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Bacterial and Parasitic Agents of Infectious Diarrhoea in the Era of HIV and AIDS - The Case of a Semi Rural Community in South Africa

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1. Introduction

Infection by the human immunodeficiency virus (HIV) is a worldwide public health concern. In the Southern African region, the HIV and AIDS pandemic has grown faster than in any other parts of the world, from infection rate, in pregnant women, of 0.8% in 1990 to 30.2% in 2005 in South Africa and 29.4% in 2009 (DOH, 2000; DOH, 2006; DOH 2010). Due to its destructive effect on the immune system, HIV infection further exposes the individual to multiple opportunistic infections. From the beginning of the HIV pandemics in the 1980s, gastrointestinal diseases have been demonstrated to be a major problem in patients with HIV and AIDS, and diarrhea is reported in up to 60% of patients with AIDS in developed countries and up to 90% in developing countries (Siddiqui et al., 2007; Silva et al., 2010). Recent studies by Bradshaw *et al*., (2005) have indicated that HIV/AIDS is the leading cause of premature mortality for all provinces in South Africa and mortality due to pre-transitional causes, such as diarrhea, is more pronounced in the poorer and more rural provinces. In Limpopo Province as well as other poorer provinces in South Africa, diarrheal diseases are the first cause of mortality after HIV/AIDS (Bradshaw *et al*., 2005). However, data on specific etiologies is sparse (Obi and Bessong, 2002) and such information will be crucial in the specific management of HIV and AIDS.

Although diarrheagenic organisms have been studied in different parts of the African continent, most research activities targeted specific organisms and their role in the production of diarrhea with little consideration to the presence of other organisms, their role in the production of inflammation which might be a considerable part of the pathogenesis of the organisms (Nel et al., 2010). Elsewhere, the combination of environmental factors, new ways of living and structural changes in the genetic material of most microorganisms have led to the appearance of emerging and re-emerging diseases (Lashley, 2006). Combined to the increasing recognition of a widening array of enteric pathogens associated with illnesses of the gastrointestinal tract, these factors highlight the growing need for the understanding

of the epidemiology and transmission of the different organisms involved in infectious diarrhea in specific settings, using the more specific and sensitive molecular tools, for a better management of these diseases and the improvement of the quality of life of the concerned populations.

Gastrointestinal infections are major causes of morbidity and mortality throughout the world and particularly in developing countries where mortality rates due to infectious diarrhea could be as high as 56% (WHO, 2004). Children and young adults are the most affected, particularly in regions with limited resources and where hygienic measures are not strictly followed (Guerrant *et al*., 2005; Opintan et al., 2010). In Africa, diarrhea has been estimated to be responsible for 25 to 75% of all childhood illnesses (Kirkwood, 1991), and episodes of diarrhea lead to about 14% of outpatient visits, 16% of hospital admissions, and account for an average of 35 days of illness per year in children less than five years old (Greenwood *et al*., 1987). Causes of diarrhea in endemic areas include a wide variety of bacteria, viruses and parasites. Intestinal parasites are associated with serious clinical disease and mortality, and are known to cause malnutrition, growth, learning and physical development impairment in children. It is thus necessary to have a fairly accurate picture of the situation in order to target intervention strategies in affected areas.

It has been suggested that intestinal parasites occur at unacceptably high levels throughout South Africa. However, accurate prevalence data for the whole country are not currently available. With the exception of mapping being undertaken in KwaZulu-Natal, the medical geography of intestinal parasitic infections is stale, very fragmented and almost useless for planning, implementing and monitoring effective interventions (Fincham et al., 1997). In Cape Town, surveys at primary schools in urban and rural communities have revealed soil transmitted helminthiasis prevalence range between 7% and 83% (Kirkwood, 1991). However, there is scanty information, if any, on the prevalence of intestinal parasitic infections in the Limpopo Province.

Bacterial organisms such as *Campylobacter spp*, *Salmonella spp*, *Shigella spp* and different groups of enteropathogenic *E. coli* are well known as causes of gastrointestinal diseases all over the world. These organisms have been demonstrated in water and stools from the Vhembe district (Obi *et al*., 2004; Larsen et al., 2011). Infections by most of these organisms can be asymptomatic, or can be treated with rehydration solutions particularly in case of viruses and some bacteria. The use of antibiotics might shorten the duration of diarrhea and limit the shedding of the organisms which otherwise might continue to pollute the environment and pose further risk of infections. Antibiotics such as erythromycin and gentamicin have been proven to be effective in some communities. However, antibiotic resistance is an overgrowing problem and there is a need to monitor the susceptibility of common bacterial isolates to drugs used in the community in order to provide guidelines for the empirical treatment of bacterial infections.

Diarrhea is a common final expression of infection with a myriad of pathogens. Appropriate management requires knowledge of the setting in which the patient became ill, the underlying disease state, presence and extent of dehydration and other clinical symptoms, travel history, known outbreaks, and pathogenic mechanism (invasive or toxigenic) and the physical findings and laboratory results at the time the patient presented with the condition (de Truchis and de Truchis, 2007; Beatty, 2010). Optimal evaluation and treatment of each of these infections (as well as of cases caused by noninfectious organisms) can limit the duration of illness, the morbidity rate, the cost of work-up, and the spread of secondary infection (Goodman and Segreti, 1999). Although the differential diagnosis of infectious

diarrhea is broad, the clinical history can help guide the clinician toward the appropriate evaluation for each patient. For those patients with diarrhea of 2-3 days' duration, work-up is rarely necessary unless fever, bloody diarrhea, or severe abdominal pain is present. A detailed history of recent travel (within 6 months), recent antibiotic use (within 6-8 weeks), and contact with individuals who are ill and specific dietary ingestions during foodborne outbreaks can suggest an infectious etiology (DuPont, 1997). Infection with HIV is also a common cause of diarrhea. Therefore, medical history should include looking for risk factors for HIV and other comorbid illness that may result in immunosuppression (eg, diabetes, liver disease, organ transplantation) (Quinn *et al*., 1983).

Clinical signs of dehydration, including dry mucous membranes, low urine output, or tachycardia, suggest severe infection. Other symptoms of severe infection include fever, severe abdominal pain, distension of the abdomen, and decreased bowel sounds. Although these findings are less helpful in determining the etiology of the diarrhea, they are helpful in deciding if the patient requires any immediate treatment or hospitalization.

Most cases of acute infectious diarrhea do not need medical evaluation or intervention because they will resolve spontaneously and rapidly (Herickstad *et al*., 2002). However, if patients have any of the following clinical signs or presentations, they should undergo medical evaluation: (1) dehydration secondary to profuse watery diarrhea or inability to tolerate oral fluids; (2) fever (temperature >= 38.5 °C or 101.3 °F); (3) stools containing blood and mucus; (4) passage of 6 or more stools in a 24-hour period or duration of illness 48 hours or longer; (5) diarrhea with severe abdominal pain in patients 50 years of age or older; or (6) diarrhea in individuals 70 years of age or older or in those with known immunosuppression (eg, AIDS, transplant patients, patients who have recently received chemotherapy) (Guerrant *et al*., 2001).

The distinction between inflammatory and non-inflammatory diarrhea has long been useful in the diagnosis of diarrhea and in the creation of treatment algorithms for managing diarrhea (Guerrant *et al*., 2001; Thielman and Guerrant, 2004). The highly inflammatory diarrheas (or overt dysenteries) are caused by cultivable and potentially treatable pathogens, such as *Shigella* species, *Campylobacter jejuni, E. histolytica, C. difficile* and more recently Enteroaggregative *E. coli* and sometimes, *Salmonella* species (Huang *et al*., 2003; Jiang et al., 2010; Hou et al., 2010). The currently available tests, microscopy for fecal leukocytes and an immunoassay for fecal lactoferrin (a simpler, quicker and more sensitive marker for the presence of fecal leukocytes), provide supporting evidence of inflammatory diarrhea and may be useful when such clinical features are equivocal (Victora *et al*., 2000; Mercado et al, 2011). Recent studies have indicated that infections with *Cryptosporidium parvum* or *Giardia* species may result in mild intestinal inflammation that leads to detectable levels of fecal lactoferrin (Alcantara *et al*., 2003). In equivocal cases, the negative predictive value of fecal lactoferrin testing may help to determine the need for routine bacteriologic culture for organisms such as *Campylobacter* spp, *Salmonella* spp, and *Shigella* spp (Thielman and Guerrant, 2004).

Stool cultures are considered to be the gold standard for the diagnosis of bacterial causes of gastroenteritis. However, their clinical use is limited to organisms that are routinely cultured (Choi *et al*., 1996). The choice of the organisms to be cultured for depends on epidemiological data available for the region as well as outbreak and travel history. For example, most laboratories only attempt to culture for *Salmonella* spp, *Shigella* spp, and *Campylobacter* spp. Culture has also been used for diagnosis purposes in cases of *Entamoeba histolytica* suspicion. However this method is cumbersome and lack both sensitivity and

specificity for the detection and identification of *E. histolytica* (Abd-Alla *et al*., 1998). Considerable savings may be achieved if cultures for bacterial enteric pathogens are restricted to samples from patients hospitalized for ≤ 3 days (Valenstein *et al*., 1996). Common organisms that can cause diarrhea, such as enteroinvasive *E. coli* and enterotoxigenic *E. coli* are not routinely looked for since these organisms can only be identified by molecular methods which are mostly restricted to research laboratories or few laboratories in developed countries. Unusual organisms such as *Yersinia* species and *Vibrio* species, which may be important in certain locations, are not routinely tested for.

Microscopy is the traditional method commonly used in developing countries for the detection of ova and trophozoites of parasites and some times can be helpful in the detection of bacterial organisms such as *Campylobacter* spp. In developed countries such as the USA, stool examination for ova and parasites is generally performed particularly if a patient is potentially immunosuppressed or returning from a developing country. However, in Africa microscopy is the mostly used method for diagnosis of parasitic infections and can be used in direct stools examination, or after staining by different methods such as the simplified Ritchie technique and Ziehl Neelsen modified coloration (Kassi *et al*., 2004). The method used also depends on the suspected microorganism. In some cases such as in cryptosporidiosis where shedding of oocysts can be intermittent, up to 3 stool specimens may be needed for diagnosis (Goodgame *et al*., 1993; Chappell *et al*., 1996). By use of a modified acid-fast stain (Kinyoun), oocysts appear as red spheres of 4 - 6μm in diameter; no other organisms should be easily confused with *Cryptosporidium* species on the basis of size and appearance. Unfortunately, acid-fast staining is relatively insensitive, requiring 10,000 oocysts/g of watery stool and 500,000 oocysts/g of formed stool to make the diagnosis. Microscopy remains the best available test for acid-fast *Cyclospora cayetanensis* infections.

Traditionally, infections by *Giardia* as well as other organisms such as *E. histolytica*, *Dientamoeba fragilis*, *Balantidium coli* and other helminthes have been diagnosed by means of ova and parasite examination of fecal or small bowel specimens (including small bowel specimens obtained using the "string" test) (Stark *et al*., 2006; Kurniawanet al., 2009). The physical characteristics of the cysts or ova may prove helpful in the differentiation of the organisms involved. For example, *Giardia* cysts are ovoid or ellipsoid and measure 11─15 μm in diameter while trophozoites are approximately the same size, with 2 anteriorly placed nuclei and 8 flagella best visualized by staining with trichrome or with the iron hematoxylin method (Shetty *et al*., 1988; El-Naggar *et al*., 2006). Although microscopy might be useful in a rural setting, its use is limited by its insensitivity and lack of specificity which might lead to over diagnosis of some infections such as those of *E. histolytica* (Kebede *et al*., 2003; Nesbitt *et al*., 2004). More sensitive methods have thus been introduced which are easier and have higher sensitivities and specificities.

The development of molecular methods has tremendously improved the detection and identification of infecting agents. A variety of PCRs have been described for the detection of different bacteria such as *Shigella, Salmonella, Campylobacter* spp, diarrheagenic *E. coli, Aeromonas* spp and *Plesiomonas* spp as well as parasitic organisms such as *Cryptosporidium*, *E. histolytica*, Micrsoporidia, *Cyclospora, Isospora* and *Giardia* species (Marshall *et al*., 1999; Sturbaum *et al*., 2001, Larsen et al., 2011) . The sensitivity of detection by PCR is greater than that by microscopy, making it of great use for detection of low numbers of parasites in stool samples (Bialek et al., 2002). PCR for the detection of Cryptosporidium species, for example, has a sensitivity of 93% and a specificity of 95%, compared with 67% and 99%, respectively, for the Direct immunofluorescence assay (DFA) assay and 68% and 58%, respectively, for

Enzyme Immuno Assay (EIA) (Bushen et al., 2004; Kar et al., 2011). In addition to identifying protozoa, the use of real-time PCR–restriction fragment–length polymorphism (RFLP) analysis can detect as few as 5 *Cryptosporidium* oocysts and can differentiate between 5 genotypes and, more recently, subtypes (Limor *et al*., 2002). PCR-RFLP analysis is more sensitive, as it may detect 50–500 oocysts/mL of liquid stool or <1 pg of DNA and <10 oocysts from environmental samples (Sturbaum *et al*., 2001). Detection of diarrheagenic *E. coli* such as EAEC has required a specific test for one of the characteristic virulence traits of this group of organisms. Because an entire cassette of potential virulence traits is regulated by the transcriptional activator AggR, some have proposed that genetic probes for this trait may be the single best test for EAEC at the present time, and such genetic probes have been incorporated into a multiplex PCR test (Cerna *et al*., 2003). With the introduction of easier, more-sensitive methods that reduce labor, time, and reagent costs, the possibility of combining assays for the detection of different targets into one assay has become a possibility. A multiplex real-time PCR and an oligonucleotide microarray may be new methods for the detection of *Campylobacter* spp, *Salmonella* spp, *Shigella* spp, *E. histolytica, Giardia lamblia,* and *C. parvum,* with excellent, perhaps unprecedented, sensitivity and specificity in either fecal or water samples (Wang *et al*., 2004; Verweij *et al*., 2004). Work on these and potential new methods to detect fecal contamination in water may help to identify and ameliorate inadequate sanitation and contaminated water that perpetuates the devastating illness burdens associated with enteric infections around the world (Dillingham and Guerrant, 2004).

The causes of infectious diarrhea include a wide array of viruses, bacteria, and parasites, many of which have been recognized only in the last decade or two (Steiner *et al*., 2006). The occurrence of the different pathogens depends on region. While enterotoxigenic *Escherichia coli* and rotaviruses predominate in developing areas, Norwalk-like viruses, *Campylobacter jejuni*, and cytotoxigenic *Clostridium difficile* are seen with increasing frequency in developed areas; and *Shigella*, *Salmonella*, *Cryptosporidium* species, and *Giardia lamblia* are found throughout the world (Taylor, 1993). Bacterial gastroenteritis generally produces more severe symptoms than viral infection, including more frequent and bloody stools and severe cramping. The importance of each pathogen depends on the region. In a study in Mozambique for example, diarrheagenic *Escherichia coli* (22%) were the most frequently isolated pathogens, followed by *Ascaris lumbricoides* (9.3%). Others detected pathogens included *Salmonella* spp. and *Giardia lamblia* (2.5% each) and *Campylobacter* spp. (1.7%). *A. lumbricoides* and *Strongyloides stercolaris* (100% versus 0%; P=0.008) were most frequently isolated in children older than 12 months of age (Mandomando *et al*., 2007).

The prevalence of *Cryptosporidium* varies widely from country to country and from one region to another. In Korea, for example, Lee *et al*. (2005) reported a prevalence of 1% (among HIV patients) while in Tanzania, Houpt *et al*. (2005) described a prevalence of 17.3% amongst HIV patients. In Guinea Bissau, *Cryptosporidium parvum* had a prevalence of 7.7% and was the second most common parasite with a marked seasonal variation, with peak prevalence found consistently at the beginning of or just before the rainy seasons, May through July. In South Africa, studies by Kfir *et al*. (1995) indicated that *Giardia* cysts and *Cryptosporidium* oocysts were found in all types of water tested including surface water, sewage or treated effluents. Studies by Moodley *et al*. (1991) in Durban, South Africa showed that *Cryptosporidium* was the second most common enteric pathogen isolated from children admitted to hospital with gastroenteritis with infection rates varying between 1.2 and 20.9% according to season with the highest prevalence in the summer months, and 10%

of the children infected with *Cryptosporidium* died. However the prevalence of *Cryptosporidium* infections is not known in Limpopo Province, and particularly in the Vhembe district.

With the advent of HIV and AIDS, it has become more important to determine the distribution of parasitic infections such as *E. histolytica* and *E. dispar* amongst HIV infected individuals. In Mexico, *E. histolytica* prevalence of 25.3% in the HIV/AIDS group and 18.5% in the HIV negative group was described using PCR (Moran *et al*., 2005). Likewise in Taiwan, persons infected with HIV were at increased risk for invasive amoebiasis and exhibited a relatively high frequency of elevated antibody titers and intestinal colonization with *E. histolytica* (Hung *et al*., 2005). Previous studies in South Africa have been based in the Durban area in the eastern coast of the country where a prevalence of 10% using the PCR has been described (Zaki *et al*., 2003). However no study to our knowledge has been conducted in the Limpopo Province and particularly in the Vhembe district. The first case of human microsporidial infection was described in 1959 and as early as 2 years after the identification of HIV as the causative agent of AIDS, the microsporidial species *Enterocytozoon bieneusi* was discovered in HIV-infected patients with chronic diarrhea (Desportes *et al*., 1985). Although infections in immunocompetent patients are usually self-limiting, infections in immune compromised host can be life threatening, especially in patients with AIDS (Desportes *et al*., 1985). Studies in Cape Town have indicated prevalence up to 22% of all *Campylobacter* spp when the filter method is used for isolation (Lastovica and Roux, 2000). In Venda, the infection level by *Campylobacter spp* was found to be around the same level (20%) amongst HIV infected individuals (Obi and Bessong, 2002). However, the isolates were not ascertained by the use of molecular methods and very few studies have determined the genetic variability of *Campylobacter* spp in Africa. Enteroaggregative *Escherichia coli* (EAEC) is an emerging diarrheagenic pathogen associated with diarrheal illnesses among patients in developed and developing countries. Recent studies have implicated EAEC in persistent diarrhea in patients infected with human immunodeficiency virus (HIV) (Wanke *et al*., 1998; Nataro *et al*., 2006).

C*lostridium difficile* is a spore-forming, anaerobic Gram positive bacillus that produces exotoxins that are pathogenic to humans. Infection can lead to asymptomatic carriage or clinical disease, ranging from mild diarrhea to life threatening pseudomembranous colitis (Cleary, 1998). *Clostridium difficile* associated disease (CDAD) is an important clinical problem that is believed to occur predominantly following hospitalisation and administration of antibiotics and especially affects the elderly (Wilcox, 1996). Community-acquired disease has been reported but the incidence is felt to be low and the rate of disease resulting in hospitalization is reported as negligible. For example a Swedish study of 5 133 cases of *C. difficile d*iarrhea defined 28% as being community acquired (Karlström *et al*., 1998). Recent events in the USA, Canada and Europe have indicated the changing epidemiology of *Clostridium difficile* associated diarrhea (CDAD) with the occurrence of serious CDAD in otherwise healthy patients with minimal or no exposure to a health-care setting (Kuijper *et al*., 2006; Reichardt *et al*., 2007). However, the occurrence of *C. difficile* in developing regions such as the Vhembe district has not been reported. In the present study, molecular biology methods were used for the detection of different emerging bacterial and parasitic organisms including *Campylobacter* spp, *Arcobacter* spp, Enteroaggregative *E. coli*, *Clostridium difficile*, *Cryptosporidium* spp, *Entamoeba histolytica* and microsporidia, in relation to their pathogenicity among HIV positive and HIV negative individuals visiting different hospitals in the Vhembe district of South Africa.

2. Material and methods

2.1 Ethical Issues

Ethical approval of this research was granted by the Health, safety and Research Ethics Committee of the University of Venda. Authorization was also sought and obtained from the Department of Health and Welfare Limpopo Province, South Africa. The different hospitals and schools were then approached and the research objectives thoroughly explained to the study participants in the local language (TshiVenda) for their consent. Informed consent was obtained from all participants either directly or through their legal and competent guardians. Only consenting individuals were accepted in the study.

2.2 Study sites and sample collection

The study was conducted in the Vhembe district, of the Vhembe district, Limpopo Province, South Africa. Thohoyandou, meaning "head of the elephant" in tshiVenda, is the former capital of the independent homeland and the proud heart of the VhaVenda people. Thohoyandou is home to the University of Venda and is also the headquarters of the Vhembe district and is the tenth most populated town in the country with 584,469 people while the population of the region is approximately 1.2 million. The Vhembe district is semi urban and agriculture is the main activity practiced by the population. Main hospitals in the region include Elim, Tshilidzini, Vhufhuli (Donald Frazer) and Siloam hospitals. These hospitals deliver care directly to the population and are referral centers for smaller clinics in the region. The Vhembe district is bounded on the north by the Limpopo River, on the west by Sand River, on the south and east by the Levubu River and the remainder of the southern boundary by the farms adjoining the south of the Sinthumule location. The bulk of the people are today concentrated in locations and crown lands approximately from longitude 29*40'E-30*50'E and latitude 22*20'S-23*10'S. In normal seasons the rain starts in October/November and from that time onwards the weather becomes moist and hot, the shade temperature ranging from 80-90 degrees and north of the mountains being 110 degrees or more.

For sample collection two groups of population were considered for the study including patients attending four main public hospitals in the region namely Elim, Vhufuli, Siloam and Tshilidzini hospitals, and pupils from two public primary schools both situated in Wuwani, locality situated at about 6km from the Tshilidzini hospital. At the primary schools, the objectives of the study were explained to the parents in a meeting with the authority of the schools who then distributed the collection bottles to the pupils whose parents had agreed to the study and signed a consent form. The pupils then brought the collection bottles home and with the help of their parents collected the stool in the bottles. The samples were collected the following morning from the schools and transported without any further delay to the Laboratory of Microbiology, University of Venda. Samples that were not analysed the same day were stored at -20^{o}C. A total of 322 stool samples were collected. 255 samples were from patients attending the three public hospitals with abdominal complaints or diarrhea while 67 were from apparently healthy pupils attending two public primary schools.

2.3 Lactoferrin latex agglutination assay

Stool supernatants were tested according to the manufacturer's specifications including appropriate kit controls (LEUKO-TEST; Tech Lab, Blacksburg, VA). Stool sample dilution

was conducted as described by the manufacturer in the following way: one drop (50 µl) of stool was added to 375 µl of diluent yielding a 1:25 dilution. Using the pipette provided with the Kit, one drop of the diluted sample was mixed with one drop of sensitized latex (lactoferrin antibody-coated latex beads) or negative latex beads for 3min and the agglutination was observed for positive samples. Each test was run in parallel with a negative control as indicated by the manufacturer. Positive controls provided with the test kits were also performed. Agglutination reaction was graded with the unaided eye from 0 (no agglutination) to 4+ (large agglutination with a clear background).

2.4 Lactoferrin quantitative assay

The lactoferrin content in the lactoferrin positive stools samples was quantified using the ELISA method with the IBD scan kit from Techlab (Blacksburg, Virginia) following the instructions of the manufacturer.

2.5 Test for occult blood

The presence of occult blood in the stool samples was tested by the Hemoccult test kit (Beckman Coulter, Inc Harbor Blvd, Fullerton, CA, USA) following the instructions of the manufacturer.

2.6 Detection of pathogenic organisms

2.6.1 DNA purification

Four different methods were used and compared for the purification of total genomic DNA from stool samples. This would then allow for the detection of most parasites from the same sample and avoid conducting several DNA extractions from the same samples for the molecular detection of different pathogens. The first method involved the treatment of 200µg of stool sample by a freeze-thaw procedure using liquid nitrogen and boiling water followed by the use of the QIAamp DNA Stool Mini Kit from Qiagen (Valencia, CA, USA) according to the manufacturer's recommendations. The second method involved the use of the QIAamp DNA Stool Mini Kit, with higher temperature (95^{o}C) for the first incubation. The third method involved the used of alkaline treatment following a modified version of the method described by Haque *et al*., (1998). Briefly, fifty microliters of 1M KOH and 18 µl of 1M dithiothreitol were added to 250mg or 250 µl of stool. The samples were mixed thoroughly by stirring with a pipette tip, followed by brief shaking. After incubation at 65°C for 15 min, the samples were neutralized with 8 µl of 25% HCl and buffered with 80 µl of 2M Tris–HCl (pH 8.3) and the suspension was mixed by briefly vortexing. The genomic DNA was then purified from the suspension using the QIAamp DNA Stool Mini Kit from Qiagen (Valencia, CA, USA) following the manufacturer's instructions. The last method was the use of glass beads in order to physically dreak open the cells, cysts, oocysts and spore that could be in the stool samples. Following the bead beating the QIA amp DNA Stool Mini Kit from Qiagen for final DNA purification.

The comparison of all the pretreatment methods showed that a combination of two pretreatment methods including one which is either the bead beating or the alkaline treatment or freeze and thaw with a surplus stool portion added untreated and the whole used in the Qiagen with an increased temperature at 95oC for 15 min gave bettwe detectionof all pathogens including bacterial and parasites. The purified DNA was stored at -20oC until further used in the different PCR and Real time PCR procedures.

2.6.2 Detection and genotyping of *Cryptosporidium*

Cryptosporidium species detection and genotyping was conducted as previously described using a real time PCR for the screening and PCR –RFLP for genotyping (Samie et al., 2006a).

2.6.3 Detection and genetic characterisation of *Entamoeba histolytica*

Entamoeba histolytica was detected from the samples using the Techlab (TechLab, Inc. Blacksburg, VA, USA) *E. histolytica* II antigen detection kit . The identification of the different species of *Entamoeba* mainly *E. histolytica* and *E. dispar* was conducted as previously described (Samie et al., 2006b). Genotyping of *E. histolytica* was conducted as previously described through the polymorphism of the serine-rich *E. histolytica* protein (SREHP) followed by enzymatic digestion (Samie et al., 2008).

2.6.4 PCR amplification for the detection of microsporidia

The PCR method described by Fedorko *et al* (1995) and further developed by Samie et al., (2007) was used with minor modification as indicated followed by restriction analysis.

2.6.5 Detection of *Campylobacters*

2.6.5.1 Culture and maintenance of reference strains

Reference strains used in this study included *Campylobacter jejuni* subsp. jejuni (ATCC 33291), *Campylobacter coli* (ATCC 33559) , *Campylobacter concisus* (ATCC 33237), *Campylobacter fetus* subsp. *fetus* (ATCC 27374), *Campylobacter hyointestinalis* (ATCC 35217), *Campylobacter upsaliensis* (ATCC 43954), *Helicobacter pylori* (ATCC 43504), *Arcobacter butzleri* (ATCC 49616), *Campylobacter jejuni* (ATCC 33560), *Campylobacter jejuni* (ATCC 81116), *Campylobacter jejuni* (ATCC 11168), *Campylobacter coli* (ATCC 33559) and *Campylobacter lari* (ATCC 35221). The cultures were sub-cultured in blood agar supplemented with 10% tryptose and 0.1% yeast extract and were preserved in Bolton broth and 25% sterile glycerol. Prior to DNA isolation, 500 µl of the preserved culture was added to 10ml of brain Heart infusion or Bolton broth and incubated in a Microaerophilic environment for 24hours and inoculated to blood agar or mCCDA. The culture method using a charcoal based media (mCCDA) was used to detect *Campylobacter* spp from 37 diarrheal stool samples collected from Donald Frazer hospital as indicated in the Cape Town protocol (Lastovica and Le Roux, 2000) and suspected colonies were confirmed using a *Campylobacter* haemagglutination kit "Campy Dry Spot" (Oxoid, England) as recommended by the manufacturer.

2.6.5.2 PCR detection of Campylobacteria

The genomic DNA purified as described above was used for the detection of *Campylobacter* spp, *Arcobacter* spp and *Helicobacter* spp by the PCR- RFLP as described by Marshall et al (1999) and Samie et al., (2007a).

Restriction profiles were generated with *Dde*I, *Taq*I, or *Bsr*I (New England Biolabs, Inc., Beverly, Mass.) in a 20-µl reaction mixture including 10 µl of the PCR amplicon with 10 U of the restriction endonuclease following conditions recommended by the manufacturer. Ten microliters of each digest was analyzed electrophoretically at 5 V/cm for 2 h with a 3% agarose gel in 1× TAE buffer. The gels were stained in ethidium bromide and photographs were taken for the analysis of the profiles. **Further specific detection and confirmation of** *Campylobacter jejuni* **and** *coli* was conducted as previously described (Linton et al., 1997; Samie al., 2007a)

Campylobacter concisus was detected from the samples using the method described by conventional and real time PCR protocols based on the methods previously described by Matsheka et al (2001).
A real time PCR for the rapid detection of *Campylobacter concisus* was developed based on the method described by Matsheka *et al*. (2001) and Samie et al (2008) using the primers pcisus1 and pcisus6 and the iQTM SYBR® Green Supermix (Bio-Rad, CA),

2.6.5.3 Specific detection of *Helicobacter Pylori*

The specific detection of H. pylori was conducted as previously described (Samie et al., 2007b) using the primers consisting of two specific 16S rRNA oligonucleotides, designated HPF and HPR, which generates a 138-bp product.

2.6.5.4 Use of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*

A multiplex PCR reaction described by Houf et al (2000) and modified by Samie et al was used to identify the three main *Arcobacter* species.

2.6.6 Detection of Enteroaggregative *E. coli* from stool samples

A quantitative real time PCR using SYBR-Green -490 (Bio-Rad, CA) based on the protocol described by Samie et al., (2007c) used to confirm the presence of the *AggR* gene of EAEC in the stool samples. Standard cultures with known numbers of EAEC cells were used as reference and positive controls, while water and *E. coli* K-12 were used as negative controls in each reaction. The level of positivity of the samples was indicated by the Ct values.

2.6.6.1 Multiplex PCR detection of EAEC virulence genes from stool samples

A multiplex PCR protocol previously described was used with modifications in order to determine the presence of three EAEC genes in the stool samples (Cerna *et al*., 2003; Samie et al., 2007c). Only the presence of the correctly sized gene PCR product(s) was interpreted as a positive test.

2.6.7 Detection of *Clostridium difficile* from stool samples

A PCR protocol targeting a species-specific internal fragment of the triose phosphate isomerase (*tpi*) housekeeping gene was used as described by Lemee *et al*., (2004) for the detection of *C. difficile* in the stool samples (Samie et al., 2008c). The presence of the binary toxin was ascertained by two different reactions using two different primer pairs for the enzymatic and the binding components of the *cdt* gene using the conditions previously described by Stubbs *et al*., (2000). The negative regulator gene was detected by using two different primer pairs as previously described by Spigaglia and Mastrantonio (2002). The first primer pair (C1 and C2) detects a fragment of the *tcdC* gene while the second primer pair (Tim 1 and Struppi 2) amplifies and internal fragment of the first PCR product. The PCR products were observed in 2% agarose gel except for the products of the second PCR for the *tcd* gene that was run in 3% agarose gel. This helped to observe any size difference that could exist in the amplification products.

2.7 Statistical analysis

All data was analysed using the statistical package for social sciences (SPSS) program (Version 13.1). The potential relationship between the presence of the different pathogens

and the pathogenesis variables such as diarrhea, intestinal inflammation (through the measurement of the intestinal lactoferrin in the stool samples) and the presence of occult blood was determined by cross tabulation and the chi squarre test, risk evaluation and the Mantel-Haenszel Common Odds Ratio Estimate was used for statistical analysis. The difference was considered significant if the p value was less than 0.05. The Pathogenicity index (PI) as well as the Inflammatory index (II) were calculated. These were the ratios of the number of samples that were diarrheal (for PI) or were positive for Lactoferrin (for II) and positive for the pathogen in consideration over the number of samples that were positive for the pathogen but not positive for lactoferrin or diarrhea. This indicates the strength of the involvement of the organisms in the specific pathogenicity (diarrhea or intestinal inflammation).

3. Results

3.1 Population demographics and characteristics of stool specimens

From a total of 322 samples from 322 individuals of whom 44 were HIV positive patients while 211 were HIV negative patients and 67 were apparently healthy school children. The age of the hospital patients varied between 2 weeks and 88 years with most patients aged between 10 and 39 years old while the school children were aged between 3 and 15 years. At the hospital 148 (58%) were females while at the schools 34 (51%) were females. Diarrhea was common among hospital patients (65%) as well as intestinal inflammation indicated by elevated lactoferrin level in the stool samples (56%), and the presence of occult blood in the stools (43%). Diarrhea was common in the age groups 0 – 2 and 2 – 5 yeas old, and also in the age groups 40 – 49 and > 60. Diarrhea was more common amongst the HIV positive group compared to the HIV negative (χ^2= 12.452, p = 0.002 < 0.05). Of the 44 samples collected from HIV positive individuals, 11 (25%) were non diarrheal, 32 (72.7%) were diarrheal and 1 (2.3%) had bloody diarrhea. Of the 44 HIV positive patients 27 (61.4%) were females. HIV positive individuals were found at all age groups but the highest percentage was among those older than 20 years.

3.2 Gender distribution of diarrheagenic organisms in the study population

There was no significant difference in the distribution of the different pathogens tested in the present study according to gender, except for *Campylobacter coli* and *H. pylori* both of which were more common in males compared to females (Table 1a). *C. parvum* was more common among females while *C. hominis* was more common among males, however, the difference was not significant (Table 1b). For *E. histolytica*, 16% of the females had *E. histolytica* DNA with about 4% E. *histolytica* single infection and 13% mixed infections with *E. dispar*, while 12.2% had *E. dispar* DNA alone. Of the 109 stool samples from males, 16 (14.7%) had *E. histolytica* with 2 (1.8%) *E. histolytica* alone and 14 (12.8%) mixed infections. Seven (6.4%) had *E. dispar* DNA alone. *Campylobacter concisus* was more common among females (although the difference was not statistically significant) unlike *C. coli* that was more common in males. Similarly, *Cryptosporidium parvum* was more common among males while *C. hominis* was more common among females. *Enterocytozoon bieneusi* and *Entamoeba histolytica* were all more common in females compared to males, but with no significant difference.

	C. jejuni	*C. coli*	*C. concisus*	*H. pylori*	*A. butzleri*	*A. skirrowii*	*A cryaerophilus*
Females	19 (10.4%)	7 (3.8%)	7 (3.8%)	76 (41.8%)	10 (5.5%)	4 (2.2%)	7 (3.8%)
Males	14 (10.0%)	14 (10%)	3 (2.1%)	75 (53.6%)	10 (7.1%)	2 (1.4%)	2 (1.4%)
Sub-total	33 (10.2%)	21 (6.5%)	10 (3.1%)	151 (46.9%)	20 (6.3%)	6 (1.9%)	9 (2.8%)
χ^2,	0.017	4.915	0.763	4.434	0.369	0.256	1.702
p value	0.897	0.027	0.382	0.035	0.544	0.613	0.192

Table 1a. Distribution of diarrheagenic pathogens by gender.

	C. parvum	*C. hominis*	*E. bieneusi*	*E. histolytica*	*C. difficile*	*EAEC*
Females	6 (4.4%)	18 (13.3%)	23 (12.6%)	22 (16.3%)	27 (14.8%)	29 (15.9%)
Males	2 (1.8%)	18 (16.5%)	13 (9.3%)	16 (14.7%)	18 (12.8%)	23 (16.4%)
Sub-total	8 (3.3%)	36 (14.8%)	36 (%11.2)	38 (15.6%)	45 (14%)	52 (16.1%)
χ^2,	1.295	0.485	0.895	0.120	0.258	0014
p value	0.255	0.486	0.344	0.729	0.612	0.905

Table 1b.

3.3 Age distribution of different pathogens in Vhembe according to sample origin

In the population studied, all age groups were affected by infections. However, patients in the age group between 3 and 5 years were the most infected particularly with organisms like *C. jejuni, H. pylori, A. butzleri* and Enteroaggregative *E. coli* for the bacterial organisms as well as *C. hominis* and *E. histolytica* among the parasites (Table 2a and 2b). Other species of *Arcobacter* did not occur among patients less than 5 years of age. *Cryptosporidium parvum* did not occur among patients aged less than 10 years (Table 2b). For *Cryptosporidium*, the age group the most affected were 2–5 years old (28.6%) 30–39 years old (23.5%), and 40–49, and 4 (27.7%). None of the samples from individuals aged >60 was positive for *Cryptosporidium*. For *E. histolytica*, the age groups most infected were 0 – 2 (33%) followed by the age group 20 – 29 (27%). *E. bieneusi* was also common among the patients aged between 3 and 5 years old. The prevalence of *A. butzleri* was lower in the older population compared to the younger populations.

3.4 Diarrhea related pathogens in the studied population

Of all the samples analyzed, 31% of diarrheal samples did not have any pathogen while 66% of the non diarrheal samples had no pathogens detected. *Helicobacter pylori* was the most commonly detected organisms using polymerase chain reaction from both diarrheal and non- diarrheal samples, However, the difference was not significant. Of the 10 bacterial organisms tested, *C. jejuni, toxigenic C. difficile*, Enteroaggregative *E. coli* and *C. coli* were the most commonly detected and associated with diarrhea among the patients in the total population. These organisms also had the highest pathogenic indexes indicating their potential involvement in diarrheal cases. Of the 4 parasitic organisms tested, *E. histolytica* and *Cryptosporidium hominis* were more common and statistically associated with diarrhea with pathogenic indexes of 8 for *E. histolytica* and 2.1 for *C. hominis*. The prevalence of the different organisms in both diarrheal and non diarrheal samples is shown in Table 3 below as well as the pathogenic indexes of the organisms. Briefly, *C. jejuni* was the most pathogenic bacterial organisms (in relation to diarrhea) while *E. histolytica* was the most diarrheagenic parasitic organism in this population.

Origin	Age group	Total	*C. jejuni*	*C. coli*	*C. concisus*	*H. pylori*	*A butzleri*	*A. skirrowii*	*A cryaerophilus*
Hospitals	0 – 2	18	2 (11.1%)	0	1 (5.5%)	9 (50%)	2(11.1%)	0	0
	3 – 5	16	3 (18.7%)	1 (6.2%)	0	9 (56.2%)	1 (6.2%)	0	0
	6 – 9	16	2 (12.5%)	0	0	8 (50%)	1 (6.2%)	0	0
	10 – 19	65	4 (6.2%)	3 (4.6%)	4 (6.1%)	29 (44.6%)	6 (9.2%)	1 (1.5%)	1 (1.5%)
	20 – 29	62	9 (14.5%)	7 (11.3%)	1 (1.6%)	26 (41.9%)	4 (6.5%)	2 (3.2%)	2 (3.2%)
	30 – 39	42	6 (14.3%)	1 (2.3%)	0	29 (69%)	4 (9.5%)	2 (4.8%)	4 (9.5%)
	40 – 49	18	4 (22.2%)	2 (11.1%)	1 (5.5%)	8 (44.4)	0	0	1 (5.5%)
	50 – 59	10	1 (10%)	2 (20%)	0	4 (40%)	0	0	0
	≥ 60	8	1 (12.5%)	2 (25%)	0	4 (50%)	1(12.5%)	0	1 (12.5%)
	Subtotal	255	32 (12.5%)	18 (7%)	7 (2.7%)	126(49.4%)	19(7.5%)	5 (2%)	9 (3.5%)
Schools	3 – 5	5	0	0	1 (20%)	4 (80%)	0	0	0
	6 – 9	4	0	0	0	1 (25%)	0	0	0
	10 – 15	58	1 (1.7%)	3 (5.2%)	2 (3.4%)	32 (55.2%)	1 (1.7%)	1 (1.7%)	0
	Subtotal	67	1 (1.5%)	3 (4.4%)	3 (4.5%)	37 (55.2%)	1 (1.5%)	1 (1.5%)	0
Total		322	33 (10.2%)	21 (6.5%)	10 (3.1%)	163 (50.6%)	20 (6.2%)	6 (1.9%)	9 (2.8%)
χ^2			7.052	0.580	0.871	0.717	4.853	0.064	2.433
P value			0.008	0.446	0.351	0.397	0.028	0.801	0.119

Table 2a

Origin	Age group	Total	*C. parvum*	*C. hominis*	*E. bieneusi*	*E. histolytica*	*C. diff*	*EAEC*
Hospitals	0 – 2	18		1 (9.1%)		3 (27.3%)	3 (16.7%)	1 (5.6%)
	3 – 5	16		4 (28.6%)	4 (25.0%)	4 (28.6%)	2 (12.5%)	5 (31.3%)
	6 – 9	16		2 (15.4%)		2 (15.4%)	1 (6.3%)	2 (12.5%)
	10 – 19	65	2 (4.3%)	5 (10.6%)	6 (9.2%)	6 (12.8%)	10 (15.4%)	11 (16.9%)
	20 – 29	62	1 (2.1%)	7 (14.6%)	11 (17.7%)	13 (27.1%)	11 (17.7%)	12 (19.4%)
	30 – 39	42	5 (14.7%)	3 (8.8%)	9 (21.4%)	5 (14.7%)	12 (28.6%)	11 (26.2%)
	40 – 49	18		2 (13.3%)	2 (11.1%)	3 (20.0%)	1 (5.6%)	5 (27.8%)
	50 – 59	10		4 (50.0%)			2 (20.0%)	3 (30.0%)
	≥ 60	8			1 (12.5%)	1 (14.3%)	1 (12.5%)	
	Subtotal	255	8 (4.1%)	28 (14.2%)	33 (12.9%)	37 (18.8%)	43 (17%)	50 (19.6%)
Schools	3 – 5	5		2 (50.0%)				2 (40.0%)
	6 – 9	4						
	10 – 15	58		6 (14.6%)	3 (5.2%)	1 (2.4%)	2 (3.4%)	
	Subtotal	67		8 (17.0%)	3 (4.5%)	1 (2.1%)	2 (3.0%)	2 (3.0%)
Total		322			36 (11.2%)	38 (15.6%)	45 (13.9%)	52 (16.1%)
χ^2								
P value								

Table 2b.

Table 2 Distribution of bacterial and parasitic agents of diarrhea in the study population according to age group.

3.5 Diarrheagenic organisms and intestinal inflammation

Intestinal inflammation was measured by the amount of lactoferrin produced in the stool samples. Previous studies have correlated the occurrence of lactoferrin in the stool samples

with leukocytes which is a marker of intestinal inflammation and even better because fecal leukocytes are generally difficult to count since they die faster once out of the body. Therefore, fecal lactoferrin is the best marker of intestinal inflammation. The inflammatory index was calculated in the same manner as the pathogenic index by dividing the prevalence of the organisms in lactoferrin positive samples by that of the organisms in lactoferrin negative samples. Of all the bacterial organisms tested *Campylobacter jejuni* was the most significantly associated with intestinal inflammation. Enteroaggregative *E. coli* was the next most inflammatory bacterial organism followed by *C. coli* and *C. concisus* (Table 4). Of all the parasitic organisms tested in the present study, *E. histolytica* was significantly associated with intestinal inflammation.

Characteristics	**Diarrheal stools**	**Non-diarrheal stools**	**Total**	**χ^2, p value**	**PI**
No Infection	53 (31.2%)	101 (66.4%)	154 (47.8%)		
All infections					
C. jejuni	29 (17.1%)	4 (2.6%)	33 (10.2%)	**18.159 (0.000)**	**6.6**
C. coli	16 (9.4%)	5 (3.3%)	21 (6.5%)	**4.934 (0.026)**	**2.8**
C. concisus	8 (4.5%)	2 (1.4%)	10 (3.1%)	1.226(0.268)	3.2
A. butzleri	14 (8.2%)	6 (3.9%)	20 (6.2%)	2.533 (0.112)	2.1
A. skirrowii	3 (1.8%)	3 (2.0%)	6 (1.9%)	0.019 (0.890)	0.9
A. cryaerophilus	4 (2.4%)	5 (3.3%)	9 (2.8%)	0.259 (0.611)	0.7
H. pylori	91 (51.7%)	60 (41.1%)	151 (46.9%)	2.652(0.103)	1.2
C. diff	34 (19.3%)	11 (7.5%)	45 (13.9%)	**9.21 (0.002)**	2.6
Toxigenic *C. diff*	20 (11.4%)	3 (2.1%)	23 (7.1%)	**10.48 (0.001)**	5.4
Non Toxigenic C. diff	14 (8%)	8 (5.5%)	22 (6.8%)	0.768 (0.381)	1.4
EAEC	36 (21.2%)	16 (10.5%)	52 (16.1%)	**6.722 (0.010)**	2.01
Parasitic organisms					
E. histolytica	34 (27%)	4 (3.4%)	38 (15.6%)	**25.544 (0.000)**	**8**
C. parvum	4 (3.2%)	4 (3.4%)	8 (3.3%)	0.009 (0.925)	1
C. hominis	25 (19.8%)	11 (9.3%)	36 (14.8%)	**5.361 (0.021)**	**2.1**
E. bieneusi	23 (13.1%)	13 (8.9%)	36 (11.2%)	1.393 (0.238)	1.5

Table 3. Prevalence of different diarrheagenic pathogens in diarrheal and non diarrheal stool samples in the general population in the Vhembe district of South Africa as detected by different Polymerase Chain Reaction methods. The pathogenic indexes show the potential association of the organisms with diarrhea.

3.6 Occurrence of organisms and occult blood in the stool samples

Occult blood was tested in the samples and correlated with the presence of the different organisms. Of all the organisms tested, 4 bacterial species were significantly associated with occult blood and these included in order of statistical importance Enteroaggregative *E. coli*, *Campylobacter jejuni*, *C. difficile* and *Campylobacter coli*. Of all the parasitic organisms tested, only *Entamoeba histolytica* showed a statistically significant correlation with occult blood. The pathogenicity index in terms of occult blood occurrence in the stool samples in association

Characteristics	Lactoferrin positive stools	Lactoferrin negative stools	Total	χ^2, p value	OR (95%CI)	II
All infections						
C. jejuni	26 (17.4%)	7 (4.0%)	33 (10.2%)	**16.586 (0.000)**	5.231 (2.2 – 12.4)	4.4
C. coli	14 (9.6%)	7 (4.0%)	21 (6.5%)	**4.122 (0.042)**	2.561 (1 – 6.5)	2.4
C. concisus	8 (5.5%)	2 (1.1%)	10 (3.1%)	**5.002 (0.025)**	5.043 (1 – 24.1)	5
A. butzleri	11 (7.5%)	9 (5.1%)	20 (6.2%)	0.803 (0.370)	1.5 (0.6 – 3.7)	1.5
A. skirrowii	2 (1.4%)	4 (2.3%)	6 (1.9%)	0.356 (0.551)	0.597 (0.1 – 3.3)	0.6
A. cryaerophilus	3 (2.1%)	6 (3.4%)	9 (2.8%)	0.539 (0.463)	0.594 (0.1 – 2.4)	0.6
H. pylori	69 (47.3%)	82 (46.6%)	151 (46.9%)	0.014 (0.905)	1.027 (0.6 - 15)	1.01
C. diff	25 (17.1%)	23 (13.1%)	48 (14.9%)			
EAEC	32 (21.9%)	20 (11.4%)	52 (16.1%)	**6.565 (0.010)**	2.189 (1.2 – 4)	1.9
E. histolytica	27 (26%)	11 (7.9%)	38 (15.6%)	**14.875 (0.000)**	4.112 (1.9 – 8.7)	3.3
C. parvum	3 (2.9%)	5 (3.6%)	8 (3.3%)	0.089 (0.766)	0.802 (0.2 – 3.4)	0.8
C. hominis	18 (17.3%)	18 (12.9%)	36 (14.8%)	0.940 (0.332)	1.419 (0.6 – 2.8)	1.3
E. bieneusi	16 (11%)	20 (11.4%)	36 (11.2%)	0.013 (0.909)	0.960 (0.4 – 1.9)	1

Table 4. Diarrheagenic organisms and intestinal inflammation as indicated by the detection of lactoferrin in the stool samples.

with the organisms was calculated using the same formula described above for lactoferrin and diarrhea. EAEC had the highest index followed by *Campylobacter jejuni*, *Campylobacter coli* and *Clostridium difficile* for the bacteria and *E. histolytica* among the parasites. The summary of these results is shown in table 4. EAEC infections were significantly associated with intestinal inflammation (χ^2=6.565, P=0.010) and 61.5% of stools that were positive for EAEC genes had elevated lactoferrin compared to 42.2% for samples negative for EAEC genes. Stool samples positive for EAEC genes were more likely to have occult blood (Odd ratio=5.069; 95%CI: 2.665 – 9.644) even when the number of cells carrying the *AggR* gene was lower in the stool. Of the samples positive for at least one EAEC gene, 69.2% had occult blood compared to only 30.7% for samples negative for EAEC genes (χ^2=27.725, P<0.00001). The occult blood pathogenicity index was higher for samples containing *AggR* compared to the other two genes. In general, most bacterial and parasitic organisms tested were more common in samples with occult blood. However, the difference was not significant (P>0.05) (Table 5).

3.7 Occurrence of infections in HIV positive and HIV negative patients with or without diarrhea

In the present study, the presence of bacterial and parasitic organisms was determined according to HIV status of the patients. In order to have a better indication on how important could a pathogen be to the HIV positive group, we calculated the HIV relatedness index (HI) by dividing the prevalence of these infections in HIV positive by the prevalence of the same organism among HIV negative patients. A higher HI indicates that the organism was more common among HIV positive patients. Generally a HI higher than 2 was a good

Characteristics	Occult blood positive stools (n=119)	Occult blood negative stools (n=203)	Total	χ^2, p value	OR (95%CI)	PI
All infections						
C. jejuni	21 (17.6%)	12 (5.9%)	33 (10.2%)	**11.233 (0.001)**	3.411 (1.6 – 7.2)	2.9
C. coli	2 (10.1%)	9 (4.4%)	21 (6.5%)	**3.929 (0.047)**	2.417 (0.9 – 5.9)	2.3
C. concisus	6 (5.0%)	4 (2.0%)	10 (3.1%)	2.35 (0.125)	2.642 (0.7 – 9.5)	2.5
A. butzleri	10 (8.4%)	10 (4.9%)	20 (6.2%)	1.557 (0.212)	1.771 (0.7 – 4.3)	1.7
A. skirrowii	2 (1.7%)	4 (2.0%)	6 (1.9%)	0.034 (0.853)	0.850 (0.1 – 4.7)	0.85
A. cryaerophilus	5 (4.2%)	4 (2.0%)	9 (2.8%)	1.275 (0.241)	2.182 (0.5 – 8.2)	2.1
H. pylori	58 (48.7%)	93 (45.8%)	151 46.9(%)	0.258 (0.611)	1.125 (0.7 – 1.7)	1.06
C. diff	26 (21.8%)	22 (10.8%)	48 (14.9%)	**7.171 (0.007)**	2.300 (1.2 – 4.2)	2.01
EAEC	36 (30.3%)	16 (7.9%)	52 (16.1%)	**27.725 (0.000)**	5.069 (2.7 – 9.6)	3.8
E. histolytica	21 (23.3%)	17 (11%)	38 (15.6%)	**6.530 (0.011)**	2.453 (1.2 – 4.9)	2.1
C. parvum	4 (4.4%)	4 (2.6%)	8 (3.3%)	0.611 (0.434)	1.7 (0.7 – 4.1)	1.7
C. hominis	17 (18.9%)	19 (12.3%)	36 (14.8%)	1.938 (0.164)	1.655 (0.8 – 3.4)	1.5
E. bieneusi	18 (15.1%)	18 (8.9%)	36 (11.2%)	2.960 (0.085)	1.832 (0.9 – 3.6)	1.7

Table 5. Diarrheagenic organisms and occult blood in the stool samples.

indication that the specific pathogen was correlated with HIV infections. Of all the organisms tested in the present study, EAEC, *C. jejuni* and *C. coli* appeared to be important bacterial pathogens in HIV positive patients while *E. bieneusi* was the most common parasitic organism among HIV positive patients.

The prevalence of EAEC infection among HIV positive individuals was significantly higher (χ^2=5.360, P=0.021) with 13 (29.5%) infections than the rest of the study population with 39 (14%) infections. Of the HIV positive patients tested, 8 were positive for *E. histolytica*. Of these individuals 5 were females and three were males. Among the HIV negative individuals, 29 (13.8%) males and 28 (13.3%) females were infected (χ^2=0.754, P= 0.385). Five samples from HIV positive patients were genotyped for *E. histolytica*. Of these, 3 (60%) belonged to the same profile mostly (3 out of 4 [75%]) found in HIV positive patients with diarrhea (2 out of 3) or without diarrhea (1 out of 3). One other profile was found mostly (7 out of 8) in HIV negative patients while one other profile was unique to a HIV positive individual. In the present study, we found a higher *Campylobacter* infection rate: 18.2% and 11.4% among HIV positive patients compared to 11.4% and 6.2% in HIV negative individuals for *C. jejuni* and *C. coli* respectively. The prevalence of these *Campylobacter's* infection among HIV positive individuals was significantly higher (χ^2=5.360, P=0.021) with 13 (29.5%) infections than the rest of the study population with 39 (14%) infections. When compared to HIV negative individuals, HIV positive individuals were more likely to have microsporidiosis (χ^2=4.414, p=0.036). In the HIV negative population, males were more infected than females. However, in the HIV positive population, females were significantly more infected than males (p<0.001). In the HIV negative subgroup, *E. bieneusi* was more

common in individuals without diarrhea (15.9%) than individuals with diarrhea (9.0%), but this was not statistically significant. In the HIV positive group, *E. bieneusi* was found only in diarrheal samples indicating the possible involvement of these organisms in the production of diarrhea in immunocompromised hosts. The prevalence of infection by *C. difficile* was generally higher in HIV negative individuals (14.4%) than HIV positive individuals (11.4%), but the difference was not significant (χ^2=0.289, p=0.591). However, all the toxigenic *C. difficile* in HIV positive patients were found in diarrheal samples, with elevated lactoferrin and occult blood while the non-toxigenic strains were found in stool samples negative for the lactoferrin test and for occult blood indicating that even though *C. difficile* infections are not more prevalent among HIV positive patients, they might be more susceptible to these infections.

		C. jejuni	*C. coli*	*C. concisus*	*H. pylori*	*A butzleri*	*A. skirrowii*	*A cryaerophilus*
HIV positive	Diarrheal	8 (21.1%)	5 (13.2%)	2 (5.3%)	19 (50%)	1 (2.6%)	1 (2.6%)	2 (5.3%)
	Non diarrheal	0	0	0	3 (50%)	0	0	0
	Sub-total	8 (18.2%)	5 (11.4%)	2 (4.5%)	22 (50%)	1 (2.3%)	1 (2.3%)	2 (4.5%)
HIV negative	Diarrheal	21 (15.9%)	12 (9.1%)	6 (4.5%)	68 (51.5%)	13 (9.8%)	2 (1.5%)	3 (2.3%)
	Non diarrheal	4 (2.7%)	4 (2.7%)	2 (1.4%)	61 (41.8%)	6 (4.1%)	3 (2.1%)	4 (2.7%)
	Sub-total	25 (9%)	16 (5.8%)	8 (2.9%)	129 (46.4%)	19 (6.8%)	5 (1.8%)	7 (2.5%)
	χ^2, p value	3.487 (0.062)	1.960 (0.162)	0.351 (0.553)	0.197 (0.657)	1.357 (0.244)	0.047 (0.829)	0.575 (0.448)
	PI	2	2	1.5	1.1	0.3	1.3	1.8

Table 6a

		C. parvum	*C. hominis*	*E. bieneusi*	*E. histolytica*	*C. difficile*	*EAEC*
HIV positive	Diarrheal	1 (3.8%)	3 (11.5%)	9 (23.7%)	4 (15.4%)	5 (13.2%)	12 (31.6%)
	Non diarrheal	0	0	0	1 (16.7%)	0	1 (16.7%)
	Sub-total	1 (3.1%)	3 (9.4%)	9 (20.5%)	5 (15.6%)	5 (11.4%)	13 (29.5%)
HIV negative	Diarrheal	2 (2.1%)	19 (20.2%)	13 (9.8%)	30 (31.9%)	28 (20.3%)	25 (18.9%)
	Non diarrheal	5 (4.2%)	14 (11.9%)	14 (9.6%)	3 (2.5%)	12 (8.6%)	14 (9.6%)
	Sub-total	7 (3.3%)	33 (15.6%)	27 (9.7%)	33 (15.6%)	40 (14.4%)	39 (14.0%)
	χ^2, p value	0.003 (0.958)	0.847 (0.357)	4.414 (0.036)	0.000 (0.993)	0.504 (0.478)	6.754 (0.009)
	PI	1	0.6	2.1	1	0.8	2.1

Table 6b.

Table 6. Distribution of bacterial and parasitic organisms among HIV positive and HIV negative patients. The statistics compare the values for the HIV positive and the HIV negative patients. The HIV relatedness index (HI) was the ratio of the occurrence of infection among HIV positive patients to the prevalence of that same infection among HIV negative patients.

4. Discussion

Intestinal bacterial and parasitic infections are common in developing countries and responsible for most acute and chronic diarrhea cases amongst HIV/AIDS patients (Silva et al., 2010). The objective of this study was to determine the prevalence and genotype distribution of bacterial and parasitic organisms in the general population including school children and among HIV positive and HIV negative individuals in the Vhembe district of South Africa; a semi urban area situated in Limpopo Province in the northern part of the country. The organisms detected include *Cryptosporidium species, Entamoeba histolytica,* Microsporidia, *Campylobacter* spp, *Arcobacter* spp, *Helicobacter pylori*, Emteroaggregative *E. coli* and *Clostridium difficile*.

According to the South African Department of Health, the HIV prevalence in the general population was 10.8% for all South Africans over the age of 2 years in 2005 (DOH, 2010). Among those between 15 and 49 years old, the estimated HIV prevalence was 16.2% in 2005. Females were more affected (13.3%) than males (8.2%). In the Limpopo Province, the prevalence in the whole population was 8%. In our study, 15.7% of the patients visiting the hospitals were positive for HIV. This is closer to the national prevalence for individuals between 15 and 49 years of age. These rates are still high compared to countries from other parts of the African continent such as Mali (1.9%), but is comparable with the rates in other countries in the Southern African sub-region such as Malawi (14.2%) and Zambia (16.5%) (Banerjee *et al*., 2004). It is well known that chronic diarrhea is one of the major AIDS-defining illnesses in WHO Classification and occurs in 60-90% of HIV infected patients in Africa and in a Swiss Cohort Study, diarrhea was found to be an independent predictor of poor survival amongst HIV and AIDS patients (Tadesse and Kassu, 2005; Humphreys et al., 2010). In our study, diarrhea was very common and was present in 74.2% of fecal specimens submitted from cases in the HIV population and is thus in agreement with data from previous studies.

Studies in other parts of the world have indicated that *Cryptosporium* spp represented by *C. Parvum* are the most common diarrheagenic parasitic organisms, however, few studies have compared rates among HIV negative and HIV positive patients. The prevalence and species distribution of *Cryptosporidium spp* vary greatly with the regions or country studied and even within specific groups of the population. This creates a complex picture of the epidemiology of infection by these organisms whose understanding will be helpful in shaping the appropriate measures for their control. In Limpopo Province, the HIV prevalence is 16.2% as determined by the report of the Department of Health and Welfare of South Africa (DOH, 2003). Previous studies in Limpopo Province have targeted different bacterial infections in HIV/AIDS patients; however no attempt has been made to isolate parasites (Obi and Bessong, 2002). This study is thus the first to use a molecular approach for the detection, genetic diversity and pathogenicity of the bacterial and parasitic infections in the region.

The real time quantitative PCR (qPCR) is a very sensitive, specific and easy to use method for the identification and quantification of organisms from a variety of sources. The qPCR used in the present study for the detection of *Cryptosporidium* has been tested for specificity and sensitivity using stools spiked with different numbers of oocysts and proved to be highly effective (Houpt *et al*., 2005; Taniuchi et al 2011). Studies in various tropical countries have demonstrated highest prevalence of cryptosporidiosis in children younger than 2 years. In rural areas children of between 2 – 5 years old are more exposed to infections since

this is the period when they begin to be active on their own. In Zimbabwe, Simango and Mutikani (2004) demonstrated that *Cryptosporidium* was common amongst children aged less than 5 years old with infection rate of 11.2%. In India, studies conducted in twin cities of Hyderabad and Secunderabad indicated that children in the age group of six months to one year were the most vulnerable with 14.3% infections compared to 8.2% among children less than five years of age while in Malaysia the prevalence was 7.5% and 33.3% in Egypt (Nagamani et al., 2007; Al-Mekhlafi et al., 2011).

It has been demonstrated in some countries such as Mexico (Javier-Enriquez *et al*., 1997), Brazil (Newman *et al.,* 1999; de Oliveira-Silva et al 2007), and Indonesia (Katsumata *et al*., 2000; Moyo et al., 2011) that *Cryptosporidium* transmission in children is usually associated with the rainy season, and waterborne transmission is considered a major route in the epidemiology of cryptosporidiosis in these areas. Although water contamination with *Cryptosporidium* has been demonstrated in other parts of South Africa, such research needs to be completed in the Limpopo Province in order to confirm the source of transmission in the region. Considering the presence of *Cryptosporidium* in the hospitals as well as in the schools, it can be hypothesized that water is a widespread transmission vector in the region. A study in Peruvian children has demonstrated that cryptosporidiosis was more frequent in children from houses without a latrine or toilet (Bern *et al*., 2002). Previous studies in Venda have also indicated poor level of hygiene in Venda (Potgieter *et al.,* 2005). However, more detailed studies need to be conducted in order to clarify the role of hygienic habits in the transmission of *Cryptosporidium* as well as other parasitic organisms in the Vhembe district.

Cryptosporidium parasitizes the small intestinal epithelium. Infection results in accelerated loss of villous enterocytes, leading to severe villous atrophy and a malabsorptive and secretory diarrhea. The most common symptom of cryptosporidiosis is watery diarrhea. Other symptoms include: dehydration, weight loss, stomach cramps or pain, fever, nausea, and vomiting. Abdel-Messih *et al*. (2005) in Egypt demonstrated that clinical findings associated with *Cryptosporidium* diarrhea included vomiting, persistent diarrhea and the need for hospitalization. Studies by Alcantara *et al*. (2003) indicated that *Cryptosporidium* was associated with inflammation as indicated by the lactoferrin test and the presence of IL8 and TNF-α. In this study, *Cryptosporidium* was also associated with inflammation and more than 59.1% of *Cryptosporidium* infections might lead to inflammation. However more detailed study is required to clarify the real impact of *Cryptosporidium* infections as well as other protozoan parasitic infections in the production of intestinal inflammation in Venda. Another study in Haiti by Kirkpatrick *et al*. (2002) indicated that malnourished children with acute cryptosporidiosis mount inflammatory (with high lactoferrin content), Th-2, and counter regulatory intestinal immune responses. Studies of Peruvian as well as Brazilian children have demonstrated malnutrition, particularly stunting with lack of growth catch-up after even asymptomatic *C. parvum* infection (Checkley *et al*., 1998; Antonios et al., 2010).

The existence of two *Entamoeba* species morphologically identical but genetically different was suggested as early as 1925 by Brumpt. However, it was not until 1993 that enough biochemical, immunological and genetic data were gathered to re-classify *E. histolytica* into 2 separate species: *E. histolytica* which can invade the gut mucosa, causes diarrhea and extra-intestinal disease, and *E. dispar*, which causes only asymptomatic colonization (Diamond and Clark, 1993). Following the reclassification of *Entamoeba histolytica*, the epidemiology of amoebiasis needed to be redefined by the use of methods that are able to differentiate between *E. histolytica* and *E. dispar.* Thus different PCR methods have been developed with

variable efficiencies. A nested PCR previously described has been successfully used to differentiate between *E. histolytic*a and *E. dispar* (Haque *et al*, 1998; Ali *et al*., 2003). Using the same method; we were able to differentiate between *E. histolytica* from *E. dispar* in samples collected from patients visiting public hospitals with gastrointestinal complaints or diarrhea; and pupils attending public primary schools in the Vhembe district. *E. histolytica* was found both in the hospital and in the Schools. However, *E. histolytica* was less common amongst primary School children aged between five and fifteen. These findings underscore the potential role of *E. histolytica* in morbidity in the study area since the association between *E. histolytica* infections and diarrhea was statistically significant ($P < 0.05$). Similar results have been found in other countries around the world such as Thailand (Haghighi *et al*., 2003).

Infection rates as well as species diversity (ratio between the occurrence of *E. histolytica* and *E. dispar*) varied tremendously from one region to the other. In Italy, more patients were found to be infected with *E. dispar* (8.3%) than *E. histolytica* (5.6%) using PCR assays (Calderaro *et al*., 2005). In Sweden, amoebiasis is a notifiable disease and 400–500 cases are reported annually to the Swedish Institute for Infectious Disease Control (SMI). The PCR analysis showed that 165 (79.7%) patients were positive for *E. dispar*, whereas only 10 (4.8%) patients were positive for *E. histolytica* (Lebbad and Svard, 2005). In contrast, higher rates of *E. histolytica* infections was found in Mexico as compared to *E. dispar* infections (13.8% versus 9.6%), using PCR (Ramos *et al*., 2005). Similarly in the Philippines, 74 cases (65.48%) were positive for *E. histolytica* and 6 cases (5.30%) positive for *E. dispar* from a mental institution (Rivera *et al*., 2006). In the Gaza strip, Palestine, *E. histolytica* was identified by PCR in 64 (69.6%) of the samples and that of *E. dispar* in 21 (22.8%) (Al-Hindi *et al*., 2005).

In the present study, we found a rate of 15.5% for *E. histolytica* which is higher than the rate found in Durban by Gathiram and Jackson (1985). This can be explained by the fact that our population was potentially ill and thus had a higher risk of been infected which was not the case in the group without diarrhea in whom there were no mixed infections and only one asymptomatic case of *E. histolytica* was found. The antigen detection test from Techlab (Blacksburg, Virginia, USA) has previously been shown to be suitable for the diagnosis of amoebiasis in endemic areas (Abd-Alla and Ravdin, 2002). In the present study, ELISA had a high specificity. It should be noted that samples positive for PCR and negative with the ELISA test were generally mixed infections with *E. histolytica* and *E. dispar*. This might have a hindering effect on the ability of the ELISA test to detect these samples and might also be related to the pathogenicity or virulence of the strains involved. It has been indicated elsewhere that when both organisms are present in an individual, *E. dispar* generally outgrows *E. histolytica*. However, since *E. dispar* is non pathogenic, the result of the infection will probably be asymptomatic. Mixed infections have also been described in Mexico where 13% of individuals were found harboring *E. histolytica* and *E. dispar* at the same time, particularly amongst HIV positive individuals (Moran *et al*., 2005).

The mechanisms of disease production following an infection by *E. histolytica* are not fully understood. Most *E. histolytica* infections remain asymptomatic. However, other studies have suggested that amebic colitis may be encountered during colonoscopic examination even in subjects who are asymptomatic (Okamoto *et al*., 2005). *E. histolytica* has also been associated with traveler's diarrhea. In a study in Sweden, when the patients were divided into immigrants and travelers, the percentages with *E. histolytica* were 3.8% and 9.5%, respectively (Barwick *et al*., 2002). In invasive amoebiasis, white blood cells can be present in the stool, and in severe cases pus can be visible, but faecal leukocyte numbers are generally

not as high as in shigellosis (Speelman *et al.,* 1984). Indeed, virulent *E. histolytica* can destroy neutrophils upon contact; hence may induce inflammation but show only pyknotic leukocytes in the stools (Guerrant *et al.,* 1981; Callendar, 1933). Such a process would be expected to cause evidence of inflammation (i.e. lactoferrin) even without morphologically clear PMNs in the stool. Inflammation occurs most often and previous studies have demonstrated that fecal lactoferrin was the best way to indicate the presence of PMN in stool samples. In our study, 85.7% of samples with *E. histolytica* DNA were positive for lactoferrin with 43% of cases presenting with high level of lactoferrin while *E. dispar* positive samples had only 1 (4.3%) case with a high lactoferrin level. This further confirms the pathogenic differences between the two species. When we excluded other detected organisms, the association of *E. histolytica* with diarrhea and with lactoferrin was even stronger. Other studies had indicated low levels of lactoferrin with *E. histolytica* and *S. hematobium* infections compared to shigellosis and other UTI infections (Aly *et al*., 2005). However, *E. histolytica* infections had not been ascertained by specific test such as PCR.

Whether risk of invasive amebiasis due to *E. histolytica* is higher among human immunodeficiency virus (HIV)-infected persons than uninfected persons remains unclear, although intestinal colonization by *E. histolytica/dispar* has been reported to be higher among HIV positive individuals (Moran *et al*., 2005). While studies in Thailand have indicated that *E. histolytica* was more common among HIV positive patients (P<0.001), studies in Mexico were not conclusive on this issue (Hung *et al*., 2005). We had recently described a much higher seroprevalence of *Entamoeba histolytica* among HIV and AIDS patients compared to HIV negative patients (Samie et al., 2010). In a study on the genetic diversity of *E. histolytica*, we found that one profile was more common among HIV positive individuals indicating that the increased susceptibility of HIV positive individual to *E. histolytica* might depend on the genetic profile of the infecting *E. histolytica* strain. In a recent study in Uzbekistan, HIV-infected patients were found to have virtually all parasites, such as *Giardia lamblia*, *Cryptosporidium parvum*, *Chilomastix mesnili, Entamoeba coli, Iodamoeba butschlii, Entamoeba histolytica/dispar, Endolimax nana, Blastocystis hominis, Enlerobius vermicularis, Ascaris lumbricoides, Hymenolepis nana*, detectable in the population of Tashkent (Nurtaev *et al*., 2006). Of special interest was the fact that in all the forms (stages) of HIV infection, the infestation with *E. histolytical/dispar* was 10 times greater than that in non HIV infected individuals.

Since their successful isolation from stools in the 1970s *Campylobacter spp* have risen from obscurity to notoriety as important food borne agents of gastroenteritis with present isolation rates superceding those of other enteric pathogens such as *Salmonella* spp. and *Shigella* spp. in most developed countries and higher prevalence among children in the developing world (Crushell *et al*., 2004; Fernández-Cruz et al., 2010). Although their implication in human infections has been described worldwide, their epidemiology varies in different regions of the world and the knowledge of their prevalence using molecular methods is essential for the designing of efficient control measures adapted to each area. Acute self-limited gastrointestinal illness, characterized by diarrhea, fever and abdominal cramps, is the most common presentation of *C. jejuni/C. coli* infection (Butzler, 2004). In this study we found a significant association of *C. jejuni* and *C. coli* infections with diarrhea and inflammation. *Campylobacter spp* other than *C jejuni/coli* have also been implicated in human and animal diseases (Lastovica and Skirrow, 2000; Moran, 2010). In this study, we detected *C. concisus* in 10 (3.1%) samples with 6 (60%) cases present in diarrheal stools indicating the

possibility of the involvement of this *Campylobacter species* in disease production in the Vhembe district. In Cape Town, studies by Lastovica and LeRoux indicated that C*. concisus* was the second most isolated *Campylobacter* after *C. jejuni* and constituted 23.55% of all *Campylobacter* isolates (Lastovica and Le Roux, 2000).

Unlike its close phenotypically related neighbour *Campylobacter*, *Arcobacter* is not currently a major public health concern, but is considered as an emerging human pathogen, and is of significance in animal health (Snelling *et al*., 2006; Kalischuk and Buret, 2010). In the present study 70% of *A. butzleri* containing samples was diarrheal and 55% with elevated level of lactoferrin indicating possible involvement in inflammatory processes. However more research needs to be conducted in order to confirm its involvement in human disease. *H. pylori* was found in 163 (50.6%) of all the samples among which 55.9% of *H. pylori* positive samples were diarrheal and that *Helicobacter pylori* was common among school children and hospital patients. These results are similar to previous studies that have indicated that *H. pylori* is *a* common human pathogen estimated to colonize 50% of the world's population (Van Der Hulst *et al*., 1996). Epidemiological evidence has suggested that *H. pylori* is spread by fecal-oral and oral-oral routes. Although there are no known environmental reservoirs for *H.* pylori, *H. pylori* has been cultured from the feces (Thomas *et al*., 1992) of infected individuals and has been detected by polymerase chain reaction (PCR) in dental plaque (Nguyen *et al*., 1993). The prevalence found in the present study was lower compared to other recent studies in Pretoria, South Africa, where *H. pylori* was found in 84% of stomach biopsies from Healthy individuals but not in dental samples (Olivier *et al*., 2006). It has been estimated that the relationship between chronic diarrhea, retarded growth, iron-deficient anaemia, and *H. pylori* infection in children especially from developing countries remains controversial (Raymond *et al*., 2005). However, more research is needed in order to determine their involvement in gastric ulcers as well as any other pathogenic features in the Vhembe district.

Over the past few years, enteroaggregative *E. coli* have been increasingly characterized in developing countries and recent data have suggested that EAEC are emerging as diarrheal agents in developed nations as well (Nataro *et al*., 2006; Opintan et al., 2010). However; the true distribution of these organisms as well as their pathogenicity is not well studied in South Africa particularly in the Vhembe district. In the present study, we detected the presence of three EAEC pathogenic genes employing a recently developed multiplex PCR. We evaluated these genes in relation to HIV status, diarrheal symptoms, intestinal inflammation, determined by elevated lactoferrin, and occult blood in a sample population composed of hospital patients with known HIV status and school children in the Vhembe district of South Africa. Different methods have been described for the detection of EAEC and have suggested the existence of two different categories of EAEC including Typical and Atypical EAEC (Jenkins *et al*., 2006). Typical EAEC carry the pAA plasmid originally detected by the AA probe. Enteroaggregative *E. coli* have also been associated with weakened immune system such as in patients with HIV and AIDS. EAEC have been described as the most common pathogen among HIV positive patients in many countries even though the rates of infection vary from country to country. In this study we found a higher rate of EAEC infection among HIV positive patients (29.5%) compared to Senegal (West Africa) where EAEC was found in 19.6 % of HIV patients and was the most common pathogen amongst these individuals (Gassama *et al*., 2001). In Switzerland, EAEC genes were detected in 22% of HIV positive patients with diarrhea while in Zambia, EAEC was

found in both HIV patients and control even though cytotoxic phenotypes were only isolated from the AIDS patients with no evidence of seasonality in the frequency of isolation, and no evidence of long-term carriage (Kelly *et al*., 2003; Crump et al., 2011).

Different markers of pathogenesis have been described in EAEC infections including fecal cytokines such as IL-8 and IL-1R, lactoferrin, and occult blood (Steiner *et al*., 1998, Greenberg *et al*., 2002). Volunteer challenge studies have demonstrated heterogeneity in the ability of EAEC isolates to cause disease and several studies have been unable to make clear associations with EAEC and diarrhea. In this study, more EAEC positive samples had elevated lactoferrin and diarrhea, and the presence of EAEC in the stools was significantly associated with occult blood ($P<0.001$). Although EAEC have been associated with bloody stool samples the relationship with occult blood has not been clearly described (Durrer *et al*., 2000). A study in Central African Republic indicated that EAEC were the most frequently identified agents in HIV positive patients with persistent diarrhea and 42.8% of the patients with EAEC as sole pathogens had bloody diarrhea (Germani *et al*., 1998). The presence of occult blood in the stools of individuals infected with EAEC was tested in a previous study that did not find a significant association between EAEC infection and the presence of occult blood in the stools since only 4 (31.1%) of EAEC positive stool samples had occult blood, while 27 (60.0%) of EAEC positive stool samples had lactoferrin (Bouckenooghe *et al.,* 2000). Our study is thus the first that found significant association between EAEC infections and occult blood in the stool and might indicate a different pathogenic manifestation of these organisms in this part of the world.

Studies elsewhere have indicated that the best characterized *E. coli* pathotypes require multiple genes to be fully/highly virulent. For example enterotoxigenic *E. coli* (ETEC) with heat-labile toxin (LT), heat-stable toxin (ST) and colonization factor antigens (CFAs) are the most virulent; Enteropathogenic *E. coli* (EPEC) with Bundle Forming Pilus (BFP) and the *eae* gene encoding the adhesin intimin, responsible for the intimate attachment of the bacteria to the epithelial cell are most virulent; Shiga-toxin-producing *E. coli* (STEC) with Shiga-like toxin (Stx) and eaeA, encoding intimin involved in attachment of bacteria to enterocytes and plasmid are most virulent (Qadri *et al*., 2000 ; Rappelli *et al*., 2001 ; Scaletsky *et al.,* 2002 ; Karch *et al*., 2006; Turner *et al.,* 2006; Medina et al., 2010). However, the presence of multiple genes has not been associated with pathogenesis in EAEC. This study has shown that strains with all the three genes were more pathogenic in terms of diarrhea production, intestinal inflammation indicated by the lactoferrin level in the stools and occult blood.

Two species of microsporidia, *Enterocytozoon bieneusi* and *Encephalitozoon* (*Septata*) *instestinalis*, are known to cause intestinal microsporidiosis. Even though *E. bieneusi* is responsible for about 90% of reported infections (Orenstein, 1994), other microsporidial species such as the Vittaforma-like species were recently described in stool samples from both HIV positive and HIV negative individuals in Portugal (Sulaiman *et al*., 2003). In our study, only *E. bieneusi* was detected in stool samples, even though the PCR method used could detect all of the *Encephalitozoon spp.* in addition to *E. bieneusi*. Other studies have also indicated that *E. bieneusi* was the most common microsporidia infecting HIV negative as well as HIV positive individuals (Sarfati *et al*., 2006) and that PCR based assays can be used successfully for microsporidian species differentiation from stool specimens, thus obviating the need for invasive biopsy procedures (Liguory *et al.,* 1997).

To date, the pathogenicity of Microsporidia is not clearly defined and the mechanisms by which Microsporidia induce diarrhea in HIV patients have not been determined. A wide

range of pathology has been associated with Microsporidia; these include inflammation and cell death, and symptoms such as shortness of breath, sinusitis, and diarrhea with wasting (Orenstein, 2003; Stark et al., 2009). In our study we found that even though HIV positive patients infected by *E. bieneusi* had more diarrhea than those non-infected, they actually had less inflammation as compared to the non-infected HIV positive individuals as demonstrated by the lactoferrin test. This could be explained by the occurrence of multiple infections in these individuals. The high level of lactoferrin could thus be due to infections by other organisms such as *Cryptosporidium* spp, *Entamoeba histolytica*, Enteroaggregative *E. coli*, *Clostridium difficile* and *Campylobacter jejuni / coli* also found in these stool samples. Compared to previous studies we have conducted in the same region, *E. bieneusi* was more common than *Cryptosporidium spp* among HIV patients (Samie *et al*., 2006a). However, HIV positive patients infected with *Cryptosporidium* had more diarrhea and more lactoferrin than those who were not infected, indicating that the expected outcome would be worse with *Cryptosporidium* than with *E. bieneusi* in this population. This observation is similar to those described by Bern *et al.* (2005) in Peru, where microsporidiosis did not appear to have a major impact on survival among AIDS patients compared to cryptosporidiosis, even though some genotypes of *E. bieneusi* caused chronic diarrhea in these patients.

E. bieneusi was not associated with intestinal inflammation in our study, as demonstrated by the lactoferrin test in HIV negative and HIV positive individuals even though most HIV positive individuals without microsporidia had elevated lactoferrin, indicating high level of intestinal inflammation. This could be due to the effect of HIV itself as previously demonstrated (Kotler *et al*., 1993; Maingat et al., 2011). This is in line with some studies where multiple small intestinal biopsies showed atrophy with acute and chronic inflammation in HIV seropositive individuals even without apparent pathogens (Orenstein *et al*., 1992; Snijders *et al*., 1995; Idris et al 2010). It thus suggests that microsporidia might be cause of secretory diarrhea in HIV patients while most HIV negative individuals remain asymptomatic in the Vhembe district.

This study also determined the prevalence of community acquired *C. difficile* toxigenic characteristics among hospital outpatients and school children and evaluated the association between different pathologic features and the presence and toxigenic profiles of the isolates. *C. difficile* was less frequent among apparently healthy school children. The two positive samples obtained from the schools were non toxigenic as opposed to the toxigenic strains obtained from the hospital outpatients. We also identified the existence of a mutation on the *tcdC* gene associated with increased virulence of associated *C. difficile* infections and this is in harmony with a previous repport (Cloud *et al*., 2007). The prevalence of *C. difficile* associated diarrhea among HIV patients have been demonstrated to vary according to different studies (Cappell 1993; Lu 1994). In a study of *C difficile* associated diarrhea among HIV positive patients in Illinois, USA, CDAD was observed in 32% of all study patients with diarrhea especially those with advanced HIV disease (Pulvirenti *et al*., 2002). Other reports have suggested that clinical manifestations and response to therapy in HIV infected patients with *C. difficile* associated diarrhea (CDAD) were similar to that of patients without HIV (DeLalla 1992; Hutin 1993; Cozart 1993) while others have noted a more severe, refractory presentation in HIV infected patients (Colarian 1988; Beaugerie 1994). In our study *C. difficile* did not appear to be associated with HIV. However like other studies, our HIV population was very little (44 patients) and was not clearly characterized in terms of CD4+ counts or HIV disease state. Thus more studies are needed to confirm the role of *C. difficile* as diarrheal agent among HIV positive patients in the Vhembe district and in South Africa in general.

Toxin-A-negative, toxin-B-positive (A- B+) *Clostridium difficile* isolates were identified in several studies (Wultanska *et al*., 2005). We found that only 3 (6.7%) of all *C. difficile* positive samples were A-B+ variants which is lower compared to those found in horses by Arroyo *et al*., (2007) and around the same level as those described by Pituch *et al*, (2003) in Poland were about 7% of the strains isolated from CDAD patients had the variant A-B+ isolates. Recent studies have also reported on the existence of cluster of A- B+ *C. difficile*, universally resistant to the fluoroquinolones tested including ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin and gatifloxacin, with MICs > 32 mg/mL, associated with a novel transversion mutation in gyrB (Drudy *et al*., 2006). The high prevalence of A-B+ *C. difficile* strains might have a negative impact on the detection of toxigenic *C. difficile* in stool samples when the ELISA test is used. This further underscores the importance of the implementation of molecular methods in the detection and characterization of *C. difficile* in specific settings.

5. Conclusions

The quantitative real time PCR using SYBR green is a simple and fast method for the detection of different infectious organisms including bacteria and parasites. Different pre-treatment methods can be used to improve DNA purification for the detection of bacterial and parasitic organisms in stool samples for molecular epidemiological studies. These include the alkaline treatment of the stool sample, the use of the freeze and thaw and the use of glass beads prior to DNA purification by the traditional phenol chloroform method or the use of different kits such as the Qiagen. This study has demonstrated a high prevalence of microsporidia, *Cryptosporidium* infections in the Vhembe district and its implications in the production of diarrhea and inflammation. *C. hominis* was more common and related to pathogenesis than *C. parvum*. HIV positive patients did not appear to be more likely to be infected by *Cryptosporidium*. However, more studies are needed using larger number of HIV positive samples. The study of antigenic profiles of these organisms will provide insight for the development of effective vaccines.

E histolytica appears to be common in the Vhembe district of South Africa. Mixed infections were especially frequent as opposed to other areas in the world such as Japan (Ali *et al*., 2003). *E. dispar* was less associated with diarrhea or fecal lactoferrin and occurred more often than *E. histolytica* in the general population. Fecal lactoferrin may provide a useful indicator of acute invasive *E. histolytica* infections and could be used as screening test for inflammatory diarrhea including *E. histolytica* in the Vhembe district considering its simplicity. This study also shows the susceptibility of females infected with HIV to *E. histolytica,* which is also commonly seen in males with or without HIV. The study of genetic and antigenic profiles will shed more light on the pathogenicity of this important protozoal infection and provide insight into improved control measures such as improved water and sanitation, vaccine and drug development.

We successfully used different PCR methods for the detection and identification of Enteroaggregative E. Coli, *Campylobacter*, *Helicobacter* and *Arcobacter* spp from stool samples. Of interest was the development of a fast and efficient real time PCR using SYBR GREEN for the detection of *C. concisus*. EAEC was an important etiological agent of diarrhea in the Vhembe district, South Africa as indicated by its high prevalence among hospital patients and particularly among HIV positive patients. Furthermore, EAEC may be a treatable cause of diarrhea in patients with AIDS (Wanke *et al*., 1998b). Toxigenic *C. difficile* was associated with pathologic conditions among the patients. Typical preventive measures against

infections by these organisms include careful personal hygiene, especially promotion of hand washing through health education programs. Major therapeutic intervention for all individuals with diarrhea consists of fluid and electrolyte therapy. However, when antimicrobial therapy is appropriate, selection of a specific agent should be made based upon susceptibility patterns of the pathogen or information on local susceptibility patterns.

Quantitative real time PCR showed that a certain threshold, related to the number of cells, was needed for the EAEC to cause pathologic symptoms such as diarrhea and inflammation. HIV positive individuals are at a higher risk of infection by EAEC and had higher level of lactoferrin when compared to HIV negative individuals. This is the first study to significantly associate EAEC with the presence of occult blood in the stools which might be due to pathogenic factors such as the plasmid encoded toxin (Pet) which is highly homologous to the EspP protease of EHEC and to EspC of EPEC as well as the protein involved in colonization (Pic) .

The current study has demonstrated that *E. bieneusi* is the most common microsporidian species occurring in the Vhembe district particularly among HIV positive patients and *E. bieneusi* is a cause of secretory diarrhea among HIV positive individuals as opposed to inflammatory diarrhea. This study has demonstrated that the pathogenicity of Enteroaggregative *E. coli* could be directly related to the genetic profile of the infecting strains. This is important in the understanding of the pathogenicity of these organisms with possible effect on the development of control methods including diagnostics, drug target molecules (genes) and vaccination procedures. This study also associated EAEC infections with occult blood which might indicate a possible relation/link between the pathogenicity of this organism and that of Enterohemorrhagic *E. coli* (EHEC) often involved in hemolytic uremic syndrome (HUS) and bloody diarrhea.

The pathogenicity index determines the importance of the infecting agent as a pathogen in a specific community. The pathogenicity index indicated that *E. histolytica*, *Cryptosporidium hominis*, *C. jejuni/coli, C. concisus*, *Clostridium difficile* and Enteroaggregative *E. coli* were the most diarrheagenic organisms in the Vhembe population while *E. histolytica*, *C. jejuni/coli, C. concisus*, *Clostridium difficile* were the most inflammatory. Enteroaggregative *E. coli* was the most associated with occult blood followed by *E. histolytica*, *C. jejuni/coli*, and *Clostridium difficile*. This further indicates the importance of the lactoferrin and occult tests as screening methods for diarrheal organisms in hospitals and will probably reduce the cost of infectious diarrheal diagnosis and improve the quality of service. HIV positive patients are more susceptible to infections, therefore, the implementation of molecular methodologies is recommended for an improved diagnosis of gastrointestinal infections among these patients and the quality of their lives. Diarrheal diseases can be prevented through access to clean, safe drinking water and through proper sanitation measures, including hand washing and safe disposal of human waste. Thus increased health education in schools as well as in the communities is highly recommended and could help prevent the transmission of diarrheal diseases in the population. Proper management and treatment of waste and waste water is recommended through increased investments in water and sanitation systems at least in fast growing areas. Such strategies could alleviate a great deal of unnecessary suffering and loss of productivity; reduce the number of lives lost to these diseases, and result in significant savings in health care costs.

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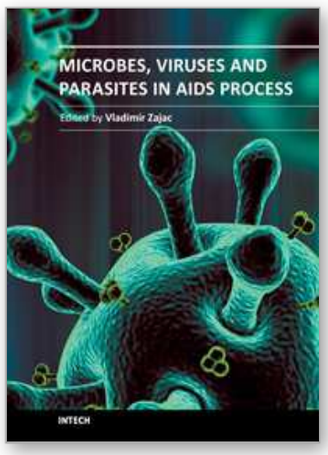

Microbes, Viruses and Parasites in AIDS Process

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The main goal in compiling this book was to highlight the situation in Africa in terms of AIDS and opportunistic diseases.Â Several chapters reveal great poverty, an apocalyptic situation in many parts of Africa.Â Global migration of people resulted in their exposure to pathogens from all over the world. This fact has to be acknowledged and accepted as African reality. New, unconventional hypotheses, not determined by established dogmas, have been incorporated into the book, although they have not yet been sufficiently validated experimentally.Â It still applies that any dogma in any area of science, and medicine in particular, has and always will hinder progress. According to some biologists, in the future, AIDS is very likely to occur in a number of variations, as a direct result of the ongoing processes in the global human society. Thus, we urgently need a comprehensive solution for AIDS, in order to be ready to fight other, much more dangerous intruders.

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