFRICA

by

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(11640355)

A Dissertation Submitted in Fulfilment of the Requirements for the Degree Masters of Science in Microbiology (MSc MB)

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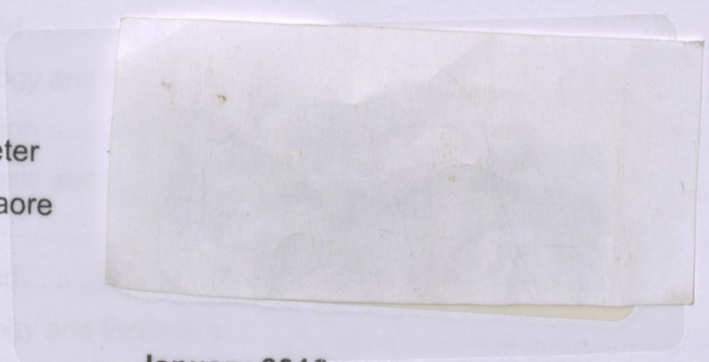
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DECLARATION

I, Solanka Ellen Ledwaba (Student no: 11640355), hereby declare that the dissertation for the Masters of Science Degree in Microbiology (MSc MB) at the University of Venda, hereby submitted by me, has not previously been submitted for a degree at this or any other university, that it is my own work in design and execution, and that all reference material contained therein has been duly acknowledged.

Signed: Ledwaba S.E

SE Ledwaba

Date: 22/April/2016

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DEDICATIONS

- I dedicate this study to my grandmother Mrs Jenet Mashaba and my parents Mr Piet and Mrs Lydia Ledwaba for supporting me and believing in me;
- My family members Martina and Juwaki for your moral support. You were pillars of strength throughout the study.

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ABSTRACT

PREVALENCE OF SELECTED BACTERIAL AND VIRAL ENTERO-PATHOGENS IN CHILDREN LESS THAN 5 YEARS OF AGE IN LIMPOPO PROVINCE, SOUTH AFRICA

by

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Supervisor: Prof N Potgieter

Co-supervisor: Dr AN Traore

Background: Enteric pathogen infections in young children less than 5 years of age are a serious health risk which leads to mortality and morbidity in several countries.

Objectives: The aim of this study was to determine the prevalence of selected bacterial (Diarrheagenic *Escherichia coli, Shigella* spp. and *Salmonella* spp.) and viral (Adenovirus 40/41, Norovirus and Rotavirus) pathogens in children from rural communities suffering from diarrhea in Limpopo province of South Africa.

Method: A cross-sectional study was conducted from July 2014 to May 2015. A total of 237 stool specimen of children less than 5 years of age presenting with diarrhea were collected from Primary Health Care facilities in various rural communities of the Vhembe district in Limpopo province, South Africa. Bacterial DNA from stool samples were extracted using QIAamp fast DNA stool mini kit; published m-PCR protocol was used to detect *E. coli, Shigella* spp. and *Salmonella* spp. Two different conformational m-PCR protocols were used to detect Diarrheagenic *E. coli* and another to detect *S. typhimurium, S. enteritidis, S. dysenteriae* and *Shigella* spp. Rotavirus Group A, Norovirus and adenovirus 40/41 were detected using a commercially available Enzyme Immune Assay (EIA) protocols.

Results: Diarrheal pathogens were detected in 62.4% of the 237 participants. The following pathogens were detected ETEC (30.8%), EAEC (26.2%), EIEC (7.6%), atypical EPEC (15.6%), typical EPEC (20.7%), EHEC (0.8%), *S. flexineri* (4.2%), *Shigella* spp., (1.3%), *S. enteritidis* (1.3%) and *S. typhimurium* (0.4%), Rotavirus (10.1%), Norovirus (8.7%), Adenovirus 40/41 (7.6%). Bacteria and bacteria co-

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infections were more prevalent (26.2%). More male participants were infected with an enteric pathogen than females (51.1% vs 43.7%). Most of the enteric pathogens were detected in children less than 2 years of age (56%). Although no clear seasonal pattern was established; bacterial infections were observed throughout the year and enteric viruses were detected in dry cooler months.

Conclusion: The findings of this study clearly revealed that enteric pathogens are etiological agents of diarrhea in children less than 5 years of age in the Vhembe district. The high detection rates of DEC and other enteric pathogens clearly indicate the need to improve sanitation and hygiene in these rural communities of Limpopo province, South Africa. Continuous epidemiological studies must be done to determine the infection and health risks of enteric pathogens.

Key words: Diarrhea, enteric pathogens, Limpopo province, Adenovirus 40/41, Rotavirus, Norovirus, Diarrheagenic *E. coli*, *S. enteritidis*, *S. typhimurium*, *S. flexneri*, *Shigella* spp.

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LIST OF ABBREVIATIONS

A	:	Abdominal pain
Ad	:	Adenovirus
D	:	Diarrhea
DEC	:	Diarrheagenic *E. coli*
Deh	:	Dehydration
E. coli	:	*Escherichia coli*
aEPEC	:	Atypical-Enteropathogenic *E. coli*
tEPEC	:	Typical-Enteropatogenic *E. coli*
EAEC	:	Enteroaggregative *E. coli*
EHEC	:	Enterohaemorrhagic *E. coli*
EIEC	:	Enteroinvasive *E. coli*
EPEC	:	Enteropathogenic *E. coli*
ETEC	:	Enterotoxigenic *E. coli*
F	:	Fever
LT	:	Heat labile toxin
m-PCR	:	Multiplex- Polymerase Chain Reaction
PBS	:	Phosphate buffer saline
RT-PCR	:	Reverse-Transcriptase- Polymerase Chain Reaction
spp.	:	Species
ST	:	Heat stable toxin
Stx	:	Shiga Toxin
V	:	Vomiting
WHO	:	World Health Organization

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Chapter 1

INTRODUCTION

1.1. RATIONALE OF THE STUDY

Gastroenteritis in children is still a major global problem and more than 800 000 children die every year due to diarrheal diseases (Liu et al, 2012). The World Health Organization (WHO) listed three main causes of deaths in children under the age of 5 years as pneumonia, diarrhea and malnutrition (WHO, 2014). In developed countries such as United States of America (USA), it has been estimated that 21 to 37 million episodes of diarrhea occur in children less than 5 years of age annually; 10% of these cases are treated as outpatients while more than 200 00 cases require hospitalization and between 325 to 425 children die from diarrhea (Glass et al, 1991). In developing countries, the incidence of childhood deaths due to diarrhea are higher than 4 million per year (Glass et al, 1991). Parashar and colleagues (2003) reported that during 1986 and 2000; an estimated 1.4 billion children less than 5 years of age were infected with diarrhea every year in developing countries of which 123.6 million cases required outpatient medical care while 9 million required hospitalization.

In sub-Saharan Africa, children are 15 times more likely to die before the age of five due to diarrheal diseases than children living in developed countries (Kotloff et al, 2013). In addition many of these children still have recurrent diarrhea every year due to exposure to enteric pathogens which occur particularly during the weaning period (Motarjemi et al, 1993) and contributes to the infection-malnutrition cycle leading to impaired growth and development (Farthing, 2000). Children less than five years of age are generally more susceptible to intestinal infections due to their less developed immune system (Stauber et al, 2013).

Mwenda and colleagues (2010) have shown that 90% of an estimated 2.2 million of deaths which are caused by diarrheal infections in children less than 5 years of age are related to poor sanitation and hygiene aspects. Other risk factors of childhood diarrhea related deaths includes: non-breastfed children, unsafe drinking-water and food, poor hygiene practice and malnutrition. Transmission of enteric pathogens

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primarily occurs via faecal-oral route (O'Ryan et al, 2006). In poor rural settings people still engage in open defecation and mostly defecate in places such as fields, forests, bushes, bodies of water which results in transmission of pathogens cause diarrheal diseases (UNICEF, 2012; Galan et al, 2013). Availability of domestic or farm animals can also contribute to the spread of enteric pathogens as young children play outside in the soil or on dung smeared floors (Rosas et al, 2006; Potgieter et al, 2007).

Most of the child deaths in developing countries are due to diseases that are preventable through affordable interventions (WHO, 2014) which includes increased breastfeeding, improved weaning practices, immunizations (Kosek et al, 2003), improved water quality, building adequate sanitary facilities for safe disposal of infectious human waste and improved hygiene behaviour such as washing hands with soap, or using alcohol-based hand sanitizer (Grimwood et al, 1995). Fluid and electrolyte replacement therapy and antibiotics are also essential in treating gastrointestinal infections (Dennehy, 2005). A recent study by Kotloff and colleagues (2013) have shown that preventative measures such as vaccination and diarrhea treatment with zinc targeting enteric pathogens in sub-Saharan Africa and Asia have reduced diarrheal burden by 40% in children less than 2 years of age. Vaccination is currently available for Rotavirus infections (Angel et al, 2007; Grimwood and Lambert, 2009; Vaccines, 2013). However, strategies still need to be developed such as implementation of new vaccines targeting other enteric pathogens that have been found to cause severe diarrhea that can lead to death in young children (Walker et al, 2005; Ngcobo and Cameron, 2012).

Few studies on the prevalence of Rotavirus (Obi et al, 2003; Potgieter et al, 2010), DEC (Obi et al, 2004), *Giadia* spp. and *Entamoeba* spp. (Samie et al, 2009) in rural communities of the Limpopo province, South Africa have been published. Consequently very little is known on the prevalence of bacterial (DEC, *Salmonella* and *Shigella*) and viral (Rotavirus, Norovirus and Adenovirus) pathogens causing diarrhea in young children less than 5 years of age in the Limpopo Province (South Africa).

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1.2. PROBLEM STATEMENT

In large areas of South Africa, factors such as poor water supply, poor sanitation and poor hygiene behaviours are the leading causes of faecal-oral diseases. The Vhembe District in Limpopo province of South Africa is a rural area that is still facing many of these challenges. Poor and unsafe water use, sanitation and hygiene behaviour could expose the vulnerable to bacterial and viral enteric pathogens that cause diarrheal diseases and could result in mortality and morbidity. In South Africa, diarrhea is the third major cause of childhood mortality in children less than 5 years of age (Bradshaw et al, 2003; StatsSA, 2012). Very few studies on the prevalence of diarrheal causing pathogens have been conducted in Limpopo province of South Africa (Obi et al, 2003; Obi et al, 2004; Potgieter et al, 2010). This study is aimed at determining the prevalence of bacterial (Diarrheagenic *E. coli, Salmonella, Shigella)* and viral (Rotavirus, Adenovirus 40/41, Norovirus) enteric pathogens that affect children less than 5 years of age and its health impact on the children in rural communities of the Vhembe district of Limpopo province, South Africa.

1.3. OBJECTIVES OF THE STUDY

1.3.1. PRIMARY OBJECTIVE

- To study the prevalence of selected bacterial and viral enteric pathogens in children less than 5 years of age with diarrhea in Limpopo province, SA.

1.3.2. SPECIFIC OBJECTIVES

- To detect diarrheagenic strains of *E. coli, Salmonella* and *Shigella* using published m-PCR protocols in stool specimen of children less than 5 years of age presenting with diarrhea
- To detect the presence of Adenovirus 40/41, Norovirus and Rotavirus in stool samples of young children less than 5 years of age presenting with diarrhea using commercially available EIA tests
- To assess the potential health risks which these pathogens might have on the children

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Chapter 2
LITERATURE REVIEW

2.1. BACKGROUND

Unsafe drinking water, poor sanitary infrastructure and poor hygiene practices contribute to approximately 1.5 million child deaths and 88% of these deaths are mainly due to diarrhea annually (Ezzati et al, 2002; Guilbert, 2003). Diarrhea is defined as a passage of three or more loose stools per day. Prevalence of acute infectious diarrhea differs with time of year, geographical location and the population studied (Dennehy, 2005). Children that are malnourished, or are HIV-positive are at increased risk of life-threatening diarrhea that can result in death (Dennehy, 2005).

Acute infectious diarrhea is caused by enteric pathogens that invade the intestines (Bushen and Guerrant, 2003). Rotavirus and Diarrheagenic *E. coli* (DEC) have been found to be major causes of severe acute diarrhea in children less than 5 years of age (Parashar et al, 1998). However other enteric pathogens have been found to also cause diarrheal infections in young children worldwide namely: *Shigella* spp., *Campylobacter* spp., *Vibrio* spp., *Salmonella spp., Yersinia spp.*, enteric Adenovirus 40/41, Rotavirus, Norovirus, Astrovirus, *Giardia lamblia, Cryptosporidium parvum, Entamoeba histolytica*, and *Cyclospora cayetanenesis* (Podewils et al, 2004). In South Africa viral enteric pathogens such as Rotavirus, Norovirus and Adenovirus have been found to cause moderate to severe diarrhea in children less than 5 years of age (Mans et al, 2010; Potgieter et al, 2010). Symptoms of enteric pathogens vary from moderate to severe complications depending on the pathogen. Watery diarrhea in children is mainly caused by Rotavirus, Norovirus or enteric Adenovirus (Farthing, 2000), while infections caused by *Shigella* spp. and EHEC are associated with bloody diarrhea (Dennehy, 2005).

2.2. VIRAL ENTERO-PATHOGENS

Gastroenteritis caused by viral pathogens occur more frequently than bacterial infections and these viruses are major causes of infections across all age groups. Enteric viruses such as Rotavirus, Norovirus and Adenovirus 40/41 have been found

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to be major causes of gastroenteritis especially in young children. Other viruses that have been found to cause gastroenteritis includes: Astrovirus, Sapovirus, Pestivirus and Cytomegalovirus (Garza and Cohen, 2011). In temperate climates, viral gastroenteritis mainly occurs during cold winter months and transmission is via faecal-oral route. This study will therefore focus on the prevalence of Rotavirus, Norovirus and Adenovirus 40/41 as they are considered major causes of viral gastroenteritis in children.

2.2.1. ROTAVIRUSES

2.2.1.1. BACKGROUND

Rotaviruses were first identified in animals in 1960's (Bishop et al, 1973; Estes and Kapikian et al, 2007). The virus was then detected in humans by electron microscopic examination of duodenal biopsies from children with acute diarrhea in Australia (Bishop et al, 1973).

Rotaviruses belong to the *Reoviridae* family; the virus is 70nm in size, non-enveloped and icosahedral in shape (Bishop et al, 1973; Estes and Kapikian, 2007). The viral genome is composed of double-stranded RNA with 11 segments that encode structural Viral Proteins (VP 1- 4, VP6 and VP7), and non-structural viral proteins (NSP1-NSP6). The VP7 is glycosylated (G genotype) while VP4 is cleaved by a protease (P genotype) and are both modified post-translationally (Estes and Kapikian, 2007). The virus is protected by three main protein layers; the inner core which encloses the genome by VP1, VP2 and VP3; the middle capsid which is formed by VP6 and is responsible for group-specific antigenic determinants; and the outer core which is composed of the VP4 and VP7 capable of producing neutralizing antibodies and forms the protein shell with protruding spikes that protect the virus (Figure 2.1).

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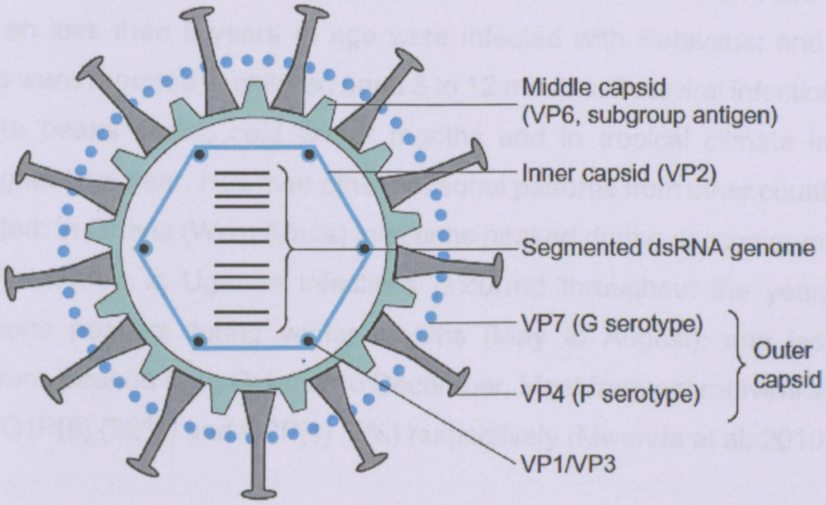


Figure 2.1: Schematic presentation of Rotavirus structure (Cunliffe et al, 2002)

Rotaviruses are classified into seven main groups based on the VP6 antigen (Groups A-G); and are further classified into different P and G types based on VP4 and VP7 antigens. Rotavirus Group A, B and C mainly causes illnesses in humans (Estes and Kapikian, 2007). Rotavirus Group A serotypes G1-G4 and G9 are associated with gastroenteritis (Banyai et al, 2012). The most common rotaviral G/P combinations affecting humans worldwide are: G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] (Banyai et al, 2012). These serotypes account to approximately 95% of pediatric Rotaviral diarrhea worldwide (Atkinson et al 2007). The G1 strains are predominant in Australia, North America and Europe accounting to 70% of infections and occurs less frequently in South America, Asia, and Africa accounting to 20%–30% of the infections (Griffin et al, 2000; Santos and Hosino, 2005). A Surveillance program conducted by the World Health Organization (WHO) in 2010 has shown that predominant uncommon strains G12P[8] and G12P[6] strains in Southeast Asia; G2P[6], G3P[6], and G1P[6] strains in sub-Saharan Africa; G1P[4] and G2P[8] strains in the Western Pacific; and G9P[4] strains in the Americas. These serotypes can be spread throughout the world and become common global strains as they are difficult to predict (WHO, 2010).

2.2.1.2. EPIDEMIOLOGY AND INCIDENCE

Rotavirus infections are responsible for approximately 2 million hospitalizations and 453 000 deaths occur in children less than five years of age worldwide; 50% of these deaths have been reported in Africa (Tate et al, 2012). A study by Mwenda et al (2010)

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reported on Rotavirus surveillance in 11 African countries and found that 40% of children less than 5 years of age were infected with Rotavirus; and hospitalization cases were reported in children aged 3 to 12 months. Rotaviral infections in temperate climate peaks during cold winter months and in tropical climate infections occur throughout the year. However other seasonal patterns from other countries have been reported: in Ghana (West Africa) infections peaked during dry cooler months (January and February); in Uganda infections occurred throughout the year; in Zimbabwe infections peaked during winter months (May to August); and lastly in Ethiopia infections peaked from October to December. Most frequent rotaviral strains detected were G1P[8] (22%) and G2P[4] (7%) respectively (Mwenda et al, 2010).

A study by Malek and colleagues (2010) found that Rotavirus infections in hospitals were higher (40%) compared to outpatient visits (23%) in the Mediterranean region. Children less than 2 years of age were found to have higher risk of developing severe gastroenteritis (Malek et al, 2010).The presence of Rotavirus in neonates has been said to be transferred during birth or nursery that has been contaminated by Rotavirus particles. These particles can be detected on the first and/or second day of life in neonates; on the third and fourth day most neonates shed the viral particles with or without diarrhea (Madeley et al, 1978). In neonates Rotavirus is asymptomatic and can result in milder symptoms. In India rotaviral infections in neonates were found mostly in symptomatic (56%) cases than in asymptomatic (45%) cases (Ramani et al, 2008). Contamination on hands of nursing and medical staff by Rotavirus is crucially important as it can result in transmission of viral particles from one infant to the other. As 1 gram of infected stool contains approximately 10^8 to 10^{11} viral particles, hands can therefore easily get contaminated. (Dennehy, 2005).

Rotavirus G12 strains were first detected in South Africa in 2004 and since then these strains have increasingly spread to other countries such as Zambia, Zimbabwe, Kenya, Ethiopia, Uganda, Cameroon, Togo and Burkina Faso (Page et al, 2009; Mwenda et al, 2010). Rotavirus G1-G4 in association with P[4] and P[8] strains have been found to be prevalent in South Africa for the past 30 years and are still predominant (Seheri et al, 2012). Potgieter et al (2010) who studied prevalence and diversity of Rotavirus strains in Limpopo province from 1998-2000 in South Africa

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found that children were infected with unusual G1P[6] followed by G1P[8], G9P[6] and G2P[6] strains.

2.2.1.3. TRANSMISSION

Transmission of Rotavirus is primarily via fecal-to-oral route that is: through contact with infected person, respiratory droplets or contact with contaminated environmental surfaces (Glass et al, 2006). The virus can also be transmitted through fomites such as children's toys or classroom objects (Biranjia-Hurdoyal and Quirin, 2012). In children's day care centers or family day care homes Rotavirus can easily be transmitted through frequent exposure of susceptible hosts. Studies have shown that settings such as pediatric and hospital nurseries are frequently overcrowded and can result in nosocomial rotaviral infections (Fischer et al, 2001). In developed countries rotaviral infections might be unrelated to sanitary conditions in the community, living conditions, or socioeconomic status of the affected countries (Bilcke et al, 2009). Therefore, the frequency of Rotavirus infection is not influenced by improvements in water supplies, sanitation and hygiene (Chanran et al, 2010). Rotavirus requires 10 to 100 viral particles to cause infection. The virus is very stable and can stay on environmental surfaces for a couple of days (Sattar et al, 1989). In shed stools the virus can survive for months under room temperature (Fischer et al, 2002).

2.2.1.4. PATHOGENESIS AND CLINICAL SYMPTOMS

Rotavirus infections mainly occur in the duodenum and the proximal jejunum. Once the virus enters the intestine, it invades and replicates in the villous epithelium causing cell death and sloughing. Loss of the villus cells results in decreased intestinal absorption of sodium, glucose, and water with decreased levels of intestinal lactase and alkaline phosphatase activity resulting in osmotic diarrhea (Atkinson et al, 2007). Rotavirus infections have also been demonstrated using mice, involving NSP4 which has been shown to mediate age-dependent intestinal secretion (Ball et al, 1996). Appearance of infected enterocytes under a dissecting microscope showed the scattered focal lesions of the mucosal surface with appearance of patches due to inflammation and production of mucosa for renewal of infected cells (O'Ryan et al, 2006). When the enterocytes recover from infection due to loss of mature enterocytes on the tips, the surface area of the intestine is decreased; causing diarrhea and loss of electrolytes (O'Ryan et al, 2006).

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Rotaviral infections predominantly occur in young children between the ages of 6 and 24 months (Brandt et al, 1983). The severity of the symptoms ranges from mild diarrhea with limited duration; to severe diarrhea (Atkinson et al, 2007). Rotaviruses have an incubation period of 1-3 days (Bernstein, 2009); accompanied by clinical symptoms such as: fever and vomiting followed by profuse, watery diarrhea and dehydration (Diggle, 2007; Wilhelmi De Cal et al, 2008). Diarrheal symptoms typically lasts for 4 to 7 days (Desselberger et al, 2009), and the virus can be shed in the feces for several weeks after onset symptoms (Center for Disease Control (CDC)), 2013).

Two Rotavirus vaccines have been administered since 2006. The monovalent RotaRix® (GlaxoSmithKline Biologicals Rixensart, Belgium) (RV1) vaccine which consists of attenuated human Rotavirus strain of G1[P8] serotype and is administered as a two dose schedule to infants (Angel et al, 2007). The RotaTeq® (Merck, Blue Bell, PA, USA) (RV5) vaccine is a live oral Rotavirus vaccine derived from five bovine-human Rotavirus strains that express human VP7 or VP4 surface proteins for G1 – G4, and P[8] antigens with genes encoding the bovine G6 and P7[5] proteins and other rotaviral bovine proteins; the vaccine is administered in a three dose schedule in infants (Clark et al, 1996). Introduction of immunization in children has shown to be more effective in reducing the disease burden (Grimwood and Lambert, 2009) and thus resulting in reduced mortalities in developing countries. The Strategic Advisory Group Experts on Immunization at WHO have suggested that Rotavirus vaccination must be part of the national immunization programme in all countries worldwide. In January 2013 the recommendations were approved and the vaccine has been introduced worldwide (Vaccines, 2013). Recently, in October 2015 WHO has introduced the Rotavirus vaccine to more than 79 countries through the GAVI Alliance including countries such as Canada, Thailand and the Philippines (PATH, 2015).

The efficacy of RV1vaccine in high income countries such as Singapore, Hong Kong, Taiwan and Japan together with six European countries, has been shown to provide protection of greater than 95% against severe diarrhea in infants during the first year of life and 90% protected during second year of life in infants (Phua et al, 2009; Kawamura et al, 2011). In Middle income countries such as Latin America and Finland the RV1 vaccine has been shown to provide protection against of 80 – 85% against

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severe diarrhea in infants during first year of life and 80% protection in the second end year in infants (Tregnaghi et al, 2011). In low income countries such as Malawi and South Africa the efficacy against severe diarrhea during the first year of life was greater than 75% in South Africa while in Malawi it was greater than 50%; during the second end of year protection in both countries was low at 35% as there was no evidence of protection (Cunliffe et al, 2012; Madhi et al, 2012). Trials of RV5 efficacy have also been determined. Severe diarrhea caused by homotypic G1, G2, G3 and G4 strains in both high- and middle-income countries have reduced by 98% during the first rotaviral season and by 88% in the following season (Cunliffe et al, 2012). In these settings hospitalization caused by Rotavirus has reduced by 90% since introduction of the vaccine (Vesikari et al, 2007). In low income countries (such as Kenya, Ghana and Mali) protection from severe diarrhea during the season was 65% and decreased to 40% over a two-year study (Vesikari et al, 2007). Detection of Rotavirus see Section 2.4.

2.2.2. NOROVIRUSES

2.2.2.1. BACKGROUND

Noroviruses were first described as "winter vomiting disease", an illness that was characterized by diarrhea and self-limited vomiting that peaked during winter months (Zahorsky, 1929). The virus was then detected by immune microscopic examination of stools in 1968 from volunteers in school form Norwalk, Ohio that were affected by gastroenteritis outbreak; the virus was then identified as Norwalk virus (Kapikian et al, 1972).

Noroviruses belong to the *Caliciviridae* family; the virus is non-enveloped, positive-sense single-stranded RNA with the genome size ranging from 7.3 to 7.4 kb. The virus is 27-38 nm in diameter with arch-like capsomeres that consist of three Open Reading Frames (ORF) (Thorne and Goodfellow, 2014). The ORF1 is a large polyprotein that is cleaved co- and post-translationally by the virus-encoded protease (NS6) for it to release six non-structural (NS) proteins. Other NS proteins that have been implicated in the replication complex formation include Viral RNA-dependent RNA polymerase (RdRp; NS7), VPg (NSp5), putative NTPase/RNA helicase (NSp3), NS 1/2 and NSp4 (Thorne and Goodfellow, 2014). The NS1/2 is processed further during the latter

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stages of viral infection due to cellular caspases that are activated by apoptosis. The ORF2 is translated and encodes VP 1 as a major capsid protein (Tan et al, 2003). The VP1 consists of a shell (S) domain which is accountable for assembly of VP1; and two protruding domains (P1 and P2); the P1 domain assists in enhancing the stability of the viral particle and the P2 domain is exposed to the surface of the viral particle and assists in interaction with the potential neutralizing antibody and histo-blood group antigen (initial binding site during infection) (Tan et al, 2003). The ORF3 is translated and encodes the VP2 as a major capsid protein, is involved in enhancing production and stability of VP1 (Bertolotti-Ciarlet et al, 2003).

Noroviruses are classified into five genogroups: GI to GV. The genogroups have been defined based on the amino acid diversity of the three ORFs, VP1 regions and RdRp. The groups are further subdivided into genotypes and the variants are based on diversity of the sequence. The genotypes are defined based on either the RdRp sequence or the capsid sequence (Dingle et al, 1996). Norovirus GI, GII and GIV mainly infects humans and the GII4 genotype accounts to 80% of infections worldwide (Zheng et al, 2006). Norovirus infections have also been detected in animals such as pigs (GII), cattle and sheep (GIII), mice (GV) (Thorne and Goodfellow, 2014), and has recently been identified in domestic dogs (GVI) (Mesquita et al, 2010). Therefore animals are also considered as reservoirs in transmission of new Norovirus strains.

A review done by Tran and colleagues (2013) has shown that Norovirus GII is the most prevalent genogroup worldwide, resulting in sporadic infections of an average of 96%; GI with an average of 3.6%; mixed infections with an average of 0.4%. For the past decade Norovirus gastroenteritis strains have drastically changed with GII.4 and GII.b/GII.3 genotypes occurring more frequently. GII.4 and GII.3 capsid genotypes have been found to account 85% of Norovirus infections in young children while GII.2 and GII.6 account for approximately 5% of the infections (Tran et al, 2013). Pandemic circulation of G11.2/2002, GII.4/2004, GII.4/2004b and GII.4/2006b with GII.4/2008 variants have been found to mainly cause childhood gastroenteritis (Tran et al, 2013).

2.2.2.2. EPIDEMIOLOGY AND INCIDENCE

Noroviruses are the second leading causes of severe childhood gastroenteritis after Rotavirus, with the prevalence range of 6 – 48% (Patel et al, 2009). The virus is

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responsible for sporadic gastroenteritis across all age groups (Glass et al, 2009) resulting in approximately 267 000 000 cases annually worldwide (Barrabeig et al, 2010). A systematic literature review by Patel and colleagues (2008) on roles of Norovirus in sporadic gastroenteritis revealed that from 1990 to 2008 Noroviruses were responsible for 12% of severe gastroenteritis in children less than 5 years of age and 12% of mild to moderate across all age groups.

Norovirus cases have been found to be low (14%) in settings with high mortality and higher (20%) in settings with low mortality (Ahmed et al, 2014). The incidence of low prevalence in high mortality countries might have been due to the presence of other pathogenic microorganisms (Ahmed et al, 2014). Prevalence of Norovirus in children less than 5 years of age or younger is 18%, and was found to be the third frequent pathogen causing hospitalizations in children (13.8%) behind Rotavirus (38.3%) and EPEC (15.5%) (Ahmed et al, 2014). Norovirus cases have been reported to be higher in countries where Rotavirus vaccine has been introduced. Based on settings, prevalence of gastroenteritis was found to be higher in the community (24%) followed by outpatient visits and lower in inpatient settings (Mans et al, 2010; Ahmed et al, 2014).

Gastroenteritis caused by Norovirus can occur in four distinct epidemiological settings with a spectrum of clinical outcomes: (i) Epidemic foodborne gastroenteritis (Parashar et al, 1998); (ii) Healthcare-associated infection occurring in closed settings such as residential/care homes and hospitals starts by introduction from a single host with continuous environmental transmission (Anon, 2007); (iii) Occurring sporadically in young children resulting in hospitalization (Nakagomi et al, 2008); (iv) Community-based infection (Widdowson et al, 2005).

In South Africa, the first Norovirus outbreaks of GI.1 and GII.1 strains as the causative in two successive outbreaks were reported in 1993 (Taylor et al, 1993). In 1995, GII.3 strain was then identified in paediatric stool specimens from sporadic cases (Wolfaardt et al, 1995). A later study in 1999, revealed Norovirus antibody prevalence levels of 94-96% among both urban and rural South African populations (Smit et al, 1999). Since then, no data on Norovirus prevalence of circulating genotypes has been reported. A recent study by Mans et al (2010) described the emerging Norovirus GII.4

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2008 variant detected in hospitalised paediatric patients in South Africa (Mans et al, 2010). Studies have shown that in South Africa, Norovirus GII.4 infections were higher in children aged less than 6 months and GI strains were predominant in those aged 7 to 12 months; infections were detected all year round with increased infections in summer (November and December) (Mans et al, 2010).

2.2.2.3. TRANSMISSION

Norovirus infections affect all age groups. In temperate climates infections are sporadic occurring all year-round with a peak during cold winter months (Glass et al, 2009). Lopman et al (2003) has shown that Norovirus outbreaks have peaked during spring and summer resulting in hospitalization of children less than 5 years of age. Transmission of Norovirus is primarily via faecal-oral route that is through contact with contaminated food or water and contact with infected persons. In closed settings such as hospitals, hotels and day care centres transmission of infectious aerosols and vomit can be transmitted by hand or mouth (Widdowson et al, 2005). Contamination of environmental surfaces has been shown to be a reservoir for Norovirus outbreaks, such as contamination of restrooms (Widdowson et al, 2005). In New Zealand a surveillance study in 2001-2007 stated that out of the outbreaks, 17.6% were due to foodborne infections, 19.9% were related to environmental sources and 61% were through contact with infected person (Lim et al, 2010).

Factors that assist in the spread of Norovirus infections are: (i) low infectious dose of <10 viral particles; (ii) difficult to control as the virus is stable in high concentrations of chlorine and at wide range of temperature; (iii) prolonged duration of viral shedding of up to 2 weeks, resulting in secondary transmission; (iv) lack of long term immunity resulting in re-infection (Patel et al, 2009).

2.2.2.4. PATHOGENESIS AND CLINICAL SYMPTOMS

Pathogenesis mechanisms of *Caliciviruses* are poorly understood due to lack of cell culture and animal models. Upon ingestion, Noroviruses enter the duodenum and jejunum infecting of the enterocytes in the villi, inducing atrophy and crypt hyperplasia. Infection results in diarrhea with malabsorption and increased secretion (Michelangeli et al, 2003).

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Noroviruses have an incubation period of 24 to 48 hours resulting in symptoms such as vomiting, nausea, abdominal cramps and watery diarrhea. The virus can also result in severe symptoms that resolve in 2 to 3 days. In severe cases such as patients affected during hospital outbreaks, duration of the illness can occur 4 to 6 days. Children less than 1 year of age are more prevalent to vomiting whereas children greater than 1 year of age develop diarrhea (Patel et al, 2009). Deaths resulting from Norovirus infection have been reported during outbreaks in nursing homes. Norovirus infections in children have been shown to be similar to those of Rotavirus resulting in mild to severe clinical symptoms (Patel et al, 2009). Norovirus infections have been found to be associated with other significant severe clinical symptoms such as seizures in infants (Patel et al, 2009), encephalopathy, necrotizing enterocolitis, pneumatosis intestinalis and disseminated intravascular coagulation (Thorne and Goodfellow, 2014).

After onset of symptoms viral shedding can occur up to 3 weeks and longer if patients are in immunocompromised. Prolonged viral shedding of up to 23 days with severe life threatening symptoms has been reported in children from paediatric oncology hospital (Simon et al, 2006). Detection of Norovirus see Section 2.4.

2.2.3. ADENOVIRUSES

2.2.3.1. BACKGROUND

Adenoviruses (Ad) were first isolated from secretions of patients with respiratory infections which were inoculated on human cell cultures resulting in cell death (Hilleman and Werner, 1954).

Enteric Adenoviruses belong to the *Adenoviridae* family (Yates, 2014). Adenoviruses are non-enveloped with icosahedral symmetry and are 70-80 nm in diameter. The genome is composed of double-stranded DNA of approximately 35kp in length (Gray and Kang, 2013). The protein capsid consists of 252 capsomeres of which 12 are pentons surrounded by five capsomeres and 240 hexones surrounded by six other capsomeres (Yates, 2014). The hexones consists of proteins II, VI, VIII and IX that

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assist in stability and assembly of the virus. The peptone proteins (III and IIIa) assist in cellular penetration; the hemagglutinin fibres assist in binding of the virus particle to the receptors. The core of the virus is made up of at least eight proteins that assist in maintaining the integrity of the genome and assist in enzymatic activities of the virus (Brown et al, 1996; Shenk, 1996).

There are 51 distinct serotypes of human Adenoviruses and are grouped into six different species (A-F) based on biologic, biochemical and immunologic differences (Gray and Kang, 2013). Adenoviruses of species F, serotype 40 and 41 are more prevalent in humans causing gastroenteritis (Wood, 1988; Wold and Horwitz, 2007). Serotypes 31, 12 and 18 of subgenus A and serotypes 1, 2, 5 and 6 of subtype C have been found to also cause rare cases of gastroenteritis (Brown et al, 1996; Horwitz, 1996).

2.2.3.2. EPIDEMIOLOGY AND INCIDENCE

The incidence of Adenovirus infection is estimated to range from 3.5 to 7.5 episodes per child per year during the first 2 years of life and 2 to 5 episodes per child per year by the age of 5 years (Brandt et al, 1983). The virus has been associated with outbreaks in hospitals, schools and day care centres (Chiba et al, 1983; Akihara et al, 2005). Adenovirus accounts to 1 – 20% of diarrheal cases reported in young children (Akihara et al, 2005). Factors such as poor sanitation and lack of access to clean drinking water have been associated with the spread of Ad. In developed countries the incidences of Ad infections are lower (1 – 8%) when compared to developing countries (2 – 31%) (Qiao et al, 1999). Nosocomial infections by Adenoviruses have been shown to increase as the virus is spread by asymptomatic patients shedding the virus through their feces (Ojeh et al, 1995). Ad 40/41 accounts to 5 – 20% of hospitalizations in children with gastroenteritis (Uhnoo et al, 1984). In South Africa several studies have been done on Ad 40/41 infections causing pediatric gastroenteritis (Steele et al, 1990; Moore et al, 1998; Moore et al, 2000).

In 1992, Van et al (1992) found that Ad 40/41 were important causes of diarrheal outbreaks in children attending day care centers in Huston (USA) from 1988 – 1991. A total of 96/613 (15%) Ad infections were detected which lasted for 7 – 14 days. The

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excretion of the viral particles were found to be 7 days before onset of symptoms and 11 days after diarrhea has stopped (Van et al, 1992).

Saderi and colleagues (2002) studied the incidence of enteric Adenovirus 40/41 gastroenteritis in Iranian children and found that most of Ad infections occurred in children aged less than 2 years of age (70%); the prevalence of Ad was found to be 8.7% (Ad 40 3.4% and Ad 41 2%). Ad 40 was more prevalent in children aged 7-18 months while Ad 41 was prevalent in children aged less than 6 months and 2 years (Saderi et al, 2002).

A Study by Aminu et al (2007) on Ad infections in children with gastroenteritis in North - Western Nigeria found that the incidence of Ad infection was higher in children aged above 25 months (29.2%) than those aged less than 24 months (21%). Enteric Ad infections were detected throughout the year with a prevalence of 23% (Ad 40/41 8%) across all age groups; co-infections with Rotavirus (14.3%) were also reported. Infections were found to be higher in outpatients (24.2%) than inpatients (13%) which indicated that the virus could be spread easily in the community. Diarrhea was accompanied by clinical manifestations such as fever, abdominal pain, vomiting and interestingly symptoms of upper respiratory tract infections were also reported and this could be an indication of other Ad serotype infection (Aminu et al, 2007).

In China and Japan, incidences of enteric Ad infections were found to range from 6.2% to 9.89% (Shimizu et al, 2007; Ren et al, 2013; Liu et al, 2014) while in African countries such as Gabon, Tanzania and Sudan; the incidences of Adenovirus infections were found to be 2% to 19.6% (Moyo et al, 2007; Elhag et al, 2013; Lekana-Douki et al, 2015). From the data collected it can be seen that that the prevalence of enteric Ad infections is more or less the same in both industrialized and developing countries.

2.2.3.3. TRANSMISSION

Adenovirus infections occur throughout the year. Infections can result in either sporadic, epidemic or endemic outbreaks (Gray and Kang, 2013). Adenovirus type 40/41 is primarily transferred via faecal-oral route and contaminated fomites. Other

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serotypes are spread by aerosols resulting in respiratory infections (Yates, 2014). Recreational waters have been found to transmit Adenovirus type 6 and 7 causing eye infections (Papapetropolou and Vantarakis, 1995). The virus is stable at pH 3 and replicates in the intestinal cells (Yates, 2014). Adenovirus requires a total of 10^5 to 10^6 viral particles to cause infection. Adenovirus infections are difficult to control as the virus can survive on environmental surfaces for a long periods of time and are resistant to disinfection; however the virus is inactivated by heat and formaldehyde chlorine (Yates, 2014).

2.2.3.4. PATHOGENESIS AND CLINICAL SYMPTOMS

Pathogenesis of Ad 40/41 is produced in the same way as Rotavirus infection. The lesion caused by Ad 40/41 in the enterocytes results in atrophy of the villi and compensatory hyperplasia in the crypts, resulting in malabsorption and loss of fluids through diarrhea (Farthing, 1997). Gastroenteritis caused by Adenovirus is accompanied by vomiting and diarrhea that lasts for 14 days. The infected person can shed the virus for one to two weeks after infection (Garza and Cohen, 2011). In infants, infection results in development of immunity of 50% by the age of 4 years (Horwitz et al, 1996). In children aged 3 months to 3 years infection can result in obstruction of the intestine taking place when one segment of the intestine invades adjacent segment of the distal intestine. This intussusception results in severe colic, vomiting and excretion of mucus and bloody stools as a result of arterial obstruction followed by bowel necrosis (Gray and Kang, 2013). Infections in immunocompromised patients (such as HIV infected patients and allogenic bone marrow transplant individuals); Ad infections can result in life-threatening complications with high mortality rate (Slatter et al, 2005). Detection of Adenovirus see Section 2.4.

2.3. BACTERIAL ENTERO-PATHOGENS

In developing countries children are exposed to different bacterial enteric pathogens from a young age, resulting in in more than 1 episode of diarrhea. Prevalence of bacterial enteric pathogens differs with geographic location, seasonal trend and age of the child. Enteric pathogens that have been found to mainly cause gastroenteritis in children includes Diarrheagenic *E. coli* (DEC) pathotypes, *Campylobacter* spp., *Vibrio* spp., *Salmonella* spp., *Shigella* spp. These enteric pathogens result in mild

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infections and have also been found to cause severe diarrhea accompanied with dehydration in certain cases (Podewils et al, 2004). This study will therefore focus on the prevalence of DEC pathotypes, *Shigella* spp. and *Salmonella* spp. they are as one of the major causes of diarrheal pathogens affecting young children.

2.3.1. DIARRHEAGENIC *E. coli* STRAINS

Escherichia coli (*E. coli*) are Gram-negative, non-spore forming bacilli that belong to *Enterobacteriaceae* family. *E. coli* are part of the normal flora of the gastrointestinal tract of warm-blooded animals and humans (Nataro and Kaper, 1998). *E. coli* colonises the mucosal layer of the large intestine within the first few hours after birth and co-exists with the human host for a lifetime with mutual benefit. However there are certain strains of *E. coli* that produce virulence factors resulting in intestinal infections (Nataro and Kaper, 1998). These pathogenic strains are based on the virulence properties, serotypes of distinct somatic "O", Flagella "H", and Capsule "K" antigens with different mechanisms of pathogenicity and clinical symptoms (Campos et al, 2004). The intestinal pathogens are referred to as Diarrheagenic *E.coli* (DEC); with six distinct pathotypes: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E.coli* (EAEC), Enterohemorrhagic *E. coli* (EHEC) and Diffusely Adherent *E. coli* (DAEC) (Vidal et al, 2005). The extra-intestinal *E. coli* pathogens are associated with neonatal meningitis, urinary tract infections and bacteremia (Campos et al, 2004).

This study will focus on Diarrheagenic *E. coli* as these pathogens have been found to be major causes of childhood diarrhea resulting in mortality and morbidity globally (Rajedran et al, 2010). Adhikari and colleagues (1985) studied an outbreak in a neonatal unit at King Edward VIII Hospital in Durban, South Africa and found that patients were infected with ETEC (17%) and EPEC (11%). In another study, Galane and Le Roux studied molecular epidemiology of E. coli in young children with diarrheal diseases and found that 26.5% of the children were infected with EPEC and 7.7% were infected with EHEC (Galane and Le Roux, 2001). In developing countries such as India, children attending tertiary care hospitals were found to be infected with EPEC (10%), EIEC (1.0%), ETEC (4.1%), EAEC (14.7%) and EHEC (2.0%) (Rajendran et al, 2010). This study will therefore focus on EPEC, EAEC, ETEC, EIEC and EHEC.

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2.3.1.1. ENTEROTOXIGENIC *E. coli* (ETEC)

ETEC produces the heat-labile toxin (*LT*) (similar to the cholera toxin) and/or the heat-stable toxin (*ST*) (Kang et al, 2014). The *LT* is a heterohexomic molecule made of a single A subunit and pantameric B subunit. The A subunit has two domains that are linked by a disulphide bridge. A1 is the active toxin molecule while A2 is the helical portion of the molecule that attaches to the B pantamer (Merritt et al, 1994). Secretion of *LT* through the outer membrane to the host is by either N-terminal signal peptides of the subunits during transportation of sec-dependent across the inner membrane to the periplasm were it assembles into in a holotoxin (Hirst and Holmgren, 1987). The general secretion pathway (GSP) is required for secretion across the outer membrane (Tauschek et al, 2002). The *LT* produces variety of effects that benefit the organism. Innate host responses are down-regulated by *LT* including the defensins and enables attachment of ETEC to the epithelial cells and colonization in the small intestine (Chakraborty et al, 2008; Fleckenstein et al, 2010) (Figure 2.2).

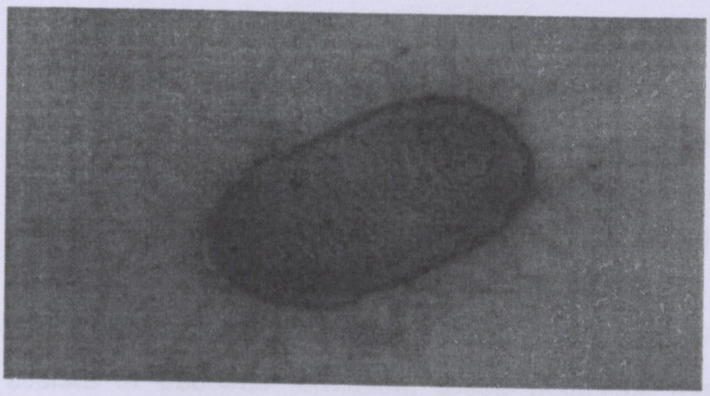


Figure 2.2: Scan electron microscopy of ETEC with fimbriae (Kang et al, 2014)

The *ST* are small cysteine-rich peptides that are released by ETEC. It attaches to the extra-cellular domain of guanylyl cyclase C (GC-C) on the brush border of the intestinal epithelium. Intracellular catalytic domain of guanylyl cyclase is activated by GC-C resulting in intracellular accumulation of cGMP (Hughes et al, 1978). The cGMP-dependent protein kinase is activated by an increase in the cGMP resulting in phosphorylation of cystic fibrosis transmembrane regulator (CFTR) causing C1-secretion and inhibition of NaCl absorption with loss of water through osmotic diarrhea (Chao et al, 1994). The *ST* peptides that have been identified in human are: STa (STI),

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ST-Ia (*ST*-P) and ST-Ib (*ST*-H) peptides. These molecules have a core structure of 13 amino acids with three disulphide bonds which are needed for biological effect. The *ST*-P peptide determines the hexametric ring forming putative GC-C binding region positioned to the outer surface promoting clustering an activation (Sato and Shimonishi, 2004). The *ST*-H and *ST*-P are encoded by the plasmid and are synthesizes initially as 72 amino acid precursor consisting of 19 residue signal peptide needed for entry into the periplasm (So and McCarthy, 1980; Fleckenstein et al, 2010). ETEC mainly causes diarrhea in developing countries that have poor sanitation (Okoh and Osode, 2008). Transmission is mainly via faecal-oral route. ETEC infections occur throughout the year but occur mostly during the rainy season (Kang et al. 2014). The infective dose is high with 10^6-10^{10}cfu (Kang et al, 2014). The bacterium mainly infects children during weaning period resulting in diarrhea. Incubation period is 1-2 days resulting in vomiting, anorexia, abdominal cramps and diarrhea occurring up to 10 times a day. The illness lasts 1-3 days and in malnourished children it can last for up to 3 weeks (Kang et al, 2014).

2.3.1.2. ENTEROPATHOGENIC *E. coli* (EPEC)

EPEC strains consists of *eaeA* (E. coli attaching effacing) gene on the chromosome located in the pathogenicity island called the locus of enterocyte effacement (LEE) (Aslani and Alikhani, 2009). The *eaeA* gene encodes intimin (94 kDa protein) which enables the bacteria to adhere to the intestinal epithelial cells resulting in local effacement of the microvilli and actin-rich pedestal formation which the bacteria resides (Sykes and Marks, 2014). EPEC secretes the Tir receptor and Tir intimin which assists in pedestal formation; these pedestals assists the bacteria to remain extracellular allowing it to not be recognized by the immune system (Ochoa et al, 2008). The histopathologic lesions on the intestinal cells is referred to as "attaching and effacing lesions". EPEC-attaching factor plasmid encodes the Bundle forming pili (*bfp*) which enables localized adherence to epithelial cells. Signal transduction of the bacteria is due to loss of tight junction integrity and change in electrolyte transportation resulting in watery diarrhea (Sykes and Marks, 2014).

EPEC that harbour the EAF plasmid are referred to as typical EPEC and those with no plasmid are referred to as atypical EPEC (Ochoa et al, 2008). Atypical and typical

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EPEC strains are from different serotypes with difference adherence patterns. The typical EPEC consists of only the localized adherence (LA) pattern, while atypical EPEC consists of localized-adherence like (LAL) pattern, aggregative adherence (AA) and diffuse adherence (DA) pattern (Aslani and Alikhani, 2009). Epidemiological studies have shown that atypical EPEC infects human and animals (such as cats and dogs) and have been considered as emerging pathogens in human (Trabulsi et al, 2002).

EPEC infections occur sporadically (Kang et al, 2014). EPEC causes acute diarrhea that mainly infects children less than 2 years of age and especially those under the age of 6 months (Podewils et al, 2004). Infective dose is low with 10^4 cfu. EPEC produces severe and prolonged diarrhea with mucus accompanied by vomiting and fever (Kang et al, 2014). In South Africa and other African countries, EPEC and ETEC have been associated with diarrhea with increased risk of deaths in children less than 12 months of age (Kotloff et al, 2013).

2.3.1.3. ENTEROHEMORRHAGIC *E. coli* (EHEC)

EHEC produces the Shiga-like toxin (*stx)* (previously known as verotoxins) which resembles the attaching and effacing lesions of EPEC. The *stx* 1 is neutralized by antiserum against Shiga toxin of *S. dysenteriae*; while *stx* 2 is not neutralized by the anti-Shiga toxin (Scotland et al, 1985). The EHEC Shiga-like toxins inhibit protein synthesis by cleaving adenosine residue from position 4324 in the 28S rRNA to inhibit elongation of factor-1-dependent aminoacyl tRNA from attaching to the 60S rRNA. Pathogenicity of the bacteria is determined by the 60-MDa plasmid that encodes for fimbrial adhesion, of which is facilitated by the LEE pathogenicity island similar to those present in EPEC (Karch et al, 1987; O'Ryan et al, 2006).

EHEC are normal enteric flora of pigs, cattle, cats, dogs and sheep, and result in no disease (Kang et al, 2014). EHEC infections were first reported in developed countries, from outbreaks caused by undercooked beef or pork and having contact with animals in the zoo. In developing countries such as Swaziland and Cameroon that have been affected by drought have resulted in large outbreaks of haemorrhagic

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coltis caused by EHEC which has been associated with open water supplies that have been contaminated by animals (Isaacson et al, 1993; Cunin et al, 1999).

EHEC infections occur sporadically, however outbreaks have been reported in day care centres, nursing homes and other institutions that have closed settings (Cohen et al, 1996). In USA *E. coli* O157:H7 is estimated to cause 10 000 to 20 000 infections and 250 deaths per year (Boyce et al, 1995). Transmission is by ingesting contaminated water or food and contact with infected person. EHEC infections have been mostly reported in infants and young children (Dennehy, 2005). The bacteria requires a dose of 10^2 cfu to cause infections in humans (Kang et al, 2014). EHEC infections results in diarrhea, haemorrhagic colitis, HUS and post-diarrheal thrombotic thrombocytopenic purpura. Illness begins with watery diarrhea and progresses to diarrhea with occult blood accompanied by severe abdominal cramps and fever is not frequent (Dennehy, 2005).

2.3.1.4. ENTEROAGGREGATIVE *E. coli* (EAEC)

EAEC are characterized by aggressive adherence (AA) to Hep-2 cells in a stacked-brick pattern (Nataro et al, 1987). The AA assists with expression of AA fimbriae located on the large AA virulence plasmid. EAEC infection results in colonization of intestinal mucosa and secretion of enterotoxin and cytotoxins. Secretion of toxins causes inflammation of cytokines in vitro resulting in growth retardation of which is characterized with prolonged asymptomatic infection. Detection of virulent EAEC is mediated by the presence of AggR activator gene (Nataro et al, 1998; O'Ryan et al, 2006).

EAEC infections occur sporadically with a dosage of 10^8. These infections result in watery diarrhea that can either be acute or persistent (Kang et al, 2014). Infections can result in malnutrition, inflammation of the intestine and symptoms can occur with or without diarrhea. EAEC has been detected in young children with infection lasting for more than 2 weeks. The pathogen infects immunocompromised patients such as HIV infected persons resulting in severe complications (O'Ryan et al, 2006).

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2.3.1.5. ENTEROINVASIVE *E. coli* (EIEC)

EIEC Pathogenicity, epidemiology and clinical manifestations are similar to those of *Shigella* species. EIEC also consists of the ipaA, B, C and ipD genes that enables signalling of epithelial cells to form cytoskeletal arrangements which allow entry of the bacteria into the host. It also consists of the IcsA gene which enables localization of the outer membrane enabling the bacteria to bind to the host. The bacteria passes through the cytoplasm by condensation of the polar actin and spreads across the enterocytes (Steiner et al, 2011).

EIEC requires a dose of 10^8 cfu to cause an infection. Infections caused by EIEC are mild when compared to *Shigella* spp. EIEC is characterized by invasion and killing of the intestinal epithelial cells resulting in inflammatory diarrhea. Infection is common in travellers from developed to developing countries, and occurs less frequently in children less than 1 year of age (Podewis et al, 2004). EIEC infections result in inflammatory diarrhea that is bloody and mucoid (Podewis et al, 2004). EIEC has been characterized with clinical symptoms such as diarrhea, vomiting, abdominal pain, fever chills, malaise and presence of blood and mucous in the stools (Podewils et al, 2004).

2.3.1.6. INCIDENCE OF DEC

Infections caused by DEC are due to poor sanitation and personal hygiene. In developing countries infection are primarily due to consumption of contaminated water from open sources such as rivers and wells (Obi et al, 2003; Dennehy, 2005; Okoh and Osode, 2008).

Rangel et al (2005) reviewed epidemiology of *E. coli* O157:H7 outbreaks in US from 1982 to 2002 and found that a total of 350 outbreaks were reported, accounting for 8598 cases of *E. coli* O157 infections. From the cases 17.4% required hospitalization while 4.1% cases experienced HUS and 0.5% resulted in death. Outbreaks peaked between 1993 and 2000 (2 to 781 cases reported); and most cased were reported from May to November.

In Bangladesh, Albert et al (1995) have found that children less than five years had acute diarrhea and was mainly caused by EPEC and ETEC. ETEC was isolated from

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children aged 0-24 months and 37-48 months with diarrhea; infections caused by ETEC peaked during wet dry months in August and EPEC infections peaked during hot dry months in February to May. In Tehran, Diarrheagenic *E. coli* strains were found to be more frequent in summer than the other season and were accompanied by vomiting especially in children less than 1 year of age (Jafari et al, 2009). The Shiga-toxin producing *E. coli,* EAEC, ETEC and EPEC were found to be more common in children. The prevalence of DEC in children with acute diarrhea was found to be: EHEC *stx*1 (5.4%), *stx*2 (11.7%), *stx*1/*stx*2 (1.8%), EAEC (16.6%), EPEC (12.6%) and ETEC: LT (2.3), ST (3.1%) and LT/ST (1.4%) (Jafari et al, 2009).

A study by Obi and colleagues from 1999 to 2002 found that DEC (20%) were the most frequent pathogen detected from diarrheal samples of children and adults visiting rural clinics in Venda (South Africa), compromising of EPEC (37%), EAEC (14%) and ETEC (13%) respectively. Most of the infections (50%) were detected in children less than 5 years old (Obi et al, 2003). Another study done by Obi et al (2004) on gene encoding virulence markers among isolates from diarrheal stool samples and river sources in rural communities of Venda (South Africa) found that open water sources (rivers used by the community) were contaminated with EPEC (34%); from diarrheal samples EPEC was found in 33.6% of the isolates with atypical EPEC being more prevalent (32.5%) followed by presence of STEC (EHEC) (16%), EAEC (16%) and ETEC (13.4%). From this study one of the study participants was infected with EHEC O157:H7 (Obi et al, 2004). Detection of DEC see Section 2.4.

2.3.2. *Shigella*

2.3.2.1. BACKGROUND

Shigella are Gram-negative, non-motile non-spore forming, facultative anaerobe bacilli that belong to the *Enterobacteriaceae* family (Niyongi, 2005; Cabral, 2010). *Shigella* are classified into four main serological groups: Group A (*S. dysenteriae* with 13 serotypes); Group B (*S. flexneri* with 15 serotypes); Group C (*S. boydii* with 18 serotypes) and Group D (*S. sonnei* with 1 serotype) (Niyongi, 2005; Percival and Williams, 2014). Serogroups A, B and C are physiologically related and *S. sonnei* are biochemically different with positive beta-D-galactosidase and ornithine decarboxylase reactions (Niyogi, 2005). *S. dysenteriae* is closely related to *E. coli* as

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it produces the *shiga* toxin associated with HUS (Nygren and Bowen, 2013). The outer polysaccharide chains of the lipopolysaccharide cell wall consists of the serotype "O" antigen and does not contain the "H" antigen. Molecular methods can be used to subdivide the serotype using the Entero-bacterial repetitive intergenic sequence based-PCR (EIRIC-PCR) typing or using the Pulse field gel electrophoresis (PFGE). Certain *E. coli* and *Shigella* spp. cannot be distinguished at polynucleotide level using DNA hybridization; virulence phenotype is the feature that can be used to differentiate the pathogens (Niyogi, 2005). *Shigella* and EIEC are biochemically similar and can induce diarrhea or dysentery. *S. dysenteriae* type 1 also known Shiga bacillus has been found as a major cause of epidemic dysentery; and for the past 40 years as a major cause of pandemic spread worldwide (Gangarosa et al, 1970; Pal, 1984).

2.3.2.2. THE SHIGA TOXIN

The Shiga toxin is a strong protein inhibitor encoded by the chromosome of *S. dysenteriae* and is involved in targeting the vascular endothelium; resulting in severe HUS (Sandvig, 2001; Bitzan et al, 2003). EHEC produces similar toxins resulting in same infections as *S. dysenteriae* (Sandvig, 2001). *Shigella* consists of 3 distinct enterotoxins namely: (i) chromosome encoded *Shigella* enterotoxin 1 (SHET1) is present in *S. flexneri* and is rarely found other serotypes (Yavzori et al, 2002); (ii) *Shigella* enterotoxin 2 (SHET2) is involved in virulence factors found in most *Shigella* strains and also in EIEC (Nataro and Kaper, 1998). The Shiga toxin consists of 3 types of effects that are encoded by the chromosomal genes with 1-A and 5-B structures similar to those of Shiga-like toxins of EHEC (Acheson et al, 1991). First is the neurotoxic effect which results in severity of the disease causing in bloody diarrhea accompanied with fever and abdominal pain (Fontaine et al, 1988). Secondly is the enterotoxic effect which attaches to the small intestine receptors preventing absorption of nutrients from the intestinal lumen of the host. Thirdly is the cytotoxic effect; in which the B-subunit of the toxin binds to the colon of host cell followed by insertion of A1 domain resulting in irreversible inactivation causing cell death, and can be seen with the presence of blood in the stools (Fontaine et al, 1988).

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2.3.2.3. EPIDEMIOLOGY AND INCIDENCE

Shigellosis (disease caused by *Shigella* spp.) is more prevalent in children in developing countries. The bacterium has been found in low income settings and overcrowded communities and is still a major cause of mortality and morbidity in these settings (Kang et al, 2014). *Shigella* harbours in closed settings such as hospitals and day care centres. *S. dysenteriae* is found mostly in South America, Asia and Africa, with infections resulting in epidemic outbreaks. Serotype 1 predominates in Malaysia, India and Guatemala while serotype 2 predominates in Nigeria and Yemen (Cabral, 2010). *S. flexneri* is found mostly were there are *Shigella* outbreaks; *S. boydii* occurs sporadically; and *S. sonnei* is found mainly in developed countries such as USA and France (Cabral, 2010). Pandemic of *S. dysenteriae* type 1 have been seen in the late 1960s in south and south East Asia (Rahaman et al, 1975), Central America (Gangarosa et al, 1970) and sub-Saharan Africa affecting populations and acting as a natural disaster causing in death (Ries et al, 1994).

Children less than 4 years of age are at increased risk being infected with *Shigella* as more than 100 000 cases of shigellosis have been reported annually (Gupta et al, 2004). In Africa and Asia increased infections of *Shigella* have been frequently been detected in infants and toddlers (Kotloff et al, 2013). Children attending day care centres are at increased risk of acquiring shigellosis than those staying at home. Outbreaks in day care centres (33-75%) are high and secondary cases have been reported (26 – 33%) in families of children attending day care centres (Kotloff et al, 1999). A study done in Bangladesh found that hospitalized children were infected with *S. flexneri* (59.1%), *S. bondii* (20.8%), *S. sonnei* (7.5%) and *S. dysenteriae* type 1 (9.4%). Most of the infants had a history of diarrhea (82%) with moderate or severe dehydration (59.8%), fever (32.7%) and abdominal cramps (19%) (Huskins et al, 1994).

A study done from 1968 to 1985 in South Africa found that hospitalized patients were mostly infected with *S. flexneri* (72%) and less frequent with *S. dysenteriae* (3%) and *S. sonnei* (2%) (Donald et al, 1987). *S. dysenteriae* type 1 was then later detected in 1994 by Pillay and colleagues; the bacterium was detected in 48 cases of patients across all age groups that had dysentery in KwaZulu-Natal (SA) (Pillay et al, 1997). In

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2001 an outbreak of food poisoning among children attending international sports day in Johannesburg (South Africa) were infected with *S. flexneri* which was detected in maize meal; out of 134 children, 53 developed diarrhea (Karas et al, 2001). A study done in Limpopo Province (South Africa), found that *Shigella spp.* were the most frequent bacterial pathogen detected in children less than 5 years in the Vhembe region (Obi et al, 2003).

2.3.2.4. TRANSMISSION

Shigella mainly infects humans and have very low survival rate outside its host (Nygren and Bowen, 2013). Transmission is primarily via faecal-oral route (Nygren and Bowen, 2013). Houseflies have also been shown to be vehicles in transmission of *Shigella* (Levine and Levine, 1991). Shigella requires dosage of 10 cfu to cause infection. Outbreaks caused by foodborne shigellosis have been reported in India and South Africa (Karas et al, 2001 Nandy et al, 2011). Factors associated with foodborne infections are due to poor hygienic behaviour of handling food by infected person, poor food storage and poor sanitary conditions were the food is being produced (Nygren and Bowen, 2013). Caretakers taking care of children at day care centres can also infect children due to poor hygiene and sanitary behaviours when handling food and water (Nygren and Bowen, 2013).

2.3.2.5. PATHOGENESIS

Invasion and spread of *Shigella* in the host cells involves four main stages: (i) cell invasion; (ii) intracellular multiplication; (iii) intracellular spread; and (iv) host cell killing (Sansonetti and Egile, 1998). *Shigella* consists of the virulence plasma, invasive plasmid antigen (*ipa*) region that has been widely studied. The ipa region consists of four genes *iapA* to *ipaD*, that encode proteins involved in cell invasion (Ingersoll et al, 2002). After the bacteria has contact with the host cell *ipaB* and *ipaC* are implanted as a single complex into the host to form 25A pore resulting in transportation of invasive proteins (Blocker et al, 1999). Apoptosis in macrophages is induced by *ipaB*; when *ipaB* combines with *ipaC* and the cytoplasmic chaperon *IpgC* results in lysis of the cell membrane for the bacteria to spread to other cells (Page et al, 1999). Movement of *Shigella* in the host cytoplasm is aided by the *icsA* (virG), virK and sopA (*icsP*) genes. The genes encode the outer membrane protein involved in intracellular spread to

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enable proper localization of *icsA* and the outer membrane protease that results in cleavage of *icsA* (Goldberg and Theriot, 1995; Ashkenazi, 2004).

2.3.2.6. CLINICAL SYMPTOMS

Shigella mainly infects the large intestine resulting in clinical manifestations such as diarrhea that can either be watery mucoid or bloody accompanied by abdominal cramps and/or fever (Dennehy, 2005). The severity of Shigellosis depends on virulence factors of the infective strain and the host's immune system (Percival and Williams, 2014). *S. sonnei* results in watery diarrhea while *S. dysenteriae*, *S. flexneri and S. boydii* results in bloody diarrhea with severe symptoms (Dennehy, 2005). *Shigella* has an incubation period of 1 – 4 days and diarrhea occurs 12 – 36 hours leading to dysentery (Niyongi, 2005; Cabral, 2010). Diarrhea firstly consists of large volume of watery stools followed by mucous bloody stools with painful defecation; in children bloody stools do not occur unless the patient has a very weak immune system (Ashkenazi, 2004). Malnourished children develop severe symptoms such as recurrent infection, growth retardation and impaired nutrition (Ashkenazi, 2004). Detection of *Shigella* spp. refer to Section 2.4.

2.3.3. *Salmonella*

2.3.3.1. BACKGROUND

Salmonella spp. are Gram-negative facultative rod-shaped bacteria belonging to the *Enterobacteriaceae* family (Garza and Cohen, 2011). *Salmonella* genus consists of three distinct species of namely: *S. enterica, S. bongori and S. subterranean. S. enterica* species is further classified into 6 subspecies (subsp): enetrica (subsp. I), salamae (subsp II), arizonae (subsp. IIIa), diarizonae (subsp. IIIb), houtenae (subsp IV) and indica (subsp. VI) (Su and Chiu, 2007).

Salmonella consists of over 50 serogroups based on the O antigen, and consists of over 2500 serotypes (each consisting of a unique arrangement of somatic O and flagella H1 and H2 antigen). All these serotypes belong to *Salmonella* subspecies enterica and these strains account to >99% of Salmonella diseases in humans (Su et al, 2004). All *Salmonella* serotypes are referred to by their genus and serotype names, for example *Salmonella* typhimurium previously referred to as *S. enteritidis*, serotype

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typhimurium (Figure 2.3). *S.choleraesuis* and *S.tyhi*, are also known to have one serotype each (Garza and Cohen, 2011). Genomic sequencing and analysis of numerous *Salmonella* genomes have closely revealed the resemblance of the core regions with evidence of recombination and rearrangement, genomic degradation and clonal diversity among the different serotypes (Liu et al, 2009). *S. enterica* serotypes have shown that demonic degradation is a common mechanism for host adaptation and re-assortment, resulting in increased virulence and development of resistance to multiple drugs (Liu et al, 2009; Chen et al, 2013).

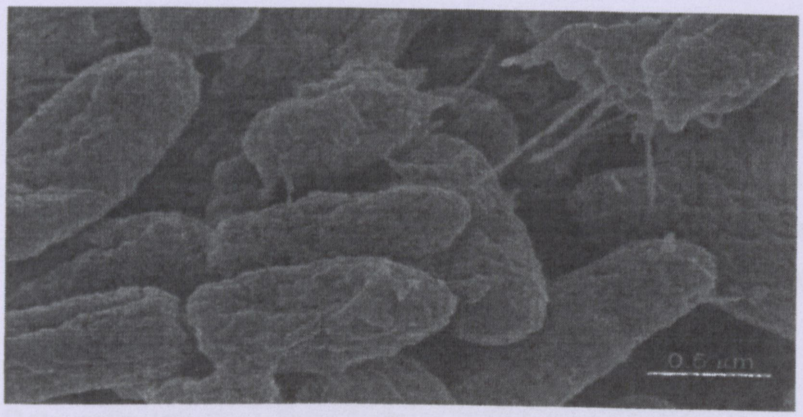


Figure 2.3: Electron microscopy of *S. enterica* subsp. *Enterica*, serotype *Typhimurium* (Sanchez-Vargas et al, 2011)

2.3.3.2. EPIDEMIOLOGY AND INCIDENCE

Salmonella infections are a health concern affecting children and adults in both developed and developing countries (Sa´nchez-Vargas et al, 2011). *Salmonella* spp. infects both animals and humans and leading to high morbidity and mortality worldwide (Sanchez-Vargas et al, 2011). Salmonellosis (disease caused by *Salmonella* spp.) cases have been found to be higher in infants and lower in children above the age of 6 years age. Non-typhoid *Salmonella* is endemic in sub-Saharan Africa, and recurrent in HIV-infected persons and children under the age of 3 years (Mandomando et al, 2009).

Majowicz et al (2010) have shown the global health impact of gastroenteritis caused by *Salmonella* spp. is high with 93.8 million cases reported, of which 80.3 million are due to foodborne infections and 155 000 results in death annually; with an occurrence

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of 1.14 episodes/100 person each year. High incidences have been found in countries such as Asia with 83.4 million cases reported, of which 137.7 thousand cases results in death with an occurrence of 4.72/100 person annually (Majowicz et al, 2010). In developing countries incidence of waterborne infection is higher compared to foodborne infections (Majowicz et al, 2010).

The Center for Disease Control and prevention (CDC) has estimated that 75% of *S. enteritidis* cases are due to consumption of raw and undercooked eggs; as the bacteria has been found to localize inside the egg (CDC, 2010). Studies now suggest that *Salmonella enterica* found in natural manure have evolved and can attach and colonize on vegetables posing a health concern (Klerks et al, 2007; Franz and van Bruggen, 2008). The CDC have also reported that there has been unusual high increase in *Salmonella* infections from 2005 to 2010 resulting in outbreaks. In 2010, and estimated 380 million eggs contaminated with *S. enteritidis* were shipped across 16 states of USA resulting in a multistate-outbreak with an estimated 1939 cases reported (CDC, 2010).

Data collected from Salm-Surv (a WHO supported foodborne disease surveillance network) from 2001 to 2005 indicated that *S. enteritidis* were the most frequent serotype detected worldwide (65%) followed by *S. typhimurium* (12%) and lastly *S. Newport* (14%) (Galanis et al, 2006). *S. enteritidis* were found to be common isolates in countries such as Asia (38%), Europe (87%) and Latin America (31%). In North America *S. typhimurium* was the most frequent pathogen (29%) followed by *S. enteritidis* (21%) (Galnis et al, 2006). In Africa, *S enteritidis* (26%) and *S. typhimurium* (25%) were found to be not significant (Galanis et al, 2006). A study by Mandomando and colleagues (2009) in Mozambican children found that children infected with *S. typhimuruim* (66%) and *S. enteritidis* (25%) had high risk of having severe malnutrition, anemia, diarrhea and pneumonia of which can lead to death. Recently, Smith et al (2014) reported that there was an unusual increase in *S. typhimurium* in children presenting with diarrhea.

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2.3.3.3. TRANSMISSION

Salmonella are transmitted by drinking water that has been contaminated by faecal matter or consuming food that are from animal origin such as beef, fish, poultry, eggs, and dairy products (Daniels et al, 2002). *Salmonella* spp. can be transmitted through food by two ways: food animals can ingest the bacteria and harbor it in intestine, it can then be spread when making meat, poultry, eggs and milk; and can be introduced into the environment by manure and litter resulting in contamination of fruits and vegetables which are eaten raw or are under cooked (Liu et al, 2011). Contamination of food sources can be through contact with an infected food handler or infected farm animals (Dione et al, 2011). Pets such as cats, lizards, turtles and chicks can also harbor *Salmonella* (CDC, 2008). Person to person infection occurs especially in cases involving infants (Daniels et al, 2002; Dennehy, 2005). A study done in infants less than 1 year of age identified the following risk factors of transmitting *Salmonella*: (i) Attending day care center with a child with a child infected with Salmonellosis; (ii) riding in a shopping cart that has been exposed to meat or poultry; and (iii) child not being breast fed (Jones et al, 2006). *Salmonella* infects children less than four years of age and mortality rates are higher during the first year of life (Dennehy, 2005). *Salmonella* are highly infective as they require low dosage to cause infections in humans (Cabral, 2010). Most *Salmonella* infections are sporadic but can also result in outbreaks especially in children less than 2 years of age. The bacteria requires infectious dose of 10^6-10^8 non-typhoid *Salmonella* to cause infections in healthy adults. In infants and immunocompromised patients smaller inoculum is required to cause an infection (Chen et al, 2013).

2.3.3.4. PATHOGENESIS

Salmonella begins with colonising the ileum and the colon by inducing an uptake mechanism that actively resembles phagocytosis (by invading enterocytes and colonocytes and proliferate in the epithelial cells and in the lamina propria) (Hansen-Wester et al, 2002). Virulence genes that are involved in invasion and required for intracellular survival are assembled in the large chromosomal DNA regions at designated *Salmonella* pathogenicity islands (SPI). SPI 1 and SPI 2 encode type III secretion systems that assists in contiguous channel across the bacterial and epithelial membranes (Dieye et al, 2007). After invasion in the host cell, the bacterium is capable

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of altering host cellular functions assisting in survival and colonization (Sanchez-Vargas et al, 2011). The bacteria may then travel to the mesenteric lymph nodes and into the systematic circulation resulting in bacteraemia. *Salmonella* then invades the enterocytes and colonocyctes resulting in enteritis with watery diarrhea, colitis and in certain cases bloody diarrhea can occur (Garza and Cohen, 2011).

2.3.3.5. CLINICAL SYMPTOMS

Food-borne*Salmonella* have incubation period of 12 to 72 hours (Cabral, 2010). Common symptoms such as nausea, vomiting, watery diarrhea, mucoid and bloody stools are also common depending on the type of strain. Symptoms are abdominal cramps, fever, chills arthralgia and headache (Thielman and Guerrant, 2004). Diarrhea is self-limited usually lasting for 3 to 7 days. Bacteremia accounts to approximately 6% of *Salmonella* infection in children and less frequent in adults. Immunocompromised patients and infants may develop non-intestinal sequelae after *Salmonella* infections such as meningitis, pneumonia and osteomyelitis (Hohmann, 2001). Shedding of infectious bacteria in stools can occur for several weeks, for example children less than 5 years of age median time of excretion is 7 weeks. In chronic carriers, *Salmonella* infections occur in the binary tract and is associated with cholelithiasis (Hohmann, 2001; Garza and Cohen, 2011). *Salmonella* infections are difficult to control as the bacteria is stable, has high tolerance to environmental stress with a widespread distribution of strains and are resistant to multiple drugs (Chen et al, 2013). Detection of *Salmonella* spp. see Section 2.4.

2.4. DETECTION OF ENTERIC PATHOGENS

2.4.1. DETECTION OF VIRAL ENTERIC PATHOGENS

There are various ways that Rotavirus can be detected in diarrheal stool specimen such as electron microscopy (CDC, 2006), Agglutination with latex particles (LA) (Bass et al, 2007) and Immunochromatography (ICG) which has high sensitivity (Dennehy et al, 1990). These techniques are commercially available, easy to perform with rapid results and are highly both sensitive (70-98%) and specific (71-100%) (Bernstein and Ward, 2004; Bass et al, 2007). Enzyme-linked immunoassay (EIA) is more sensitive with high specificity detection for Rotavirus Group A (Dennehy et al, 1990; Moradi-Lakeh et al, 2014). The Rotavirus monoclonal antibody tests are used in a sandwich-

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type methodology. The monoclonal antibodies act against the VP6 gene which is applied in the microwell plate. The stool suspension together with negative and positive controls are transferred into the wells of the microplate and are incubated at room temperature with the conjugate (biotinylated monoclonal anti-Rotavirus antibodies). After the wash step, polystreptavidin peroxidase conjugate is added to form a sandwich complex of immobilized antibodies. After addition of the substrate the attached enzyme complex will result in colour (blue) change determining if the test is positive (RIDASCREEN, Rotavirus). Rotavirus EIA is sensitive with high specificity detection for Rotavirus Group A (Dennehy et al, 1990; Moradi-Lakeh et al, 2014). Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time PCR are used mostly in molecular laboratories using Rotavirus-specific primers and probes that are complementary to the VP4 and VP7 regions and are used for detection and characterization of Rotavirus strains (Seheri et al, 2010). Due to availability of resources Rotavirus RIDASCREEN EIA was used to detect Rotavirus from stool specimen.

Noroviruses can be detected in faecal samples with a median of 4 weeks (high viral load during onset symptoms) and up to 8 weeks after viral infection; the virus has also been detected in asymptomatic cases. Enzyme immunoassay (EIA) is commercially available suitable for detecting specific antigens in faecal specimen (Dennehy, 2005). RT-PCR is more advanced assay allowing molecular analysis of Norovirus in both clinical and environmental samples (Vinje et al, 2003). RT-PCR enables detection of Norovirus genogroups, the viral RNA can further be analysed by nucleotide sequencing to determine the specific genotype and assist in phylogenetic analysis; and has been a useful tool in epidemiological studies (Vinje et al, 2003, Moyo et al, 2007; Mans et al, 2010). Due to availability of resources Norovirus RIDASCREEN EIA was used to detect Norovirus in the stool specimen.

Enteric Adenoviruses cannot grow on cell culture; therefore diarrhea caused by Adenovirus of Group F serotypes 40 and 41 can be detected using EIA and PCR techniques (Saderi et al, 2002; Garza and Cohen, 2011; Gray and Kang, 2013). Due to availability of resources Adenovirus 40/41 were detected from stool specimen using Premier Adenoclone 40/41 EIA. Adenoclone uses the same technique as RIDASCREEN in which a microplate is used to place the negative, positive and the

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diluted stool specimen in the microwells. Interpretation of positive Adenovirus 40/41 is by observing a blue colour in the wells and also adding the stop solution in order to read the results using spectrophotometer.

2.4.2. DETECTION OF ENTERIC BACTERIAL PATHOGENS

Salmonella spp. can be isolated from bone marrow, blood, urine or stools depending on type of infection (Cabral et al, 2010). Identification on stool and blood cultures can be performed using conventional microbiological identification and serology diagnostic testing. In cultures *Salmonella* can be grown on selective media such as *Salmonella-Shigella* agar and Xylose lysine deoxycholate (XLD) in which black colonies on both plates are formed due to their ability to produce Hydrogen sulfide. Blood cultures have sensitivity of 40-60% while stool cultures are positive for 30-35% and urine with 7-10% sensitivity. Multiplex-PCR techniques have been shown to detect multiple genes of *Salmonella* spp. with a high sensitivity than culture methods (Radhika et al, 2013). In this study Multiplex-PCR was used to detect *S. enteritidis* with *sefA* (Soumet et al, 1999); S. *typhimurium* with *fliCI* (Soumet et al, 1999), other primers were *st11*(Soumet et al, 1999) and *invA* (Salehi et al, 2005) of which are virulent genes.

Shigella spp. can be detected in stool cultures, however this technique has limitations as the bacteria cannot grow if the child infected is given antibiotics before collection of samples (Edwards, 1999). The bacteria can be detected using conventional culture techniques. *Shigella* on nutrient agar appear as circular, translucent glistening, slightly opaque colonies. The colonies differ in size depending on the serotype *(S. dysenteriae* are smaller while *S. sonnei* are larger colonies with irregular edges). The bacteria can grow on blood agar with no hemolysis. A study has shown that bacteria can also be detected by microarrays using the gyrB genes (Kakinuma et al, 2003). Molecular detection is more sensitive with the use of PCR techniques (Radhika et al, 2013); sensitivity is increased by 50% when compared to culture methods. In this study *S. flexneri* were detected by multiplex-PCR for detection of Shiga toxin 1 (*shetlA*) and Shiga toxin 2 (*sheltB*) (Thong et al, 2005); *Shigella* spp. were detected by *ipaH* (Kong et al, 2002) and *ial* (Paton and Paton 1998) virulent genes.

E. coli can be detected from stool specimen using culture methods such as MacConkey agar in which *E. coli* appear as pinkish or red colonies; Motility Indole

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Ornithine (MIO) media and other various biochemical tests. Detection of pathogenic *E. coli* pathotypes can be detected via molecular techniques (Omar et al, 2010). Molecular techniques such as multiplex-PCR require detection of pathogens using specific primers forward and reverse primers (Omar and Barnard, 2014). In this study multiplex-PCR was used to detect DEC. Genes for detection of EPEC are *bfp* and *eaeA* (Aranda et al, 2004); EAEC are *eagg* (Pass et al, 2000); ETEC are *LT* and *ST* (Pass et al, 2000); EIEC *ial* (Lopez-Saucedo et al, 2003); and EHEC are *stx1* and *stx2* (Moses et al, 2006). Commensal *E. coli* can be seen by mdh genes (Tarr et al, 2002).

2.5. SUMMARY OF LITERATURE REVIEW

Many rural areas in the Vhembe district of the Limpopo province in South Africa still do not have access to safe, clean drinking water and water is collected from sources which are often contaminated with animal excreta (Samie et al, 2012). Several studies have shown that water from contaminated places with poor sanitation and hygiene conditions in rural areas can result in diarrheal outbreaks (Eisenberg et al, 2007; Badowski et al, 2011). Usually children younger than five years of age are mostly affected by diarrheal outbreaks (Kotloff et al, 2013). In addition poor sanitation such as lack of access to latrines; poor hygiene behaviour such as not washing hands after having contact with contaminated surfaces before eating and after using the latrine can lead to diarrhea and respiratory infections (Badowski et al, 2011; Brown et al, 2013).

As shown from the literature review, enteric pathogens (Rotavirus, Norovirus, Adenovirus, *Shigella* spp. *Salmonella* spp. and DEC) are some of the main causes of gastroenteritis in children under the age of 5 years. Norovirus has been considered one of the leading causes of gastroenteritis after introduction of Rotavirus vaccine followed by Adenovirus 40/41 (Patel et al, 2009; Mans et al, 2010; Ahmed et al, 2014). These viruses have been found to cause diarrhea mainly in children less than 2 years of age. DEC strains (ETEC, EAEC, and EPEC) have also been found to cause diarrhea in children (Albert et al, 1995). *Shigella* spp. and *Salmonella* spp. have also been reported especially in children attending day care centres, in which transmission is be through sharing of toys and lack of hygiene of caretakers (Jones et al, 2006; Nygren and Bowen, 2013)

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Most of the studies in Limpopo province especially the Vhembe district have focused mainly of parasitic infections, while infections involving Rotavirus were last reported in 2010 (Potgieter et al, 2010). Bacterial infections such as DEC and *Salmonella* spp. have been reported in 2004 (Obi et al, 2004). It is therefore important to determine the aetiology of enteric pathogens that might play a role in childhood gastroenteritis. Therefore this study is aimed at determining the prevalence of enteric pathogens (*Shigella* spp., *Salmonella* spp., DEC, Rotavirus, Norovirus and Adenovirus 40/41) affecting children less than 5 years of age in rural communities of the Vhembe district.

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Chapter 3
METHODOLOGY

3.1. ETHICAL CONSIDERATION

The project is registered at the University of Venda (SMNS/14/MBY/28) and ethical clearance was obtained from the ethical board (SMNS/13/MBY/0212) of Univen. Informed and signed consent forms were obtained from the participant parents and/or primary caretakers before collection of stool samples.

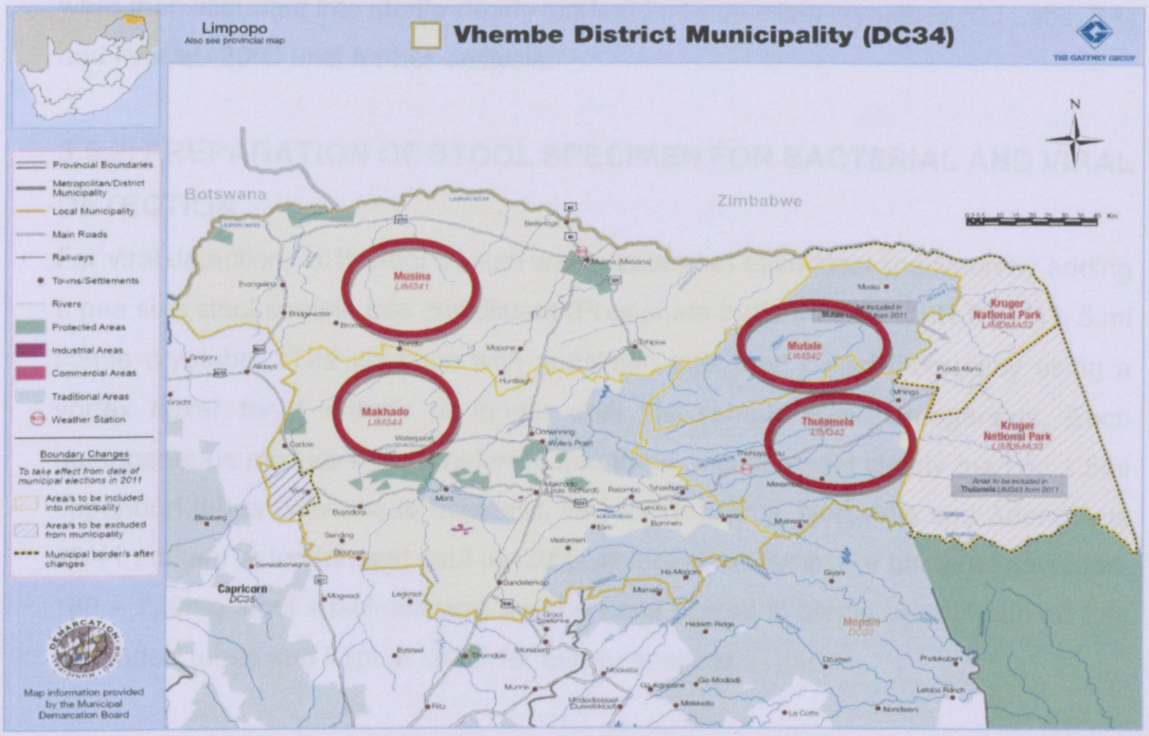
3.2. STUDY DESIGN

This was a prospective study to determine the prevalence of selected bacterial and viral entero-pathogens affecting infants and young children with diarrhea attending outpatient Primary Health Care facilities (clinics) in the Vhembe District, Limpopo province.

3.3. STUDY SITE

Stool samples were collected from children less than 5 years of age with diarrhea visiting Primary Health Care facilities from the four municipalities (Elim, Makhado, Mutale, Musina and Thulamela) (Figure 3.1) in the Vhembe district of the Limpopo Province. A total of 30 clinics were randomly recruited to be part of the study. The study area shares borders with Zimbabwe and Botswana in the north-west and Mozambique in the south-east of Kruger National Park. The size of the Vhembe district is approximately 25 597/km^2 and consists of 821 local communities (villages) with a population size of 1 472 615. The Vhembe district lacks improved water quality, sanitation infrastructure and still have poor hygiene practices.

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Figure 3.1: The Vhembe district indicating the four municipalities used in the study site

3.4. SAMPLE COLLECTION

A total of 237 stool samples of children under the age of 5 years of age presenting with diarrhea were collected from clinics in the Vhembe District. In this study diarrhea was defined as a passage of three or more loose stools within 24 hours. A questionnaire was also used to collect demographic and clinical data of the study participants (Age, gender, clinical symptoms, type of stool specimen, etc.) (Appendix 1) with assistance from the nurse and legal guardian of the child. The stool specimen was collected from the child by a qualified nurse in 20ml stool collection bottles (Sterlin, UK). The samples were then stored at -4°C and transported to the University of Venda laboratory for further analysis.

3.5. METHODOLOGY APPLIED IN THE STUDY

3.5.1. LABORATORY PROCEDURE

Upon arrival at the laboratory all the stool specimen were given a unique laboratory number for identification to protect the identity of the study participants. The specimens

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were then aliquoted into sterile clearly marked 2ml screw cap cryotubes (CJ Labs, SA) and kept at -20^{0}C until further analysis.

3.5.2. PREPARATION OF STOOL SPECIMEN FOR BACTERIAL AND VIRAL DETECTION

For viral detection, 10% stool dilution was prepared of each stool specimen by adding a pea size stool sample into 3ml diluent (Phosphate buffered saline, pH 7.4) in a 5 ml sterile cryotube. The cryotube with specimen was then mixed thoroughly using a vortex mixer for 1 minute or longer until the sample was homogenous. Each homogeneous mixture was transferred into three sets of sterile clearly marked 1.5ml eppendorf tubes (Whitehead Scientific, SA) for Rotavirus, Norovirus and Adenovirus 40/41 antigen detection and kept at -20^{0}C till further analysis. For bacterial detection, 180 – 220mg stool specimen was weighed and placed in sterile clearly marked 2ml eppendorf tubes and kept at -20^{0}C for further analysis

3.5.3. DETECTION OF VIRAL ENTERO-PATHOGENS USING ENZYME IMMUNOASSAY (EIA)

Identification of Enteric Adenovirus type 40/41 was determined using the Premier Adenoclone 40/41 kit (Meridian Bioscience, Europe) according to the manufacturer's instructions. Identification of Rotavirus was determined using the RIDASCREEN Rotavirus EIA kit (R-Biopharm Germany) according to the manufacturer's instructions. Identification of Norovirus was determined using the RIDASCREEN Norovirus 3rd Generation EIA kit (R-Biopharm Germany) according to manufacturer's instructions. Positive and negative controls for all the EIA assays were provided by the kits. For interpretation of Adenovirus 40/41, Rotavirus and Norovirus results, absorbance was measured spectrophotometrically at 450nm with a Versa Max tunable microplate reader (Separations). Samples were considered positive if the absorbance was greater than 0.150nm.

3.5.4. DETECTION OF BACTERIAL ENTERO-PATHOGENS USING PUBLISHED m-PCR PROTOCOLS

Bacterial DNA was isolated from all stool specimen using the QIAamp Fast DNA stool mini kit according to the manufacturer's instructions (Qiagen, CA, USA).

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IM of *ial*, *eagg*, *astA*, *bfp* and *gapdh* primers (F and R), 0.3 IM of eaeA and stx2 primers (F and R), 0.5 IM of *stx1* and *st* primers], 2 µl of DNA sample 1 µl of *gapdh* cDNA and 5 ul of PCR grade water. The reactions were subjected to an initial activation step at 95^{0}C for 15 min, 35 cycles that consisted of denaturation at 95^{0}C for 45s, annealing at 55^{0}C for 45s, extension at 68^{0}C for 2 min and final elongation at 72^{0}C for 5 min (Omar and Barnard, 2014).

Table 3.2: Primers used for identification of DEC (Omar and Barnard, 2014)

Pathogen	Primer	Sequence (5'-3')	Size (bp)	Reference
E. coli	*mdh*	GGT ATG GAT CGT TCC GAC CT GGC AGA ATG GTA ACA CCA GAG T	304	Tarr et al (2002)
EIEC	*Ial*	GGT ATG ATG ATG ATG AGT CCA GGA GGC CAA CAA TTA TTT CC	650	López-Saucedo et al (2003)
EHEC/Atypical EPEC	*eaeA*	CTG AAC GGC GAT ACG ATC CAG CCA GAC GAT ACG ATC CAG	917	Aranda et al (2004)
Typical EPEC	*bfpA*	AAT GGT GCT TGC GCT TGC TGC TAT TAA CAC CGT AGC CTT TCG CTG AAG TAC CT	410	Aranda et al (2004)
EAEC	*eagg*	AGA CTC TGG CGA AGC ACT GTA TC ATG GVT GTC TGT AAT AGA TGA GAA C	194	Pass et al (2000)
EHEC	*stx1*	ACA CTG GAT GAT CTC AGT GG CTG AAT CCC CCT CCA TTA TG	614	Moses et al (3006)
	stx2	CCA TGA CAA CGG ACA GCA GTT CCT GTC AAC TGA GCA CTT TG	779	Moses et al (2006)
ETEC	*LT*	GGC GAC AGA TTA TAC CGT GC CGG TCT CTA TAT TCC CTG TT	360	Pass et al (2000)
	ST	TTT CCC CTC TTT TAG TCA GTC AAC TG GGC AGG ATT ACA ACA AAG TTC ACA	160	Pass et al (2006)
E coli toxin	*astA*	GCC ATC AAC ACA GTA TAT CC GAG TGA CGG CTT TGT AGT C	106	Kimata et al (2005)
External control	*gapdh*	GAG TCA ACG GAT TTG GTC GT TTG ATT TTG GAG GGA TCT CG	238	Mbene et al (2009)

3.5.4.3. m-PCR PROTOCOL TO IDENTIFY SPECIFIC *Salmonella* spp. AND *Shigella* spp. STRAINS IN STOOL SAMPLES

The m-PCR protocol published by Mieta (2010) was used to detect *S. flexneri*, *Shigella* spp., *S. enteritidis* and *S. typhimurium* on specimen that were positive in the first PCR run in Section 3.6.4.1. All m-PCR reactions were performed in a Biorad Mycycler™ Thermal cycler in a total volume of 20 µl. The genes used are shown in Table 3.3. Each reaction consisted of 1X Qiagen® PCR multiplex mix (containing HotstartTaq® DNA polymerase, m-PCR buffer and dNTP mix) (Whitehead Scientific, SA); 2 µl of the primer mixture [0.lµM of *St11* and *fiiCl* primers, 0.2µl of *sefA* and *Inva* primers, 0.3µM of *lpaH* and *Ial* primers, 0.5µM of *shetlA* and *shetlB* primers, 4µl of DNA sample and

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4µl of PCR grade water. The reactions were subjected to an initial activation step at 95^{0}C for 15 min, 35 cycles that consisted of denaturation at 95^{0}C for 45s, annealing at 55^{0}C for 45s, extension at 68^{0}C for 2 min and final elongation at 72^{0}C for 5 min (Mieta, 2010).

Table 3.3: Primers used for identification of *Shigella* and *Salmonella* spp. (Mieta, 2010)

Pathogen	Primer	Sequence (5'-3')	Size (bp)	Reference
Salmonella 1	*st11*	GCC AAC CAT TGC TAA ATT GGC GCA GGT AGA AAT TCC CAG CGG GTA CTG G	429	Soumet et al (1999)
S. typhimurium	*fliC*	CGG TGT TGC CCA GGT TGG TAA T ACT CTT GCT GGC GGT GCG ACT T	559	Soumet et al (1999)
S. enterItidis	*sefA*	AGG TTC AGG CAG CGG TTA CT GGG ACA TTT AGC GTT TCT TG	312	Soumet et al (1999)
Salmonella 2	*Inva*	GTG AAA TTA TGC CCA CGT TCG GGC AA TCA TCG CAC CGT CAA AGG AAC	284	Salehi et al (2005)
Shigella spp	*IpaH*	CCT TGA CCG CCT TTC CGA TA CAG CCA CCC TCT GAG GTA CT	606	Kong et al (2002)
Shigella spp	*Ial*	GGT ATG ATC ATG ATG AGT CCA GGA GGC CAA CAA TTA TTT CC	630	Paton & Paton (1998)
Shiga Toxin 1	*shetIA*	TCA CGC TAC CAT CAA AGA TAT CCC CCT TTG GTG GTA	309	Thong et al (2005)
Shiga Toxin 2	*shetIB*	GTG AAC CTG CTG CCG ATA TC ATT TGT GGA TAA AAA TGA CG	147	Thong et al (2005)

3.5.5. ELECTROPHORESIS AND VISUALIZATION OF DNA

Bacterial DNA was analysed using a 2.5% (w/v) agarose gel in TAE buffer (40mmol-1 Tris acetate; 2mmol-1 EDTA, pH 8.3) with 0.5µgml-1 Ethiduim Bromide. DNA was electrophoresed for 1-2h in an electric field strength of 8V cm-1 gel. The DNA was then visualized using UV light (Gene Genius Bio Imaging system, Vacutec®). The relative sizes of the DNA fragments were estimated by comparing their electrophoretic mobility with that of the standards run with the samples on each gel, either 1kB or 100bp markers (Fermentas®) as provided by previous studies (Mieta et al, 2010; Omar and Barnard 2014).

3.6. DATA ANALYSIS

Data from the EIA and PCR samples were entered into Microsoft Excel (2010) spread sheets. Data from questionnaires were entered into separate excel spread sheets. The average and median of the samples were calculated using Microsoft Excel (2010) when needed.

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Chapter 4

RESULTS

4.1. DEMOGRAPHIC DATA OF STUDY POPULATION

A total of 30 primary health care clinics in the Vhembe district were recruited to be part of the study from August 2014 to May 2015. From these clinics a total of 237 stool specimen were collected from children less than 5 years of age presenting with diarrhea. Based on gender distribution 44.3% (105/237) of the samples were from females and 51.5% (122/237) of the samples were from males. A total of 4.2% (10/237) of the samples collected did not have the demographic data and these samples were labelled as Unknown (Figure 4.1). The average age of the study participants was 14 months with a median of 10 months. Within this age group 58.6% (139/237) were in the age group of 0 - 12 months; 21.9% (52/237) were in the age group of 12 - 24 months; 6.8% (16/237) were in the age group of 24 - 36 months; 3.0% (7/237) were in the age group of 36 - 48 months, 5.1% (12/237) were in the age group of 48 - 60 months; and 4.2% (10/237) were the Unknown age group (Figure 4.1).

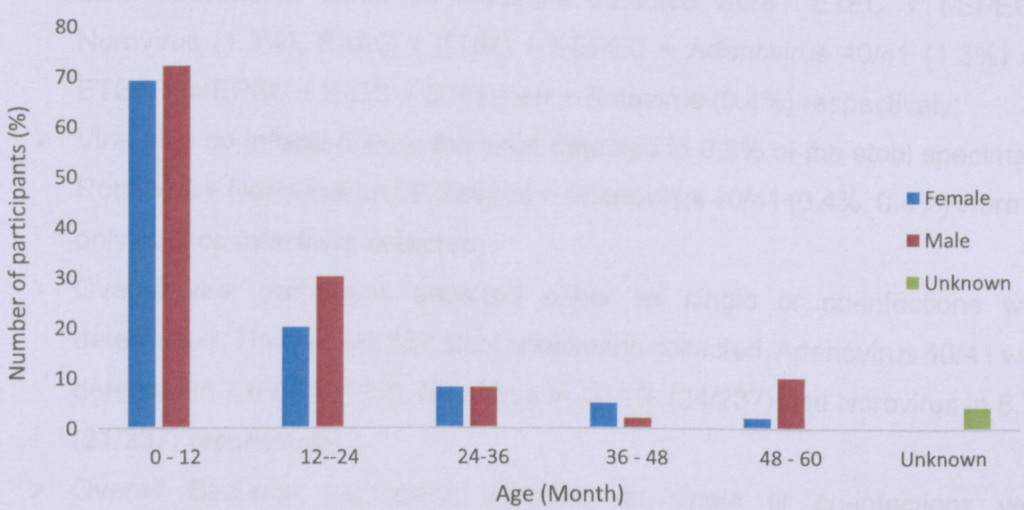


Figure 4.1: Gender distribution of study participants according to age group

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4.2. PREVALENCE OF ENTERIC PATHOGENS IN THE STUDY

Occurrence of enteric pathogens was observed as follows (Table 4.1):

- At least 1 pathogen was detected in 149 of the 237 (62.9%) diarrheal specimens;
- Due to simultaneous occurrence of 2 or more pathogens in the stool specimen; more than 149 enteric pathogens were detected;
- Single infections caused by enteric viruses: Adenovirus 40/41, Norovirus and Rotavirus (2.5%, 2.5%, 4.2%) were detected respectively;
- Single infections caused by enteric bacteria: ETEC, EAEC, a-EPEC and t-EPEC (3.0%, 5.5%, 0.8%, and 0.8%) were also detected respectively;
- Bacteria-bacteria co-infections were detected in 26.2% of the stool specimen. Of these, ETEC + t-EPEC and ETEC + EAEC were the highest co-infections detected each accounting to 4.2% of the stool specimen. More than two co-infections were also detected in some of the isolates such as: EAEC + ETEC + t-EPEC in 3.0% of the stool specimens; ETEC + t-EPEC + EIEC in 1.3% of the stool specimens; and a-EPEC + t-EPEC + EIEC + *Shigella* spp. in 0.4% of the stool specimens;
- Bacteria-viral co-infections were detected in 15.2% of the stool specimen. EAEC + Rotavirus were the highest co-infections to be detected in 2.5% of the stool specimens. Other co-infections detected were ETEC + t-EPEC + Norovirus (1.3%), EAEC + ETEC + t-EPEC + Adenovirus 40/41 (1.3%) and ETEC + a-EPEC + EIEC + *S. flexneri* + Rotavirus (0.4%) respectively;
- Viral-viral co-infection were the least detected in 0.8% of the stool specimens. Rotavirus + Norovirus and Rotavirus + Adenovirus 40/41 (0.4%, 0.4%) were the only viral co-infections detected;
- Overall viral pathogens detected either as single or co-infections were determined. That is from 237 stool specimens collected, Adenovirus 40/41 were detected in 7.6% (18/237), Rotavirus in 10.1% (24/237) and Norovirus in 8.7% (21/237) respectively;
- Overall Bacterial pathogens detected as single or co-infections were determined. That is from 237 stool specimen collected, ETEC were detected in 30.8% (73/237), EAEC in 26.2% (62/237), Atypical EPEC in 15.6% (37/237), Typical EPEC in 20.7% (49/237), EIEC in 7.6% (18/237) and EHEC in 0.8%

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(2/237) of the stool specimen respectively. *S. Flexneri* were detected in 4.2% (10/237%), *Shigella* spp. in 1.3% (3/237), *S. enteritidis* in 1.3% (3/237) and *S. typhimurium* in 0.4% (1/237) of the stool specimen respectively;

- *Vibrio* spp. were detected in 0.4% (1/237) of the stool specimen although the pathogen was not included as part of this study.

Table 41: Occurrence of diarrheal pathogens detected in children with acute gastroenteritis

Diarrheal pathogen	Cases (n=237)
Number of specimen positive for any diarrheal pathogen	**149 (62.9%)**
Positive for only one diarrheal pathogen	**46 (19.4%)**
Adenovirus 40/41	6 (2.5%)
Norovirus	6 (2.5%)
Rotavirus	10 (4.2%)
ETEC	7 (3.0%)
EAEC	13 (5.5%)
a-EPEC	2 (0.8%)
t-EPEC	2 (0.8%)
EIEC	0 (0%)
EHEC	0 (0%)
S. flexneri	0 (0%)
Shigella spp.	0 (0%)
S. enteritidis	0 (0%)
S. typhimurium	0 (0%)
Positive for more than one bacterial pathogen	**62 (26.2%)**
ETEC + t-EPEC	10 (4.2%)
EAEC + ETEC	10 (4.2%)
EAEC + t-EPEC	5 (2.1%)
ETEC + EHEC	1 (0.4%)
ETEC + a-EPEC	4 (1.7%)
a-EPEC + t-EPEC	3 (1.3%)
EAEC + a-EPEC	1 (0.4%)
ETEC + *S. flexneri*	1 (0.4%)
EIEC + *S. flexneri*	1 (0.4%)
EIEC + *Shigella* spp.	1 (0.4%)
EIEC + t-EPEC	1 (0.4%)
a-EPEC + *S. flexneri*	1 (0.4%)
EAEC + ETEC + t-EPEC	7 (3.0%)
ETEC + a-EPEC + t-EPEC	3 (1.3%)
ETEC + t-EPEC + EIEC	1 (0.4%)
EAEC + ETEC + EIEC	1 (0.4%)
EAEC + ETEC + a-EPEC	3 (1.3%)
EIEC + *S. flexneri* + *S. enteritidis*	1 (0.4%)
ETEC + EIEC + *S. flexneri*	1 (0.4%)
EAEC+ a-EPEC + EIEC	1 (0.4%)
EAEC + EIEC + *S. flexneri*	1 (0.4%)
EAEC+ ETEC + a-EPEC + t-EPEC	2 (0.8%)
EAEC + t-EPEC + EIEC + *S. flexneri*	1 (0.4%)
ETEC + t-EPEC + EIEC + *S. flexneri*	1 (0.4%)
a-EPEC + t-EPEC + EIEC + *Shigella* spp.	1 (0.4%)

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Positive for both bacterial and viral pathogen	**36 (15.2%)**
EAEC + Rotavirus	6 (2.5%)
EAEC + Adenovirus 40/41	1 (0.4%)
EAEC + Norovirus	1 (0.4%)
ETEC + Adenovirus 40/41	1 (0.4%)
EIEC + *Shigella* spp. + Rotavirus	1 (0.4%)
a-EPEC + EIEC + Rotavirus	1 (0.4%)
ETEC + t-EPEC + Norovirus	3 (1.3%)
ETEC + a-EPEC + Rotavirus	1 (0.4%)
EIEC + Rotavirus	1 (0.4%)
EAEC + a-EPEC + Norovirus	1 (0.4%)
a-EPEC + t- EPEC + Norovirus	1 (0.4%)
EAEC + a-EPEC + Adenovirus 40/41	1 (0.4%)
a-EAEC + Rotavirus + Norovirus	1 (0.4%)
EIEC + Rotavirus + Adenovirus 40/41	1 (0.4%)
EAEC + ETEC + a-EPEC + Norovirus	2 (0.8%)
EAEC + ETEC + t-EPEC + Adenovirus 40/41	3 (1.3%)
ETEC + a-EPEC + t-EPEC + Norovirus	3 (1.3%)
ETEC + a-EPEC + EIEC + *S. flexneri* + Rotavirus	1 (0.4%)
EAEC + ETEC + EIEC + Adenovirus 40/41	1 (0.4%)
EAEC + ETEC + EHEC + Norovirus	1 (0.4%)
ETEC + + a-EPEC + t-EPEC + Adenovirus 40/41	1 (0.4%)
ETEC + a-EPEC + EIEC + Norovirus	1 (0.4%)
EAEC + EIEC + *S. flexneri* + Adenovirus 40/41	1 (0.4%)
t-EPEC + *S. enteritidis* + *S. typhimurium* + Adenovirus 40/41	1 (0.4%)
Positive for more than one viral pathogen	**2 (0.8%)**
Rotavirus + Norovirus	1 (0.4%)
Rotavirus + Adenovirus 40/41	1 (0.4%)

4.2.1. ASSOCIATION BETWEEN ENTERIC PATHOGENS AND GENDER DISTRIBUTION OF THE STUDY POPULATION

From the samples that tested positive for Adenovirus 40/41, 61.1% (11/18) were seen in male participants and 38.9% (7/18) were seen female participants (Table 4.2). Rotavirus was detected in 54.2% (13/237) of the male participants compared to 41.7% (10/237) of the female participants (41.7%). Norovirus was detected in 52.4% (11/21) of the male participants and 42.9% (9/21) of the female participants.

ETEC were detected in 59.0% (43/237) of the male participants and in 41.1% (30/73) of the female participants. EAEC were detected in 61.3% (38/62) of the male participants compared to 37.1% (23/62) of the female participants. Atypical EPEC were detected in 56.8% (21/37) of the male participants and in 43.2% (16/37) of the female participants. EIEC were detected in 55.6% (10/18) of the male participants compared to 44.4% (8/18) of the female participants (Table 4.2). EHEC were detected

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in male participants 50% (1/2). *S. flexneri* were detected in 50% (5/10) of the male participants and 50% (5/10) of the female participants. *Shigella* spp. were detected more in 66.7% (2/3) of the female participants than in 33.3% (1/3) of the male participants. *S. enteritidis* and *S. typhimurium* were only detected in 100% (3/3) and 100% (1/1) of the male participants. (Table 4.2).

Table 4.2: Distribution of diarrheal causing microorganisms based on gender of the study population

Diarrhea causing Microorganism (n= total positive specimen)			Gender (n=237)		
			Female	Male	Unknown
Viruses	Adenovirus 40/41 (n = 18)		7 (38.9%)	11 (61.1%)	-
	Norovirus (n =21)		9 (42.9%)	11 (52.4%)	1 (4.8%)
	Rotavirus (n = 24)		10 (41.7%)	13 (54.2%)	1 (4.2%)
Bacteria	DEC	ETEC (n=73)	30 (41.1%)	43(59.0%)	-
		EAEC (n=62)	23 (37.1%)	38 (61.3%)	2 (3.2%)
		a-EPEC (n=37)	16 (43.2)	21 (56.8%)	-
		t-EPEC (n= 49)	26 (53.1%)	23 (46.9%)	-
		EIEC (n=18)	8 (44.4%)	10 (55.6%)	-
		EHEC (n=2)	-	1 (50%)	1 (50%)
	Salmonella spp.	*S. enteritidis* (n=3)	-	3 (100%)	-
		S. typhimurium (n=1)	-	1 (100%)	-
	Shigella spp.	*S. flexneri* (n=10)	5 (50%)	5 (50%)	-
		Shigella spp. (n=3)	2 (66.7%)	1 (33.3%)	-

4.2.2. ASSOCIATION BETWEEN ENTERIC PATHOGENS AND AGE DISTRIBUTION OF THE STUDY POPULATION

The association between enteric pathogens and age distribution of the study population is shown in Table 4.3. Enteric Ad 40/41 infections were detected across all age groups except children aged 36 – 48 months. Rotavirus infections were detected across all age groups. Norovirus infections were detected mostly in children aged 0 – 12 months and 12 – 24 months.

The most prevalent bacterial pathogens across all age groups were DEC strains (Table 4.3). Children aged 0 – 12 months, were mostly infected with ETEC 30.9%

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Table 4.3: Distribution of diarrheal causing microorganisms based on age of population

Diarrhea causing Microorganism (n= total positive specimen)			Age (number of samples)					
			0 – 12 Months (n=139)	12 – 24 Months (n=52)	24 – 36 Months (n=15)	36 – 48 Months (n=9)	48 – 60 Months (n=11)	Unknown (n=11)
Viruses	Adenovirus 40/41 (n=18)		12 (8.6%)	2 (3.8%)	3 (20.0%)	-	1 (9.1%)	-
	Norovirus (n=21)		5 (3.6%)	11 (21.2%)	-	-	1 (9.1%)	4 (36.4%)
	Rotavirus (n=24)		7 (5.0%)	9 (17.3%)	1 (6.7%)	1 (11.1%)	3 (27.2%)	3 (27.2%)
Bacteria	DEC	ETEC (n=73)	43 (30.9%)	17 (32.7%)	7 (46.7%)	2 (22.2%)	3 (37.3%)	1 (9.1)
		EAEC (n=62)	36 (25.9%)	15 (28.8%)	4 (26.7%)	2 (22.2%)	3 (27.3%)	1 (9.1)
		a-EPEC (n=37)	26 (18.7%)	9 (17.3%)	1 (6.7%)	1 (11.1%)	-	-
		t-EPEC (n=49)	34 (24.5%)	11 (21.2%)	3 (20.0%)	-	1 (9.1%)	-
		EIEC (n=18)	7 (5.0%)	4 (7.7%)	3 (20.0%)	3 (33.3%)	1 (9.1)	-
		EHEC (n=2)	-	1 (1.9%)	-	-	-	1 (9.1%)
	Salmonella spp.	*S. enteritidis* (n=3)	2 (3.8%)	1 (1.9%)	-	-	-	-
		S. typhimurium (n=1)	-	1 (1.9%)	-	-	-	-
	Shigella spp.	*S. flexneri* (n=10)	5 (3.6%)	2 (3.8%)	2 (13.3%)	1 (11.1%)	-	-
		Shigella spp. (n=3)	1 (0.7%)	-	1 (6.3%)	1 (11.1%)	-	-

4.2.3. Clinical data of the study participants

A questionnaire was used to obtain clinical data of the study participants (Appendix 1). Of the 149 children that tested positive for an enteric pathogen, 53.7% (80/149) patients were reported to have diarrhea only; 10.7% (16/149) patients were reported to have diarrhea accompanied with vomiting; 8.1 % (12/149) patients were reported to have diarrhea accompanied with fever (Table 4.4). Severe symptoms were observed in 2.7% (4/149) patients were reported to have diarrhea accompanied with dehydration; and in 2.7% (4/149) of the patients reported to have diarrhea accompanied with vomiting and dehydration. A Bristol stool scale was used to classify

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the type of stool specimen collected (Appendix 2). A total of 42.3% (63/149) of the study participants had watery stools while 38.3% (57/149) had sausage stools and 19.5% (29/149) reported to have mushy stools (Table 4.4).

Table 4.4: Clinical data obtained from the questionnaire

Clinical Data		No of cases (n = 149)
Symptoms	D	80 (53.7%)
	D + V	16 (10.7%)
	D + F	12 (8.1%)
	D + A	7 (4.7%)
	D + Deh	4 (2.7%)
	D + V + A	1 (0.7%)
	D + V +F	8 (5.4%)
	D + A + F	7 (4.7%)
	D + V + A + F	6 (4.0%)
	D + V + Deh	4 (2.7%)
	D + F + Deh	2 (1.3%)
	D + A + Deh	1 (0.7%)
	D + V + F + Deh	1 (0.7%)
Stool type	Watery	63 (42.3%)
	Sausage	57 (38.3%)
	Mushy	29 (19.5%)

Key: D = Diarrhea; V = Vomiting; F = Fever; A = Abdominal cramps; Deh = Dehydration

From the single enteric pathogens that were detected in Table 4.1, clinical data was observed. That is Rotaviruses were detected in 4.2% (10/237) of the specimen, the most frequent clinical symptoms observed were diarrhea accompanied with vomiting in 30% (3/10) and diarrhea accompanied with fever in 30% (3/10%) of the specimen followed by diarrhea accompanied with vomiting, fever and dehydration in 10% (1/10) of the specimen (Figure 4.2). Watery stools were frequently observed in 50% (5/10) of the specimen followed by mushy stool in 40% of the specimen and sausage stools in 10% (1/10) of the specimen.

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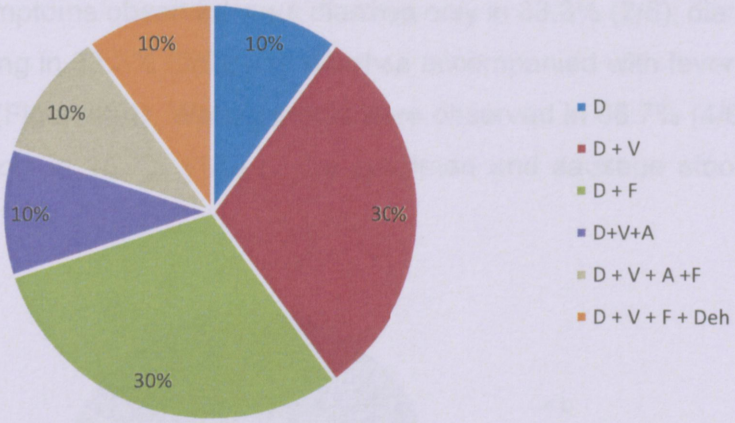


Figure 4.2: Clinical symptoms of Rotavirus infected children under the age of 5 years

Key: D = Diarrhea; V = Vomiting; F = Fever; A = Abdominal cramps; Deh = Dehydration

Single infections of Norovirus were detected in 2.5% (6/237) of the specimen. Clinical symptoms observed were diarrhea only in 66.7% (4/6); and diarrhea accompanied with abdominal cramps and fever in 16.7% (1/6) of the specimen (Figure 4.3). Watery stools were observed in 66.7% (4/6) of the specimen and sausage stools in 33.3% (2/6) of the specimen.

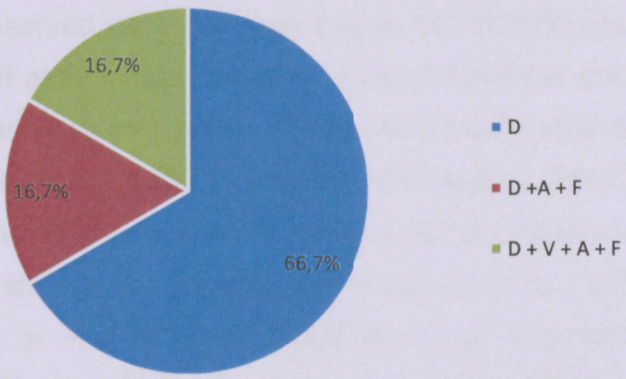


Figure 4.3: Clinical symptoms of Norovirus infected children under the age 5 years

Key: D = Diarrhea; V = Vomiting; F = Fever; A = Abdominal cramps; Deh = Dehydration

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Single infections of Adenovirus 40/41 were detected in 2.5% (6/237) of the specimen. Clinical symptoms observed were diarrhea only in 33.3% (2/6); diarrhea accompanied with vomiting in 33.3% (2/6); and diarrhea accompanied with fever 16.7% (1/6) of the specimen (Figure 4.4). Watery stools were observed in 66.7% (4/6) of the specimen, mushy stools in 16.7% (1/6) of the specimen and sausage stools in 16.6% of the specimen.

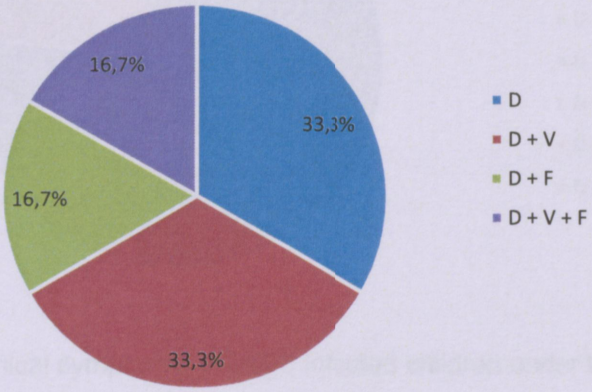


Figure 4.4: Clinical symptoms of Adenovirus 40/41 infected children under the age of 5 years
Key: D = Diarrhea; V = Vomiting; F = Fever; A = Abdominal cramps; Deh = Dehydration

Single infections of ETEC were detected in 3.0% (7/237) of the stool specimen. Clinical symptoms observed were: diarrhea only in 85.7% (6/7) and diarrhea accompanied with fever and abdominal cramps in 14.2% (1/7) of the study participants. Sausage stool specimen were observed in 57.1% (4/7); Mushy stool specimen in 28.6% (2/7); and watery stools in 14.2% (1/7) of the participants. EAEC were detected in 5.5% (13/237) of the stool specimen. Clinical symptoms observed were: diarrhea only in 53.8%; diarrhea accompanied with vomiting in 7.7% (1/13) and severe diarrhea accompanied in vomiting and dehydration was observed in 7.7% (1/7) of the participants (Figure 4.5). Mushy stools were seen in 30.8% (4/13); sausage stools in 46.2% (6/13); and watery stools in 23.1% (3/7) of the specimen. Typical EPEC were detected in 0.8% (2/237) of the stool specimen; diarrhea was observed in 100% (2/2) of the participants. Watery stools were observed in 50% (1/2) and mushy stools in 50% (1/2) of the specimen. Atypical EPEC were detected in 0.8% (2/237) of the stool specimen. Clinical symptoms observed were Diarrhea accompanied with vomiting in

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50% (1/2) and diarrhea accompanied with vomiting and abdominal cramps in 50% (1/2) of the participants. Sausage stools were observed in 50% (1/2) and watery stools in 50% (1/2) of the specimen.

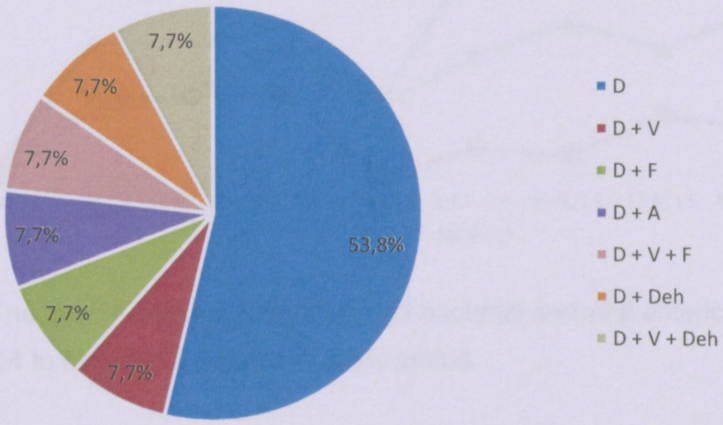


Figure 4.5: Clinical symptoms of EAEC infected children under the age of 5 years
Key: D = Diarrhea; V = Vomiting; F = Fever; A = Abdominal cramps; Deh = Dehydration

4.2.4. SEASONAL DISTRIBUTION OF ENTERIC PATHOGENS

During the study period (July 2014 to May 2015), a very faint seasonal pattern of enteric pathogens in the Vhembe District was observed. Overall diarrheal cases were reported throughout the year with increase in infections from August 2014 to October 2014, reaching a peak from December 2014 to March 2015. Bacterial infections were detected throughout the year. Enteric viruses were detected throughout the year except in November 2014. The viral infections increased from January to March 2015 reaching a peak from August to October 2014. In summary, seasonal pattern of enteric viruses was detected mostly in spring (hot and windy and dry months) and autumn (warm and dry months) while enteric bacteria was detected in spring (hot and windy months), in summer (hot and rainy months) and in autumn (warmer and dry months) (Figure 4.6).

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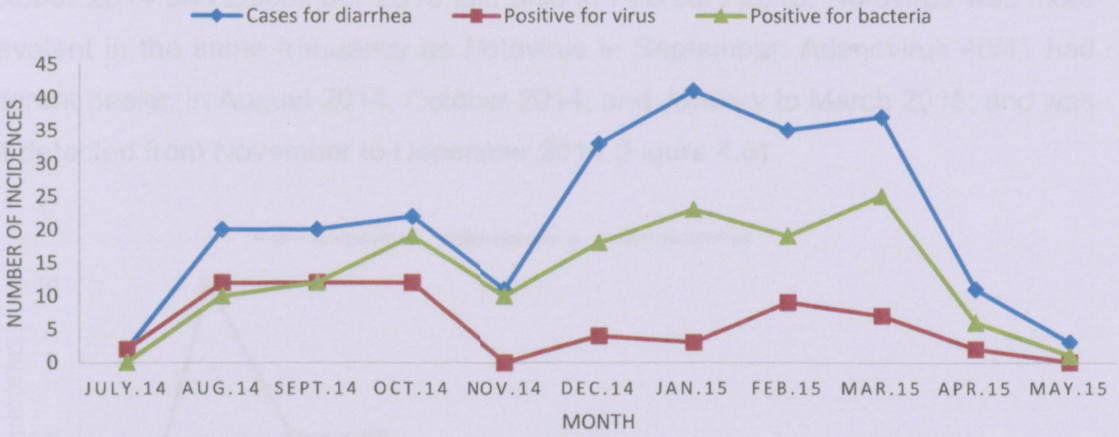


Figure 4.6: The overall seasonal distribution of bacterial and viral enteric pathogens detected from July 2014 to May 2015 during the study period

Specifically looking at enteric bacterial infections, DEC were the most frequent enteric pathogens occurring throughout the year with highest peaks reported from December 2014 to March 2015. *S. flexneri* had a slight increase from January to March 2015 of which is considered to be warm and dry months. Other enteric pathogens such as *S. typhimurium* and *Shigella* spp. did not really show any seasonal trend (Figure 4.7).

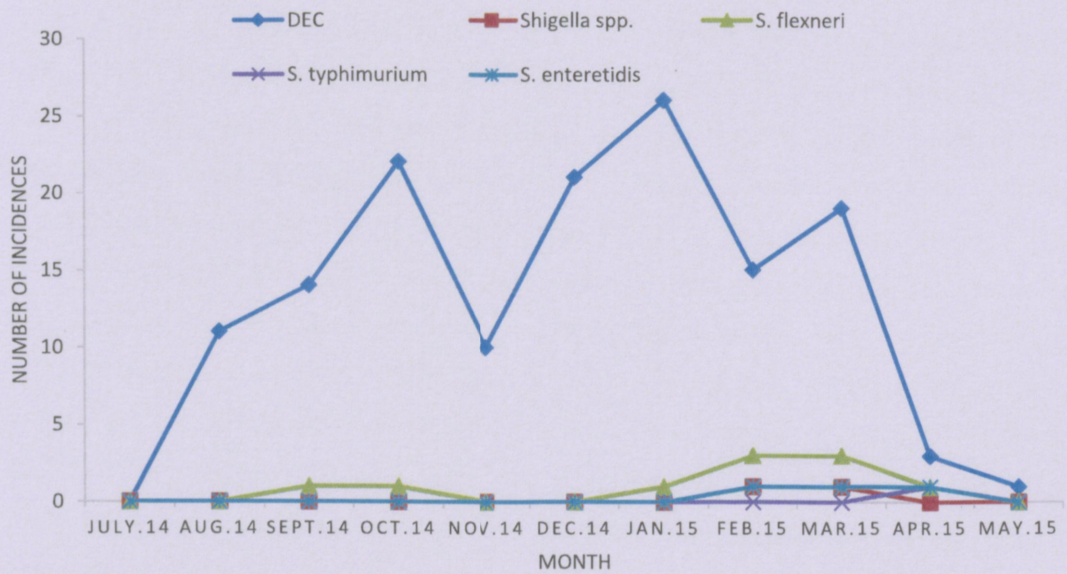


Figure 4.7: Seasonal distribution of enteric bacteria

Rotavirus infections had the highest incidences from August 2014 and infections were detected throughout until October 2014. Norovirus peaked in September 2014 to

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October 2014 and December 2015 and also in February 2015. Norovirus was more prevalent in the same frequency as Rotavirus in September. Adenovirus 40/41 had different peaks, in August 2014, October 2014, and January to March 2015; and was not detected from November to December 2014 (Figure 4.8).

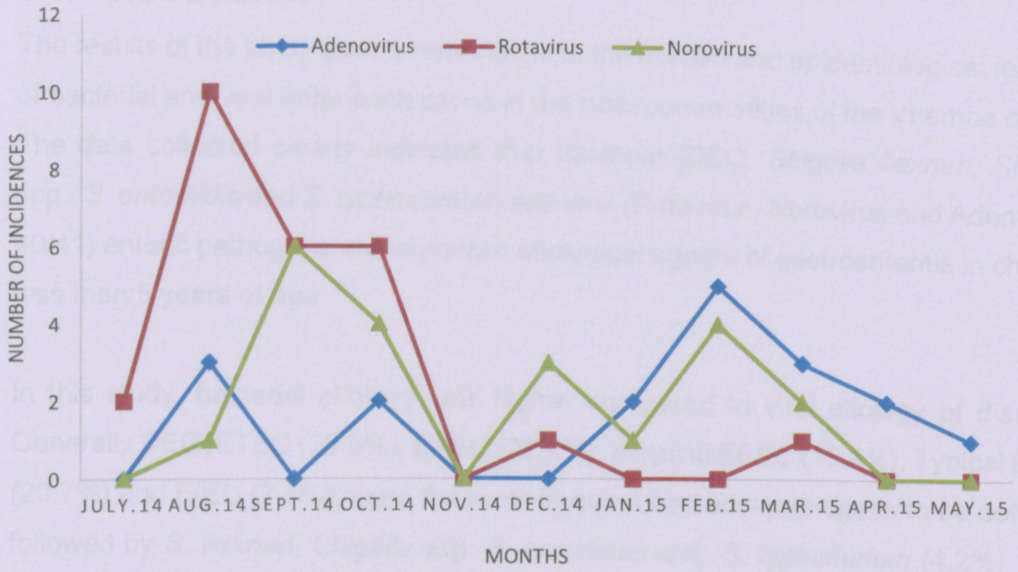


Figure 4.8: Seasonal distribution of enteric viruses

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Chapter 5

DISCUSSSION AND CONCLUSIONS

5.1. DISCUSSION

The results of the study gave a new insight in the burden and epidemiological features of bacterial and viral enteric infections in the rural communities of the Vhembe district. The data collected clearly indicated that bacterial (DEC, *Shigella flexneri, Shigella* spp., *S. enteritidis* and *S. typhimurium*) and viral (Rotavirus, Norovirus and Adenovirus 40/41) enteric pathogens are important etiological agents of gastroenteritis in children less than 5 years of age.

In this study, bacterial etiology was higher compared to viral etiology of diarrhea. Generally DEC [ETEC (30.8%), EAEC (26.2%), Atypical EPEC (15.6%), Typical EPEC (20.7%) and EIEC (7.6%)] were the most frequent bacterial pathogens to be detected followed by *S. flexneri, Shigella* spp. *S. enteritidis* and *S. typhimurium* (4.2%, 1.3%, 1.3%, 0.4%) respectively. Prevalence rates of DEC have been reported in rural settings to be 45.2% in Bangladesh (Albert et al, 1995), 12.5% in Tehran (Jafari et al, 2009) and 27.1% in Uruguay (Varela et al, 2015) affecting children less than 5 years of age. In this study, ETEC (30.8%) were the most frequent DEC strains to be detected followed by EAEC (26.2%) respectively. Varela et al (2015) in Uruguay also identified EAEC as the second leading cause of diarrhea in children less than 5 years of age. Few studies have reported on EAEC as an emerging enteric pathogen causing paediatric diarrhoea, especially persistent diarrhea in developing countries (Huang et al, 2006; Rahouma et al, 2011). EPEC has also been recognized as the most frequent DEC causing diarrheal pathogen in children less than 5 years of age (Yousseff et al, 2000; Bodhidatta et al, 2010; Nitiema et al, 2011; Varela et al, 2015). In the present study, atypical EPEC were detected in 15.6% of the isolates and typical EPEC in 20.7% of the isolates. The reasons for changes in exposure of DEC strains in children could be due to exposure to animals in the rural communities and introduction to weaning food (Varela et al, 2015). In the present study, EHEC were detected in only 0.8% of the isolates. A study by Ranjedran et al (2010) in India have reported EHEC in only 2% of young children attending primary health care facilities.

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In the present study, *S. flexneri*, *S. enteritidis*, *Shigella* spp. and *S. typhimurium* (4.2%, 1.3%, 1.3%, and 0.4%) were seen. Studies have reported diarrheal infections caused by *S. flexneri* to be higher in South Africa with 72.0% (Donald et al, 1987) and in Bangladesh with 59.1% (George et al, 2015). In this study *S. flexneri* occurred at low frequencies. Nitiema et al (2010) in rural communities of Burkina Faso also reported *S. flexneri* at low frequency of 1.9% causing diarrhea in children under the age of 5 years. Infections caused by *S. enteritidis* and *S. typhimurium* have been reported to be as a result of consumption of contaminated food eaten raw or undercooked and these infections have been found to occur sporadically (Daniels et al, 2002). In Africa, *S. enteritidis* has been reported to account for 26% diarrheal infections while *S. typhimurium* is 25% (Galanis et al, 2006). In Mozambique, *S. enteritidis* has been reported to account for 25% and 66% for *S. typhimurium* (Mandomando et al, 2009). As mentioned in the literature review sporadic infections caused by bacterial infections such as *S. flexneri*, *Shigella* spp., *S. enteritidis* and *S. typhimurium* could be due to factors such as geographic location, seasonal trend of the pathogens (Podewils et al, 2004).

Rotavirus (10.1%), Norovirus (8.4%) and Adenovirus 40/41 (7.8%) were also detected. The low detection rate of enteric viruses in this study could have been influenced by the methodology used. In this study, EIA's were used for detection of Rotavirus, Norovirus and Adenovirus 40/ 41 mainly due to costs of PCR techniques. EIA has been reported to have low sensitivity when compared to molecular analysis such as PCR techniques (Gray et al, 2007). A study by Kirby and colleagues (2010) reported on RIDASCREEN EIA used for detection of Norovirus and found the sensitivity of 63% with specificity of <98%. The sensitivity of EIA to Genogroup (G) I strains was reported to be low with high sensitivity of GII.4 strains (Kirby et al, 2010). Gray and colleagues (2007) evaluated the sensitivity of RIDASCREEN EIA test against RT-PCR, the results clearly indicated RT-PCR was more sensitive than EIA (96.37% vs 43.81%). From the above information it can clearly be seen that the incidence of enteric viruses can actually be higher than is documented in this study irrespective of the type of EIA used.

Rotavirus has been found to be a major cause of acute gastroenteritis in children less than 5 years of age worldwide (Atkinson et al, 2007; Tate et al, 2012). In this study,

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Rotavirus (10.1%) was prevalent. These findings are consistent with a number of studies conducted in other countries that reported the prevalence of Rotavirus as follows namely: 9.0% in Bangladesh (Albert et al, 1999), 18.1% in Tanzania (Moyo et al, 2007), 24.2% in South Africa (Mans et al, 2010), 18% in Thailand (Bodhidatta et al, 2010), 19% in United Kingdom (Borrows and Turner, 2014), 27.1% in Gabon (Lekana-Douki et al, 2015) and 5.1% in Uruguay (Varela et al, 2015). Since the introduction of the Rotavirus vaccine, infections have reduced by 98% in developed countries and by 65% in developing countries (Vesikari et al, 2007). In South Africa, efficacy of Rotavirus vaccine against severe diarrhea during the first year of life was found to be greater than 75% and lower efficacy of 35% during the second year of life (Cunliffe et al, 2012).

Noroviruses have been reported to be the second leading causes of non-bacterial gastroenteritis after Rotavirus (Patel et al, 2009). As reported in the literature review, Noroviruses have been found to be major causes of sporadic infections accounting to 12% in children less than 5 years of age (Patel et al, 2008). In the present study, Noroviruses were detected in 8.9% of children less than 5 years of age with diarrheal infections. The findings are consistent with other studies reported on the prevalence of Norovirus such as: 13.7% in Tanzania (Moyo et al, 2007), 14.3% in South Africa (Mans et al, 2010), 12.0% in UK (Borrows and Turner, 2014), and 3.4% in Uruguay (Varela et al, 2015).

In developed countries incidences of Adenovirus 40/41 infections are lower (1-8%) when compared to developing countries (2-31%) (Qiao et al, 1999). In this study, Adenovirus 40/41 was detected in 7.8% of diarrheal infections in children less than 5 years of age. The prevalence rate of Adenoviruses in other countries have been reported to be 15% in the USA (Van et al, 1992), 8.7% in Iran (Saderi et al, 2002), 23% in Nigeria (Aminu et al, 2007), 9.6% in South Africa (Mans et al, 2010), and 5.5% in Democratic Republic of Congo (Mayindou et al, 2015).

The prevalence of co-infections in this study were reported to be higher for bacterial – (26.2%) than viral co-infecions. ETEC + t-EPEC, EAEC + ETEC and EAEC + ETEC + t-EPEC co-infections (4.2%, 4.2%, 3.0%) were the most frequent co-infections reported. Youseff et al (2000), in Jordan also detected ETEC + EAEC combinations in

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3.7% of children aged less than 5 years. Bacterial-viral co-infections were the second most co-infections to be reported in 15.2% of the isolates. EAEC + Rotavirus, EAEC + ETEC + t-EPEC + Adenovirus 40/41 and ETEC + a-EPEC + t-EPEC + Norovirus co-infections (2.5%, 1.3%, 1.3%) were reported to be the most frequent co-infections to be detected in the study participants. Vilchez et al (2009) also detected DEC co-infections in children with diarrhea, ETEC +EAEC (2.9%), ETEC + EPEC (1.3%), ETEC + a-EPEEC (1.3%) and EPEC + ETEC + EAEC (0.8%) respectively. Virus-virus co-infections were also detected, Adenovirus 40/41 was detected in all the viral co-infections that is: Rotavirus + Norovirus and Rotavirus + Adenovirus 40/41 co-infections accounting to 0.4% of the isolates.

Among the children from the Vhembe district that were enrolled in this study, male participants were more likely to be infected with a diarrheal causing pathogen than female participants (51.1% vs 44.7%). Enteric virus infections were slightly more prevalent in males (14.8%) than in females (11.0%). Rotavirus were seen in 54.2% male participants and in 41.7% female participants, with similar trends for Norovirus and Adenovirus 40/41. The incidence could mean that male participants under the age of 5 years are more susceptible to enteric virus infections than females of the same group but this is not conclusive due to the small sample size. Similar findings were observed in Burkina Faso (Nitiema et al, 2011), Morocco (Qazoui et al, 2014), UK (Borrows and Turner, 2014) and Gabon (Lekana-Douki et al, 2015) in which the incidence of enteric viruses were reported to be higher in male than female participants under the 5 years of age. DEC pathotypes were seen in male participants than female participants, with the same trend for *S. flexneri*, *Shigella* spp. Diarrheal infections caused by *S. enteritidis* and *S. typhimurium* had only male participants. Similar studies in Thailand (Bodhidatta et al, 2010), Burkina Faso (Nitiema et al, 2011), Libya (Rahouma et al, 2011) and Uruguay (Varela et al, 2015) have also reported on male participants being more likely to be infected with enteric bacteria than female participants. The incidence of high detection rate of enteric pathogens in male participants is not clearly understood (Varela et al, 2015).

Prevalence rates of enteric pathogens have been reported in children less than 5 years of age to be 74.8% in Bangladesh (Albert et al, 1999), 66.4% in Jordan (Youssef et al, 2000), 57.9% in Burkina Faso (Nitiema et al, 2011), 60.9% in Gabon (Lekana-Douki

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et al, 2015) and 51% in Uruguay (Varela et al, 2015). In this study, enteric pathogens were also detected across all age groups. Enteric viruses, Adenovirus 40/41, Rotavirus and Norovirus diarrheal infections were more prevalent in children aged less than 2 years of age (24.1%). The findings of this study are similar to other studies in African countries on the prevalence of enteric viruses that showed higher incidences of 37% - 82.7% in children less than 2 years of age to be infected than other age groups (Moyo et al, 2007; Mans et al, 2010; Bonkoungou et al, 2013; Ren et al, 2013; Qazoui et al, 2014; Lekana-Douki et al, 2015). In this study, prevalence of DEC infections were higher in children less than 2 years of age. ETEC and EAEC were the most frequent strains in children aged 0 – 24 months. Infections caused by a-EPEC, t-EPEC, EIEC also followed the similar trend, with infections recorded mostly in children aged 0 – 24 months. In Bangladesh, infections mainly caused by ETEC, EPEC, and EAEC occurred frequently in children less than 2 years of age (Albert et al, 1995). *S. flexneri* and *S. enteritidis* infections were also reported in children aged 0 – 24 months. Studies in Burkina Faso and Libya have detected higher incidences of bacterial enteric infections in children aged less than 2 years (Rahouma et al, 2011; Nitiema et al, 2011).

A questionnaire was used to determine clinical symptoms of the study participants that were positive for an enteric pathogen. Mild to moderate symptoms were reported in 53.7% to have diarrhea only, diarrhea accompanied with fever were reported in 8.1% and diarrhea accompanied with vomiting were reported in 10.1% of the study participants. Severe diarrhea accompanied with dehydration (2.7%) and diarrhea accompanied with vomiting and dehydration (2.7%) were also observed. Study participants infected with ETEC, EAEC and typical EPEC were seen to have clinical symptoms of diarrhea only (85.7%, 53.8%, 100%). In this study, sausage stools were observed in infections caused by ETEC (57.1%), EAEC (46.1%) and atypical EPEC (50%). Vilchez et al (2009) also reported children under the age of 5 years to have symptoms of diarrhea, vomiting and fever.

In this study, Rotavirus infections were found to be diarrhea accompanied with fever (30%) and diarrhea accompanied with vomiting (30%); with only 10% reported to have severe diarrhea accompanied with vomiting fever and dehydration. Norovirus infections were found to have moderate symptoms of diarrhea only (66%). Adenovirus

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infections were detected to have diarrhea accompanied with vomiting (33.3%). Children that have been diagnosed with diarrhea accompanied with severe dehydration require oral rehydration therapy together with zinc solution and should be administered as soon as possible in order to avoid more complications that can result in death (Podewils et al, 2004). Infections caused by Adenovirus 40/41, Rotavirus and Norovirus in children results in watery diarrhea (Farthing, 2000). In this study, watery stools were observed in infections caused by Rotavirus (50%), Norovirus (66.7%) and Adenovirus 40/41 (66.7%) respectively.

Enteric pathogens especially viruses are very stable and remain in the environment over long periods of time, also prolonged shedding of viral and bacterial pathogens through an infected host plays a critical role in re-transmission of these enteric pathogens through faecal-oral transmission route (Fischer et al, 2002). In settings such as day care centres, children often play in confined areas resulting in spread of enteric pathogens by contaminated toys, fomites or through infected care takers (Widdowson et al, 2005; Biranjia-Hurdoyal and Quirin, 2012). In rural households lack of sanitary infrastructure and poor hygiene plays a critical role in transmission of diarrheal pathogens. In some rural areas pit latrines are still used. Children less than 5 years of age often defecate in open spaces outside the house as the parents are afraid that the child might fall in the pit latrine (Lindsay et al, 2013). The child's faeces are often rolled up in sand and deposited in the latrine or the faeces are placed with the compost (Lindsay et al, 2013). These faeces are often exposed to flies during defecation and this could result in flies acting as vectors of diarrheal pathogens. The flies are also attracted to raw meat and can easily contaminate foodstuffs (Lindsay et al, 2012). When children play on the floor or in the soil they can easily get exposed to faecal coliforms and other microorganisms (Badowski et al, 2011). Pickering et al (2011) found that hands are also important vectors in transfer of pathogens in Tanzanian mothers. Bacterial levels can be increased by not properly washing hands after using the latrine, cleaning up child's faeces, cleaning dishes and preparing food. Most of the households use sand instead of soap to wash their hands and the utensils (dishes), and the sand could be a source of contamination as it contains faecal coliforms and some pathogens (Pickering et al, 2011). Badowski et al (2011) found that inadequate sanitary facilities, hand and dish contact with the ground could also lead to transmission of faecal contamination (Badowski et al, 2011).

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The distribution of enteric bacteria occurred throughout the year. Bacterial infections slightly reduced during July 2014, April 2015 and Maay 2015. DEC infections were the most frequent enteric pathogens detected and occurred throughout the year with a slight decrease in November 2014. Infections caused by *S. flexneri* were the second to be detected, from the data collected infections were reported in February and March 2015 (warm and dry months), in autumn. Seasonal distribution of *S. enteritidis, Shigella* spp. and *S. typhimurium* could not be determined as very few cases were reported. Even though infections caused by *S. enteritidis* were low, all the cases were reported from February to April 2015 in warm and dry months (autumn).

The distribution of enteric viruses have been shown to occur during August to October 2014 and also in February and March 2015. Viral infections increased mainly during warm dry months (spring season) and in hot and dry months (autumn). Infections of enteric bacteria were reported throughout the year, with a slight decrease in November 2014 and April 2015. A study by Bonkoungou et al (2013) in Burkina Faso observed that viral infections occurred mostly during dry season while bacterial infections occurred during rainy season. In temperate climates Rotavirus infections have been reported to occur mainly in cool, drier months of the year. In this study, incidence of Rotavirus infections reached the highest peak in August 2014 with continued infections in September and October 2014 with few cases reported in December 2014 and March 2015. Therefore Rotavirus infections in this study were found to occur during warm dry months (late summer and autumn season). A study in South Africa (Gauteng province), observed Rotavirus infections and found that the peak incidence of Rotavirus occurred during late winter months (August and September) (Seheri et al, 2010). Another study in South Africa also observed the seasonal trend of Rotavirus infections and found that the peak incidence of Rotavirus occurred from April to August in colder dry months (Late autumn and winter) (Mans et al, 2010). In temperate climates, Norovirus causes sporadic infections that have been reported to occur all year-round (Glass et al, 2009). In the present study, Norovirus infections were also observed; the highest peak was reported in September and October 2014, with other peaks reported in December 2014 and February 2015. Lopman et al (2003) observed that Norovirus outbreaks have peaked during spring and summer resulting in hospitalization of children less than 5 years of age. In South Africa, Mans et al (2010)

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also studied the seasonal distribution of Noroviruses and found that infections caused by Norovirus occurred throughout the year with high peaks observed in November and December. Enteric Adenovirus 40/41 infections in temperate climates occur mainly throughout the year resulting in sporadic infections (Gray and Kang, 2013). In this study, incidences of Adenovirus 40/41 were reported in August 2014, October 2014, and January to March 2015. The highest peak was therefore reported in hot rainy months (summer to autumn).

5.2. CONCLUSIONS

The majority of people living in the rural communities in South Africa in Limpopo province (Vhembe district) still do not have access to improved water, sanitation and hygiene conditions. This results in children being exposed to enteric pathogens causing diarrheal outbreaks. A prospective study was conducted in order to determine the prevalence of bacterial and viral enteric pathogens in children less than 5 years of age. Children presenting with diarrhoea from rural clinics of the Vhembe district as outpatients were recruited to be part of the study.

The following observations were made with regard to the prevalence of enteric pathogens

- Rotavirus (10.1%) infections followed by Norovirus (8.7%) and Adenovirus 40/41 (7.6%) infections were detected from 237 stool specimen. Rotaviruses were not further tested to determine whether they were vaccine strains since the vaccine was introduced in SA and this will be part of another study;
- ETEC (30.8%) infections followed by EAEC (26.2%), Atypical EPEC (15.6%), Typical EPEC (15.6%), EIEC (7.6%), EHEC (0.8%), *S. flexneri* (4.2%), *Shigella* spp. (1.3%), *S. enteritidis* (1.3%) and *S. typhimurium* (0.4%) infections were detected from 237 stool specimen;
- Co-infections were seen as follows: bacteria-bacteria co-infections (26.2%); bacteria-virus co-infections (15.2%) followed by virus-virus co-infections (0.8%) respectively;
- The higher detection rate of DEC compared to other bacterial and viral enteric pathogens, suggested infections caused by DEC are major causes of diarrhea in children less than 5 years of age in the Vhembe district;

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- Most enteric viral and bacterial infections were reported mainly in children less than 2 years of age;
- ETEC, EPEC, EAEC were the most frequent DEC strains to be detected across all age groups;
- Unusual DEC strain combinations were observed which suggest possible exchange of pathogenic genes between *Enterobacteriaceae* isolates;
- In the Vhembe district, seasonal distribution of Norovirus were observed all year round; Rotaviruses were prevalent during warm and dry months and Adenovirus 40/41 were detected during cool dry months and;
- Seasonal distribution of DEC strains were observed throughout the year with a slight decrease in November; *S. flexneri* and *S. enteritidis* strains were observed in warm dry months; and *S. typhimurium* strain occurring only in April.

The objectives of the study were achieved. Prevalence of enteric viruses and bacterial pathogens was determined. DEC and Rotavirus infection were more prevalent diarrheal infections posing health risk to children less than 2 years of age in the Vhembe District, Limpopo Province.

5.3. RECOMMENDATIONS

- Enteric pathogens are primarily transmitted through faecal-oral route, therefore improving sanitation and hygiene in the households can play a major role in reducing the disease burden of these pathogens. Policy makers should therefore assist in improving water and sanitation aspects in rural communities of the Vhembe district;
- Rotavirus vaccine has already been introduced in South Africa and incidences caused by the virus has greatly decreased over the past years, this intervention results in children being exposed to other enteric pathogens such as Norovirus and Adenovirus. It is therefore recommended vaccines against these viruses be implemented to reduce disease burden;
- In this study enteric pathogens were discovered in approximately 62.4% of the diarrheal cases reported; it is therefore necessary to expand and determine other diarrheal pathogens causing diarrhoea such as parasites;
- To determine the actual disease burden of enteric viruses (Adenovirus, Norovirus and Rotavirus); sensitive molecular techniques such as RT-PCR,

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APPENDICES

APPENDIX 1: QUESTIONNAIRE USED FOR SAMPLE COLLECTION

Department of Microbiology, School of Mathematical and Natural Sciences

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RESEARCH PROJECT DATA CAPTURE FORM S:

Subject Number:.....

Consultation Details:

Date:.....Visit Number:.....Site / Clinic name:.....

Patient Information:

Name:.....Date of Birth:.....Gender:.....

Contact Details:.....Parent status:.....

Medical History:

Date of Onset:..... Rota Vaccine dose received: ☐

Source of Contamination: Community ☐ Outbreak ☐ Hospital ☐

Symptoms: Diarrhea ☐ Vomiting ☐ Fever ☐ Abdominal pains ☐

Dehydration ☐ Reported clinical immunodeficiency ☐

Sample Collection:

Date of Collection:.....

Type of Stools: Watery ☐ Sausage ☐ Mushy ☐

Treatment:

Current:.....

Previous:.....

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APPENDIX 2: BRISTOL STOOL SCALE

Bristol Stool Scale

Type 1		Seperate hard lumps, like nuts (hard to pass)
Type 2		Sausage-shaped but lumpy
Type 3		Like a sausage but with cracks on it's surface
Type 4		Like a sausage or snake, smooth and soft
Type 5		Soft blobs with clear-cut edges (passed easily)
Type 6		Fluffy pieces with ragged edges, a mushy stool
Type 7		Watery, no solid pieces. Entirely liquid

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