University of the Witwatersrand

Department of Epidemiology and Biostatistics

Aetiology and pathogen-specific risk factors for diarrhoea among children under the age of 5 years

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DECLARATION

I, Simbulele Onesimo Mdleleni declare that this Research Report is my own, unaided work. It is being submitted for the Degree of MSc Epidemiology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

somdleleni

(Signature of candidate)

__1st_day of__October__2020___in_Johannesburg_

DEDICATION

This research is dedicated to my parents, Lindiswa Nonkwelo and Bayete Mdleleni. Thank you for all the support and love throughout the years. I love you.

Sbwl uThixo to bless and keep y'all ♥

PRESENTATIONS ARISING FROM RESEARCH REPORT

Poster presentation at the 12th African Rotavirus Symposium 2019: Aetiology and pathogen-specific risk factors for diarrhoea among children under the age of 5 years.
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ABSTRACT

Background

Children under five years of age are most affected by diarrhoea, and co-infections may lead to more severe disease. Rotavirus vaccine introduction has altered the aetiology of diarrhoea in some high-income countries but limited data are available for low-middle income countries. The aim of this study was to investigate the aetiology of acute diarrhoea and possible risk factors associated with rotavirus diarrhoea post-rotavirus vaccine introduction in hospitalised children.

Methods

A secondary analysis was conducted, using data from a diarrhoeal sentinel surveillance program, which enrolled children hospitalised for acute diarrhoea at nine hospitals in South Africa (SA). A TaqMan array card was used to detect multiple enteric pathogens from stools collected during 2015-2016.

The prevalence of enteropathogens was described using a polymerase chain reaction cycle threshold (Ct) of ≤35 and specific Ct cut-offs for selected pathogens. Logistic regression was performed to investigate risk factors associated with rotavirus detection in the stool of children who received at least one dose of rotavirus vaccine.

Results

The analysis consisted of 793 children <5 years. Using Ct cut-off of ≤35, prominent diarrhoeal pathogens were: Enteroaggregative *Escherichia coli* (EAEC, 33,2%), adenovirus (28,6%), *Shigella*/Enteroinvasive *E. Coli* (EIEC, 24,1%), *Cryptosporidium* (23,6%), and rotavirus (22,1%). Using specific Ct cut-offs, rotavirus (20,7%), *Shigella*/EIEC (15,4%), and *Cryptosporidium* (13,0%) had the highest detection frequencies.

The highest detection of rotavirus was among children 6-11 months old (35,3%), while *Shigella*/EIEC was highest amongst older children (24-59 months old) (50,5%) (Ct ≤35). The most prominent co-detections with rotavirus identified were Helicobacter *pylori* (4,9%) and *Shigella*/EIEC (3,7%). Children 6-11 months old (OR=4.27; 95%CI:2.022 – 9.020), admitted in winter (OR=29.57; 95%CI:13.212 – 66.164) or spring, (OR=7.05; CI:3.214 – 15.483), and hospitalised at the Red Cross Children's Hospital (OR=2.92; 95%CI:1.139 – 7.511) had higher odds of rotavirus detection in stool despite being vaccinated against rotavirus. Participants from Klerksdorp Hospital had lower odds of rotavirus hospitalisation (OR=0.15; 95%CI:0.400 – 0.611).

Conclusion

Rotavirus was still a prominent cause of diarrhoea in hospitalised children <5 years old in 2015 and 2016. The prevalence of *Shigella*/EIEC was higher in children 24-59 months old while rotavirus was most frequently detected in younger children (6–11 months old). Development of the new rotavirus vaccines and vaccination schedules and the development of vaccines against bacteria such as *Shigella* would help decrease the prevalence of diarrhoeal disease in children <5 years of age.

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All the glory be to my Lord and Saviour and Best Friend – Jesus Christ.

DEFINITION OF TERMS

AGE	Acute Gastroenteritis
AF	Attributable Fraction
AMR	Antimicrobial Resistance
CDAD	*Clostridium difficile*-associated diarrhoea
CHBAH	Chris Hani Baragwanath Academic Hospital
CI	Confidence Interval
Ct	Cycle Threshold
DD	Diarrhoeal Diseases
EAEC	Enteroaggregative *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
EPI	Expanded Programme on Immunisation
GEMS	Global Enteric Multicentre Study
HIV	Human Immunodeficiency Virus
HR	Hazard Ratio
HREC	Human Research Ethics Committee
IQR	Interquartile range
LT-ETEC	Enterotoxigenic *Escherichia. coli* producing heat-labile toxin
MAL-ED	Malnutrition and Enteric Disease Study
M/M	Matikwana and Mapulaneng
MSD	Moderate-to-severe diarrhoea
NICD	National Institute for Communicable Diseases
OR/aOR	Odds Ratio/ adjusted Odds Ratio
PCR	Polymerase Chain Reaction
P-value	Calculated probability
RCCH	Red Cross Children's Hospital
RTHC	Road To Health Card
RVV	Rotavirus Vaccine
SA	South Africa
SES	Social Economic Status
STEC	Shiga-toxigenic *Escherichia coli*

ST-ETEC	Enterotoxigenic *Escherichia coli* producing heat-stable toxin
TAC	TaqMan Array Card
WHO	World Health Organization

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1.1 Background

The World Health Organization (WHO) defines a case of diarrhoea as the passage of three or more loose or liquid stools per day, or more frequently than is normal for the individual (1). It is a symptom of infection in the intestinal tract caused by bacteria, viruses or parasitic organisms (2). Diarrhoeal diseases remain an important public health problem, not just in Africa but worldwide. In 2016 diarrhoea was the fifth leading cause of death amongst children ≤5 years old (3), with approximately 90% of diarrhoeal deaths occurring in South Asian and Sub-Saharan African countries (4). Research conducted in 12 sub-Saharan African countries in children under five years of age showed that the prevalence of diarrhoea in Cameroon was 16% (2011), 16% in Ethiopia (2011), 12% in the United Republic of Tanzania (2010), 15% in the Democratic Republic of Congo (DRC; 2013-14), and 12% in Uganda (2011) (5). In Senegal, diarrhoeal diseases accounted for 15% of all deaths in children below 60 months and were the third leading cause of childhood death (6). South Africa (SA) is also one of the African middle-income countries significantly affected by diarrhoeal diseases, accounting for nearly 19% of deaths of children under the age of 5 years in the country in 2010 (7). The high rate of mortality in low-income countries is due to insufficient health resources, poorer access to healthcare facilities, and co-morbidities such as Human Immunodeficiency Virus (HIV) and undernutrition (8–11).

Viruses such as rotavirus and norovirus, bacterial pathogens and parasitic organisms can cause diarrhoea (3). In a review by Lanata et al., it was found that rotavirus, calicivirus and enteropathogenic and enterotoxigenic *Escherichia coli* (ETEC) were found to cause more than half of diarrhoeal cases in children <5 years (12). A large Global Enteric Multicenter Study (GEMS) found that rotavirus, *Cryptosporidium*, enterotoxigenic *Escherichia coli* producing heat-stable toxin (ST-ETEC; with or without co-expression of heat-labile enterotoxin), and *Shigella* were enteric pathogens most associated with moderate-to-severe (MSD) diarrhoea in children aged 0-59 months in African and Asian countries (13).

In patients with dysentery, *Shigella* was detected in 16,6 % of infants (0–11 months), 66,0% of toddlers (12–23 months), and 78,4% of children (24–59 months) (13). A small proportion (<5%) of MSD diarrhoea was attributable to adenovirus 40/41 during infancy, and during the second year of life (13). According to the GEMS 2012 study, other pathogens that had smaller contributions to MSD diarrhoea included norovirus (GII genogroup), sapovirus,

Enteroaggregative *Escherichia coli* (EAEC), typical Enteropathogenic *Escherichia coli* (EPEC), nontyphoidal *Salmonella*, and *Entamoeba histolytica* (13). *Giardia* was not found to be associated with MSD diarrhoea but was, found more frequently in controls than in cases aged 12-59 months with the disease (13).

The geographical area of a country or province contributes to the prevalence of diarrhoea-causing pathogens (14). Pathogens causing diarrhoea often show seasonal trends, demonstrating a link between climate and enteric disease (15). In SA, a high number of diarrhoeal cases are due to bacterial enteropathogens in the summer months and rotavirus in the winter months (16). Water, sanitation and hygiene, and nutrition also play an important role in the prevalence of diarrhoea (17). This is especially true for bacterial pathogens, where improvements in water and sanitation have led to decreased incidence of bacterial diarrhoea. Studies show that even though diarrhoea-related deaths are less common in high-income countries, the incidence of rotavirus disease does not differ in high-income and low-income countries but is often more severe in the latter (17). This shows that improvements in water and sanitation may not impact significantly on rotavirus disease, and the most cost-effective approach to the prevention of rotavirus disease is vaccination.

South Africa was the first African country to introduce the oral rotavirus vaccine, Rotarix® into the Expanded Programme on Immunisation (EPI) in August 2009 (18). The first dose of the vaccine was scheduled at 6 weeks of age and the second at 14 weeks. By the following year, 2010, 67% of children <1 year had received a complete 2-dose series of Rotarix®, and 80% of the same cohort had received at least one dose and the effectiveness of the vaccine against rotavirus hospitalisation was found to be 57% (95% CI 40–68) for two doses and 40% (95% CI 16–57) for one dose (18,19). During the pre-vaccine era, between 2003 and 2005, it was estimated that 1 in every 43-62 children less than 2 years old in South Africa would be hospitalised for rotavirus-attributable diarrhoea and since the introduction of the Rotarix®™ vaccine, results obtained between 2007 and 2010 show that the numbers of rotavirus diarrhoea hospitalisations have substantially decreased (18,20). The reduction in rotavirus diarrhoea hospitalisations in South African children <12 months old was 45-50% greater than non-rotavirus diarrhoea hospitalisations in 2011, after the introduction of the rotavirus vaccine (21).

Rotavirus diarrhoeal infections caused over 200,000 deaths annually, mostly in low-income countries even after the introduction of the rotavirus vaccine (22). To lower the burden of MSD

diarrhoea, interventions focusing on the aforementioned pathogens would have a significant impact (13).

It is crucial to determine which enteric pathogens are prevalent in the post-rotavirus vaccination era. This will guide the future enteric diseases research agenda and allow appropriate allocation of resources for preventative measures. The purpose of this study was to assess the aetiology of diarrhoea in South African hospitalised children under 5 years of age post rotavirus vaccine introduction, from 2015 to 2016. In addition, risk factors associated with rotavirus hospitalisation in children who had received at least one dose of the rotavirus vaccine were investigated.

1.2 Literature Review

This section reviews the epidemiology of diarrhoea pre- and post-rotavirus vaccine introduction, including the effectiveness of the vaccine and factors that influence diarrhoeal infections in children under the age of five years.

1.2.1 Diarrhoea aetiology

Diarrhoea can be caused by various infectious agents (bacteria, viruses, and protozoa) whose contribution to diarrhoeal morbidity deaths and differs (15). The incubation period, the occurrence of symptoms after exposure, and the duration the pathogen is excreted in the stool after symptoms subside differs with each enteric pathogen (23). These differences make it difficult to accurately identify the cause of the diarrhoea when multiple diarrhoea-causing organisms are found in the patient's stools (24).

Stool testing techniques used to investigate the aetiology of diarrhoea include microscopy, serology, enzyme immunoassays and culture (25,26). Other techniques with wider detection abilities have been employed to detect various diarrhoea-causing agents. Novel methods such as multiplex polymerase chain reaction (PCR) can detect pathogens that remain unidentified by conventional techniques (26).

Real-time PCR has been shown to reduce labour time, reagent costs and the risk of cross-contamination (26). Another advantage offered by PCR diagnostic methods is the ability to test for multiple targets in a single multiplex reaction (26). PCR has also demonstrated high sensitivity and specificity with species-specific DNA from stool samples. Compared to an enzyme-linked fluorescent assay (VIDAS CDA2; bioMe´rieux) and an enzyme-linked assay [Premier Toxins A and B (PTAB); Meridian], using 540 samples, researchers concluded that

real-time PCR was the preferred diagnostic method for detecting *Clostridium difficile*-associated diarrhoea (CDAD) in stool samples because it had the highest concordance with toxinogenic culture (25).

In a review of studies using traditional methods, done by Lanata et al. 2013, it was estimated that rotavirus, calicivirus, EPEC and ETEC were the cause of the majority of all diarrhoea deaths in children <5 years of age (12). The GEMS found rotavirus, *Cryptosporidium*, ETEC, and *Shigella* to be responsible for most cases of MSD (using different diagnostic techniques for different pathogens) (13). However, a reanalysis of the GEMS study using quantitative molecular analysis improved the characterization of the causes of diarrhoea, identifying *Shigella*, rotavirus, adenovirus, enterotoxigenic E. *coli*, *Cryptosporidium* and campylobacter as the most prominent diarrhoea-causing agents (27).

A study conducted in the Central African Republic (CAR) using commercial enzyme immunoassays for viruses, Merthiolate iodine formaldehyde concentration technique for parasite diagnosis and conventional methods and/or PCR assays for bacterial pathogens, found that when adjusting for the presence of other pathogens, rotavirus, norovirus, astrovirus, *Shigella*/Enteroinvasive Escherichia *coli* (*Shigella*/EIEC), *Cryptosporidium parvum/hominis*, were positively associated with diarrhoea (28).

Using conventional, immunological and molecular detection methods in India, Nair et al., found that among bacterial pathogens V. *cholerae* O1 (26%), EAEC (6.3%), *Shigella* spp (6.1%), C. *jejuni* (4.7%), and ETEC (4.5%) had the highest overall isolation rate (29). Viruses that had a high prevalence in children <5 years old were rotavirus (19,6%) followed by human adenovirus (5%), astrovirus (2.3%), norovirus (3.1%), and sapovirus (1,6%) (29). Among these, *Giardia lamblia* was most predominant (11,2%), followed by *Cryptosporidium* sp. (6.3%) and E. *histolytica* (3.3%) (29).

Even though the overall burden of gastroenteritis has decreased since the introduction of rotavirus vaccines, the role of other pathogens in diarrhoeal diseases such as norovirus has increased in high-income countries such as the United States of America (USA) (30). In some African countries and the Americas, rotavirus still contributed the most to diarrhoeal disease post-vaccine introduction (31,32). Other diarrhoea causing pathogens in Africa post-vaccine introduction include *Cryptosporidium* and norovirus GII (31).

1.2.2 Rotavirus

Rotavirus is found in stools of infected individuals and it is transmitted by hands, nappies, or objects such as toys (33). It spreads easily as very small amounts of stool are sufficient to transmit the disease. Rotavirus is easily spread amongst families, nursery schools, and even in hospitals (33).

Rotavirus infections can be asymptomatic or symptomatic, and diarrhoea is the main clinical manifestation of the virus (34). Other rotavirus infection symptoms include vomiting, fever, and stomach pain due to inflammation in the intestines. Clinical symptoms may occur 1 to 3 days after infection and may last 3 to 8 days (34).

Diarrhoea, especially in infants and children, often leads to dehydration (35). Therefore, diarrhoea can be treated through replacement of lost body fluids with oral rehydration solution, or intravenously in severe cases (35).

1.2.3 Rotavirus burden prior to vaccine introduction

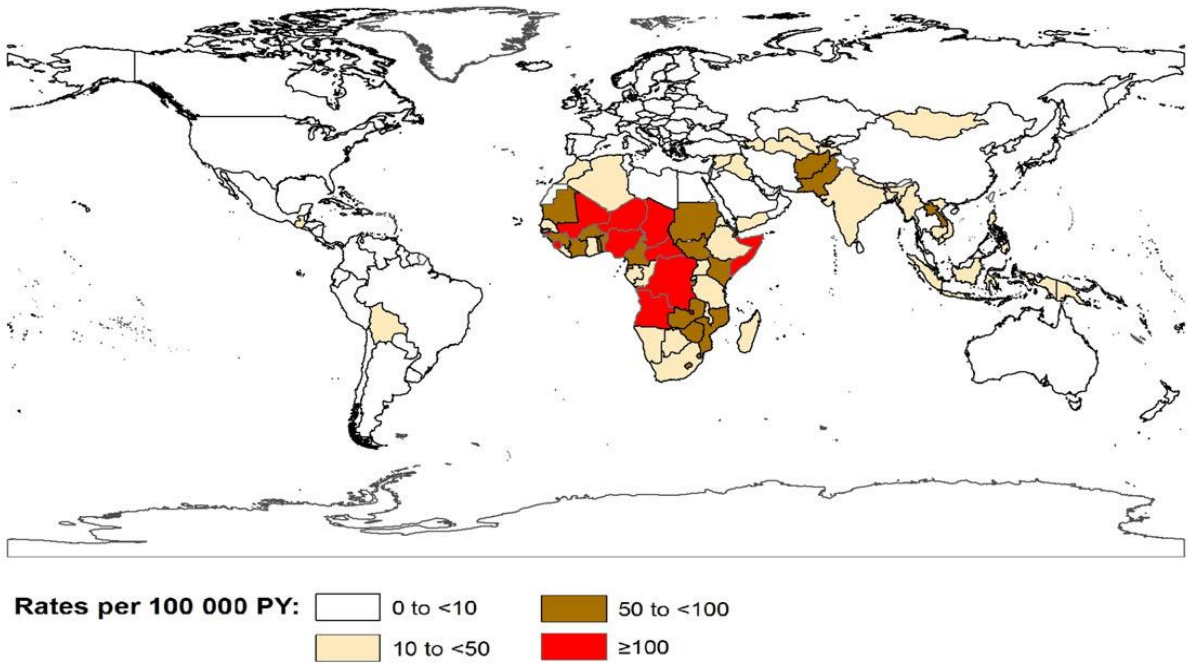


Figure 1.1 Rates of deaths attributed to rotavirus diarrhoea in children <5 years by country, in 2013. Image from Tate et al (22)

Rotavirus has been found to be a leading cause of diarrhoea among children from both high and low-income countries (9). Prior to vaccine introduction, rotavirus was associated with approximately 40% of diarrhoea deaths in children <5 years old, worldwide (22). In 2008, prior

to the widespread use of the rotavirus vaccine, rotavirus infections were estimated to account for over 453 000 deaths in children, globally, most deaths occurring in economically disadvantaged countries (9). In 2013, post-rotavirus vaccine introduction, rotavirus diarrhoea still occurred mostly in low-income countries in Africa and Asia even after the introduction of the vaccine in some of these countries, as shown figure 1.1 above (22).

In hospitalised American children younger than three years old, the proportion of rotavirus was estimated to be greater than 49%, prior to the introduction of the rotavirus vaccine (36). Post-vaccination era, rotavirus was detected in less than 25% of children hospitalisations (36). In South Africa prior to rotavirus vaccine introduction, rotavirus diarrhoea was responsible for one in every 43–62 severe diarrhoea cases in children 0 to 24 months (18). In 2010 and 2011, after the introduction of the vaccination, there was a decrease of 54% and 57%, respectively, in rotavirus diarrhoea hospitalisations in South African children under five years old. It was also estimated that approximately 20% of children <5 years old hospitalised with diarrhoea were infected with rotavirus (20). In a Tanzanian hospital, it was recorded that pre-vaccine (2009-2013) introduction, rotavirus positivity rate among infants was 41% but had decreased to 14% post-vaccine (2014-2015) introduction (37). In Madagascar, prior to rotavirus vaccine introduction, diarrhoea accounted for a median of 26% in hospitalisations of children younger than five years, however, during the post-vaccine era, diarrhoea was responsible for 16% of hospitalisations (38).

1.2.4 Rotavirus Vaccine

Two oral rotavirus vaccines are available globally, Rotarix® developed by GlaxoSmithKline (GSK) and RotaTeq® by Merck (39). The live-attenuated rotavirus vaccine, Rotarix®, is a P1A[8]G1 strain and therefore represents the most common human rotavirus antigens – VP7 and VP4 (40). The vaccine has shown to provide 85 – 95% protection against MSD caused by G1 and non-G1 rotavirus serotypes (G3, G4, and G9) in some trial settings (41,42). RotaTeq®, a pentavalent human-bovine (WC3), contains five live reassortant rotaviruses (40). The VP7 human rotavirus protein is expressed by G1, G2, G3 or G4 reassortant viruses, and the attachment protein P7[5] represents the bovine rotavirus parent strain WC3 (40). The fifth reassortant virus in Rotateq expresses the attachment protein P1A[8] from the human rotavirus parent strain and the outer capsid protein G6 from the bovine rotavirus parent strain (40).

Both vaccines have shown comparable protective efficacy (39). However, the efficacy of the vaccines in preventing rotavirus disease among children has been shown to be higher in high-income countries from Europe, Asia, and America (72-100%) (39). Lower efficacy rates were observed in low-income countries of Africa and Asia (49-72%) (39).

In South Africa, Rotarix® (an oral, live-attenuated vaccine) was introduced into the South African Expanded Program on Immunization in August 2009 administered at 6 weeks and 14 weeks of age (43). In the years 2010 and 2011, after the introduction of the vaccine, at least 13 000 to 20 000 hospitalisations in children <2 years old were prevented (43). The vaccine was shown to be 54% effective against hospitalisation for rotavirus in children less than a year old, and 61% effective in children between one and two years old (19). Factors that may cause the lower effectiveness of the vaccine in low-income countries include interference from placental or breast milk antibodies, comorbidities such as HIV, undernutrition, host genetic factors, or a high prevalence of enteric coinfections, although more research is required (44).

Rotavirus vaccines do not prevent all forms of diarrhoea in children, but they do aim at preventing children from developing severe dehydrating rotavirus-associated diarrhoea that requires hospitalisation, and thus lower childhood mortality (45).

1.2.5 Rotavirus Strains

To better understand the effectiveness of the rotavirus vaccine in Africa, it is also important to understand the distribution and diversity of rotavirus strains pre- and post-vaccine introduction (46). Rotavirus is a member of the Reoviridae family and is a medium-sized (70-100 nm) non-enveloped virus (47). The virus is divided into seven groups (A-G) (48). The most prevalent group since the discovery of the virus has been group A, while group B and C have not been found epidemiologically important in many countries other than China (49). Rotavirus group A has also been found to be the leading cause of severe dehydrating gastroenteritis in children younger than 5 years, and may sometimes lead to fatal diarrhoea (50,51).

Africa has a high genetic diversity of rotavirus strains and there have been reports of untyped strains found on the continent (46,52). A study showed that the most predominant rotavirus strain in the Eastern and Southern regions of Africa from 2010 to 2015 was G1P[8] (23.8%) (53). In Northern America, Europe, and Australia the G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] represent >90% genotypes circulating (54,55). It is possible that other strains of the

virus (and other enteropathogens) are responsible for the re-infections in children, hence further investigation is needed. Figure 1.2 below shows commonly circulating strains of rotavirus in six African countries post-vaccine introduction.

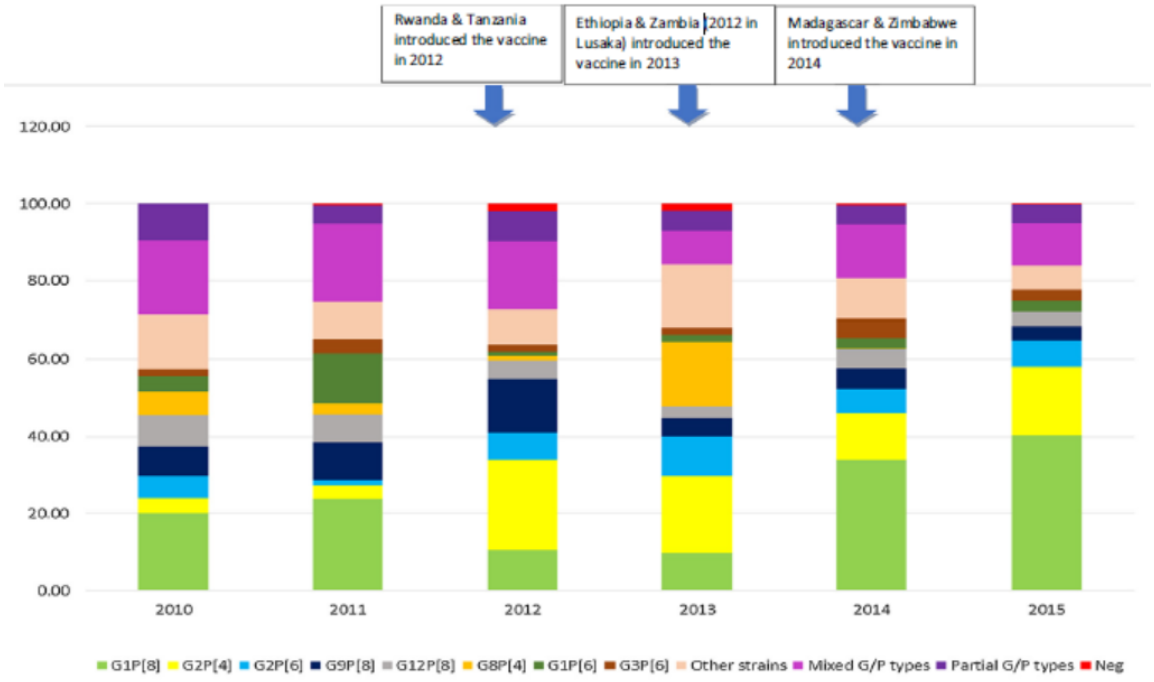


Figure 1.2 Temporal appearance of circulating strains before and after vaccine introduction in six African countries (Rwanda, Tanzania, Ethiopia, Zambia, Madagascar and Zimbabwe) (53)

1.2.6 Co-infection

Studies have found that detection of the rotavirus often occurs with other enteric pathogens, including other viruses, bacteria, and protozoa that are associated with diarrhoeal diseases (56,57). Enteropathogen co-infections have been shown to be more common in African and Asian countries (13,15). One study showed that the severity of a diarrhoeal case increases when there is a rotavirus-E. *coli* co-infection but other studies have not demonstrated this synergy (56,58). In a study conducted by Bhavnani and colleagues, co-infections with rotavirus and *Giardia* were found in all age categories (59). The study also found interactions between rotavirus and E. *coli*. The coinfections led to a greater chance of developing diarrhoea than would be expected in single pathogen infections (59). Co-infections pose difficulties in the detection of the cause of diarrhoea in a patient and impede progress in overcoming the high burden of the disease (24).

In a comparison between rotavirus diarrhoea cases and non-diarrhoea controls, Breurec et al. found that mixed bacterial and viral infections were found in 10% of cases, and viruses were the most prevalent pathogenic agents causing diarrhoea, identified in 55% of cases (28). A study conducted in Niger found mixed infections associated with rotavirus or any other pathogen in 10.3% of young children (60). In SA, multiple infections, with the detection of rotavirus and at least one other enteropathogen (39%) were found in 80% of children less than 12 months old (61).

HIV infection has also been identified as a comorbidity of diarrhoea (62). A cohort study showed that rotavirus infections had a higher incidence in HIV-positive children compared to children who were HIV negative, even though the infections did not more commonly lead to diarrhoea (63). Studies demonstrate that the overall prevalence/burden of rotavirus disease in HIV-positive children may be greater than in HIV-negative children (64). HIV-exposed-uninfected children were shown to have a higher risk of morbidity and mortality from diarrhoeal disease, but there are limited data on rotavirus-specific diarrhoea in these children (65,66).

No standard methodology has been developed to identify the true cause of an episode of diarrhoea when more than one pathogen is identified in the stool and, therefore, diarrhoea cannot be directly attributed to a particular pathogen isolated from a specimen (12,60). However, the characterization of circulating agents still provides important information for the prevention and control of these infections (60). Detection of rotavirus in stool samples, without testing for other enteric pathogens, may result in attribution of the diarrhoeal episode solely to rotavirus. Thus, co-detection of other enteric pathogens is important when considering "rotavirus vaccine failures".

1.2.6 Diarrhoeal Risk Factors

Although deaths in under-5s due to diarrhoea have decreased over the last twenty years, the reductions have been unsatisfactory in most low-income countries (17). Understanding the determinants of diarrhoea for targeted planning and implementation of prevention strategies is then of paramount importance as it will aid in decreasing the burden of global disease.

1.2.6.1 Doses of Rotavirus Vaccine

The immune response to the rotavirus vaccine and its efficacy is dose-dependent (67). Thus, decreasing or increasing the number of doses of the vaccine may affect the immunogenicity and efficacy of the vaccine (67).

A study conducted in SA and Malawi showed that three-dose rotavirus vaccine at six, ten, and 14 weeks of age could be more effective than the two-dose option administered at ten and fourteen weeks of age (68). A Ghanaian study comparing two and three doses of rotavirus vaccine showed that three doses of the vaccine led to improved antirotavirus immunoglobin A (IgA) seroconversion frequencies and geometric mean concentrations (GMCs) compared to two doses (69). However, a similar study conducted in Pakistan showed no significant difference in IgA seroconversion and GMCs in children who received either two or three doses of rotavirus vaccine (70). Further investigation of the effect of the number of doses and schedules of administering the human rotavirus vaccine are still needed.

1.2.6.2 Age

Enteropathogenic infection begins soon after birth and the detection of enteropathogens varies by age (13,15). Both the number of new cases of diarrhoea and the number of enteric pathogens detected per stool increased significantly during the first year of life in the Malnutrition and Enteric Disease (MAL-ED) study (15). A number of diverse enteropathogens were detected from diarrhoeal stools, with 22 pathogens in the first year of life and 25 in the second year of life (15). In the GEMS study, generally, two pathogens per age stratum were noted whose incidence markedly exceeded the others: rotavirus and *Cryptosporidium* in infants (0-11 months); rotavirus and *Shigella* in toddlers (12-23 months); and *Shigella* and rotavirus in older children (24-59 months) (13).

Global epidemiological studies show that all children are infected with rotavirus before the age of 3 years (17). In SA, prior to vaccine introduction, a burden of disease study indicated that 90% of children younger than the age of 2 years hospitalised or visiting the outpatient department were infected with rotavirus (20). The highest prevalence of rotavirus diarrhoea in SA occurred among children aged 3-17 months old whereas in European countries the highest incidence was found in children 6-23 months (20,71,72).

In the GEMS study, it was found that rotavirus had the highest attributable fraction (AF) during infancy but it diminished with increasing age (13). *Cryptosporidium* had the second-highest attributable fraction during infancy which persisted in importance in children who were 2 years

old (14). Unlike rotavirus, the adjusted AF of *Shigella* increased with increasing age (13). In the 2013 GEMS study, pathogens associated with increased risk of mortality were ST-ETEC (hazard ratio [HR] 1·9; 0·99–3·5) and typical enteropathogenic E. *coli* (HR 2·6; 1·6–4·1) in infants, and *Cryptosporidium* (HR 2·3; 1·3–4·3) in toddlers (13). Across all sites in the MAL-ED study, norovirus GII, rotavirus, *Campylobacter* spp, astrovirus, and *Cryptosporidium* spp were responsible for most diarrhoea cases in children one years old and less, and *Campylobacter* spp, norovirus GII, rotavirus, astrovirus, and *Shigella* spp were most highly detected in children who were in their second year of life (15).

1.2.6.3 Hygiene and Sanitation

In sub-Saharan Africa, reduction in diarrhoeal mortality has been "unsatisfactory", as Das et al have described even with the introduction of RVV (6,17). A possible explanation for the unsatisfactory decline in the sub-continent in diarrhoeal morbidity is that risk factors associated with insufficient water, sanitation and hygiene (WASH), insufficient promotion of breastfeeding, and under-nutrition remain very high (73,74). As the "urban health penalty" hypothesis suggests, these risk factors are more prevalent in poor urban areas and other underprivileged settings (rural areas), and together with the global uneven distribution of healthcare, make children living in these conditions more prone to pathogen infections and consequently, diarrhoeal diseases (6,75). Results from the GEMS study showed that poor water sanitation occurred mostly amongst diarrhoeal patients compared to non-diarrhoea controls who were of a higher wealth quantile and had access to clean water (13).

1.2.6.4 Seasonality

The WHO estimates that because of climate changes, we can also expect changes in diarrhoeal disease incidence and, therefore, it is important to understand climate variability as a risk factor for diarrhoeal diseases (76). The relationship between climatic patterns and diarrhoea can be expected to change with different causal agents such as rotavirus, norovirus, *Giardia*, *Cryptosporidium* and pathogenic *Escherichia coli*, *Campylobacter* and *Salmonella* (77).

Evidence from several studies has shown that there is a link between the peak season of diarrhoea and climatic factors, especially rainy seasons and high temperatures in developing countries (78–82). In Zimbabwe, prior to rotavirus vaccine introduction, yearly seasonal peaks occurred during winter months, May and June but since the introduction, seasonal peaks have shifted toward September and October and "were substantially blunted" (83). Evidence from

the Americas shows the effectiveness of the vaccine is lower in children less than a year born during the rotavirus season than those born outside the rotavirus peak months (84).

A study done in Cape Town showed that there was a link between an increase in minimum and maximum temperature, and the incidence rate of diarrhoea in <5 years of age (85). In SA, pre-vaccination era, the onset of rotavirus season was in April, but after the vaccine was introduced the onset period has been delayed and is observed in May (18). It has also been observed that the rotavirus peaks in the colder months are more pronounced but of shorter duration in the post-vaccination period than in the pre-vaccination period (18).

1.2.6.5 Socioeconomic Status

Thiam and colleagues identified a number of factors associated with diarrhoea in children under the age of 5 years in the cross-sectional study they conducted in Mbour, Senegal, in 2017 (6). The mother's occupation was associated with diarrhoea - being a housewife was associated with higher diarrhoea risk for the children than working in the public or private sector (6).

Higher socioeconomic status is associated with better access to household amenities and healthcare access which can reduce the risk and sequelae of diarrhoea (6,86). However, as Root suggested, there exist other diarrhoeal factors that even wealthy parents have no control over, such as a contaminated environment, lack of water, or crèche/school surroundings (86). Thus, even children from wealthier backgrounds are still at risk of diarrhoeal infections.

Diarrhoeal epidemics have been linked to overcrowding in rapidly developing cities in African countries (87). A number of cross-sectional studies have observed that households with more than one child <5 years old were associated with a higher prevalence of diarrhoea (6,88–90). This suggests that a larger gap between births and exclusive breastfeeding in the first 2 years of life might have a possible impact on diarrhoea prevention as children are allowed to breastfeed for longer periods (6).

1.2.6.6 Nutrition

Nutrition and diarrhoea are interlinked, and thus the importance of improving childhood nutrition to reduce diarrhoea mortality, cannot be overlooked (17). Nutritional risk factors include not exclusively breastfeeding infants younger than 6 months, inappropriate complementary feeding until 2 years of age and vitamin A and zinc deficiency (91).

Lack of breastfeeding is associated with an increase in diarrhoea incidence in infants aged 0-11 months old (92). Lack of breastfeeding is also associated with an increase in diarrhoea-

related mortality among infants aged 0-11 months compared to those who were exclusively breastfed (92). It is recommended to commence breastfeeding an hour after birth, to exclusively breastfeed infants until 6 months of age, and to continue breastfeeding until at least 2 years of age (93). Improved rate of breastfeeding is associated with reduced diarrhoea morbidity and mortality (17,91).

Das and colleagues found an inverse relationship between acute malnutrition and being underweight with childhood rotavirus infection among children <5 years old in Dhaka after controlling for a different climate, socio-demographic and sanitation practices (94). Malnutrition is a suspected risk factor for *Salmonella* infection, however, a temporal disconnection between the *Salmonella* peak season and the annual malnutrition peak was found in Niger (60,95).

Contaminated weaning foods also play a crucial role in childhood diarrhoea, especially in low-income countries (96). Enteropathogens transmitted through food, have found to cause up to 70% of diarrhoeal episodes, therefore, it is important that the strategies for preventing diarrhoea in children associated with malnutrition are not only limited to the promotion of breastfeeding or improving water supply and sanitation (97). Educating food handlers, particularly mothers and helpers, will have a significant impact since they play a crucial role in the children's diet (96,97).

1.2.6.7 Drug resistance

Many studies conducted in Africa have shown that there has been an emergence of multidrug-resistant organisms over the years, especially with resistance to cotrimoxazole, amoxicillin, and chloramphenicol (98–100). Other drugs to which diarrhoea-causing pathogens have been reported to be resistant include fluoroquinolones and extended-spectrum cephalosporin (ESCS) (101–103). *Shigella spp.* has also been reported to show resistance against amoxicillin; *Shigella sonnei* was most sensitive to the drug (55, 99, 100). In Africa, there is a low prevalence of resistance to ESCs and fluoroquinolones as opposed to the prevalence of resistance found in Asia (55, 100, 101). Even though 90% of EPEC isolates and more than 50% of all Enterobacteriaceae were found to be sensitive to other antibiotics tested, they were found to be resistant to amoxicillin and cotrimoxazole in Niger, Africa (60). It is, therefore, important to determine whether potentially drug-resistant enteric pathogens such as *Shigella* are prevalent in children hospitalised with diarrhoea in South Africa.

Additional risk factors that play a role in diarrhoeal infections in African children <5 years old are shown in Table 1 below.

Table 1.1 Potential risk factors associated with diarrhoea disease in African children below the age of five years. The table was adapted from Mutama et al. (107)

Potential Risk Factors	Pearson chi-square	df	p-value
Behavioural risk factors			
Hand washing	6.214	1	0.013
Bottle feeding	10.078	4	0.039
Latrine utilization	13.351	3	0.004
Breastfeeding child	0.823	1	0.364
Length of breastfeeding	0.223	1	0.637
Disposal of infant faeces	5.257	1	0.022
Method of water storage	16.213	1	0.000
Socio-demographic risk factors			
Level of Education	20.694	15	0.147
Religion of caretaker/mother	37.897	10	0.000
Age of mother/caregiver	2.111	3	0.550
Age of the child	17.896	5	0.003
Number of children <5	0.416	1	0.519
Gender of child	4.608	1	0.032

1.3 Problem Statement

Diarrhoeal diseases remain a major public health problem globally and in SA (108). Paediatric diarrhoea can affect physical and cognitive development that may lead to costly impairment of human potential and productivity (14). Investigation and understanding of current aetiology and trends of diarrhoeal diseases will help in identifying potential interventions to decrease the prevalence of diarrhoeal disease in SA.

The introduction of rotavirus vaccines has led to substantial reductions in diarrhoeal morbidity and mortality, yet sub-optimal vaccine coverage and lower efficacy of the vaccines in low-income compared to high-income countries are reasons for concern. It is thus important to monitor the contribution of rotavirus to diarrhoeal hospitalisations after vaccine introduction and to understand the role that other enteric pathogens such as norovirus, caliciviruses, sapovirus, bacterial pathogens and parasites play at the population level as they are significant contributors to acute childhood diarrhoea (109,110).

This information will be vital in determining the enteric disease research agenda and where funding is best spent. If rotavirus remains an important cause of diarrhoea post-vaccination,

the development of new RVV and novel vaccine schedules will need to be pursued. If other enteric pathogens are found to have become more prevalent post rotavirus introduction, then vaccines against these pathogens should be prioritised.

Risk factors that predispose vaccinated children to rotavirus infections, the so-called "vaccine failures", are unclear and need further investigation. Age and seasonality might play a role in predisposing vaccinated children to rotavirus infections. Evidence suggests that the effectiveness of the vaccine may be lower in children less than a year born during the rotavirus season than those born outside the rotavirus peak months (84). The use of novel quantitative molecular techniques allows for better detection of enteric pathogens and can assist in understanding the role of enteric co-infections (111,112).

1.4 Justification

Many other pathogens besides rotavirus have been implicated as causes of diarrhoeal-induced deaths, yet there has not been enough research to determine the importance of all common pathogens in South Africa, especially after the introduction of rotavirus vaccine (9,12,113).

This study is important in that it aims to identify prominent pathogens that cause acute childhood diarrhoea post-rotavirus vaccine introduction. Understanding the aetiology of childhood diarrhoea is necessary to direct diarrhoea treatment and prevention efforts (114). Identifying pathogens responsible for diarrhoea post rotavirus vaccine introduction will provide a platform for putting in place mitigation measures that will reduce the morbidity and mortality rates attributed to acute diarrhoea.

Understanding some of the reasons for the detection of rotavirus in children who have received rotavirus vaccination, will guide efforts in improving the impact of the rotavirus vaccine.

1.5 Question

How has the introduction of the oral rotavirus vaccine influenced the aetiology of acute diarrhoea in hospitalised children <5 years in South Africa?

1.6 Aim

To investigate the aetiology of acute diarrhoea and possible risk factors associated with rotavirus diarrhoea post-rotavirus vaccine introduction in hospitalised children <5 years old.

1.7 Objectives

Objective 1

To describe the aetiology of acute diarrhoeal hospitalisations in South Africa from 2015 to 2016, after the introduction of routine rotavirus vaccination, among children <5 years of age.

Objective 2

To describe enteric co-infections among children <5 years of age hospitalised with acute diarrhoea in South Africa from 2015 to 2016.

Objective 3

To investigate factors associated with rotavirus-positivity on stool testing among South African children <5 years of age who received rotavirus vaccination.

Chapter 2 – Methodology

2.1 Primary Study

The National Institute of Communicable Diseases (NICD) (Wits HREC approval M091018) established a prospective Rotavirus Sentinel Surveillance Programme in April 2009 at three sentinel sites in two provinces in South Africa. The sentinel sites included Chris Hani Baragwanath Academic Hospital (CHBAH), Soweto, Gauteng; Dr George Mukhari Academic Hospital (DGMAH), Ga-Rankuwa, Gauteng; and Matikwana and Mapulaneng (M/M) Hospitals, Bushbuckridge, Mpumalanga. Additional sites were added during the course of the surveillance programme: Edendale Hospital, Pietermaritzburg, KwaZulu Natal; Red Cross War Memorial Children's Hospital, Cape Town, Western Province; Kimberley Hospital, Northern Cape; Polokwane Hospital, Limpopo; Pelonomi Hospital, Bloemfontein, Free State; Dora Nginza Hospital, Port Elizabeth, Eastern Cape; and Klerksdorp Hospital, North West.

Children <5 years of age hospitalised for acute diarrhoea were screened for enrolment during normal working hours from Monday to Friday. An acute diarrhoeal episode was defined as ≥3 loose stools in a 24-hour period, with <7 days duration. Written informed consent was obtained from the parent or guardian for the child's participation. Demographic, socioeconomic and clinical information was collected by means of a questionnaire administered to the parent/guardian of the child. The child's vaccination card and medical records were also assessed, and vaccination status, clinical assessment, treatment and outcome recorded on a case report form. Standard of care of children admitted with acute diarrhoea generally included rehydration, either orally or intravenously, correction of electrolyte abnormalities and early feeding. Stool samples were not routinely collected from children hospitalised with acute diarrhoea.

As part of the surveillance programme, a stool sample was collected within 48 hours of hospital admission. Testing of stool samples for rotavirus was performed at the NICD in Johannesburg and the Medical Research Council/Diarrhoeal Pathogens Research Unit, University of Limpopo using the Prospect Rotavirus ELISA (Oxoid, Basingstoke, UK). Quantitative molecular testing was retrospectively performed at the NICD on selected stool samples from 2015 and all available stool samples from 2016 using the Taqman Array Card (TAC) (112). The TAC is custom-designed and compartmentalises probe-based quantitative PCR assays for multiple enteropathogens (112).

2.2 Current study design and participants

This was a secondary data analysis using data from children <5 years of age, enrolled in the Rotavirus Sentinel Surveillance Programme during 2015 and 2016 who had a stool sample tested by quantitative molecular methods. A cross-sectional study design was used to assess pathogens detected in the stool, including co-infections, and risk factors associated with rotavirus positivity at the time of hospitalisation. Oral rotavirus vaccination was part of the EPI since August 2009, and all children enrolled during 2015 and 2016 would have been eligible to have received two doses of Rotarix® at 6 and 14 weeks of age.

2.3 Sample Size

A sample size calculation was not performed as the size of the study population had already been determined, based on the availability of sample test results. A total of 793 participants had a molecular stool result available and were included in the analysis.

2.4 Detection of enteric pathogens using Taqman Array Card (TAC)

2.4.1 TAC design

The Houpt Laboratory at the University of Virginia developed a custom enteric TAC, containing 384 wells, that detects multiple targets in eight samples on one card (Appendix C). The enteric TAC targets included viruses, bacteria, fungi, protozoa, and helminths. MS2 and phocine herpes virus (PhHV) were added to the stool samples during nucleic acid extraction as extrinsic controls to monitor extraction and amplification. A universal bacterial 16S assay was also included on the card to be used for normalization of pathogen burden to total bacteria load. Full details of the testing are not provided in this research report as testing had already been conducted prior to this secondary data analysis.

Testing was conducted by Prof Nicola Page and her staff at the NICD, according to Standard Operating Procedures provided by the University of Virginia Houpt Laboratory. A modified TAC card was used and baseline adjustments and interpretation of the raw results were done by Prof Page. The final results of the TAC testing were provided in Microsoft Excel format, including study number and cycle threshold (Ct) values for each of the targets.

2.4.2 Pathogen Detection

Detection of a pathogen: targets with Ct values ≤35 were considered positive, and Ct values > 35 were considered negative. This definition was used for all targets and pathogens listed in

Table 2.1. The use of this generalised Ct cut-off may lead to positive detection of a pathogen which is not necessarily causing diarrhoea in the child.

Table 2.1 TAC targets that were used to define the enteric pathogens

Pathogen	**Target**	**Reference**
Viruses		
Adenovirus	Hexon[a]	(115)
Astrovirus	Capsid	(115)
Enterovirus	5'UTR	(115)
Norovirus GI	ORF1-ORF2	(115)
Norovirus GII	ORF1-ORF2	(115)
Rotavirus	NSP3	(115)
Rotavirus strain P[4], P[6], P[8], P[9], P[10], P[11]; G1, G2, G3, G4, G8, G9, G10, G12	Unpublished	
Sapovirus	RdRp	(115)
Bacteria		
Aeromonas	*Aerolysin*	(115)
Bacteroides fragilis	*EGBF*	(115)
Campylobacter spp *Campylobacter jejuni/coli*	*Cpn60* *hipO/GlyA*	(115) (115)
Clostridium difficile	*tcdB*	(115)
Enteroaggregative E. coli (EAEC)	aaiC, aatA	(115)
Atypical *Enteropathogenic E. coli* (EPEC)	eae without bfpA, stx1, or stx2	(115)
Typical *Enteropathogenic E. coli* (EPEC)	bfpA with or without eae	(115)
Enterotoxigenic E. coli (ETEC) ST-ETEC LT-ETEC	STh, STp or LT STh or STp, with or without LT, LT only	(115) (115)

Shiga-toxigenic E. coli (STEC)	eae without bfpa and with stx1 or stx2	(115)
Helicobacter pylori	*UreC*	(115)
Mycobacterium tuberculosis	*IS6110*	(115)
Shigella/enteroinvasive E. *coli* (EIEC)	*ipaH*[b]	(115)
Salmonella	*ttr*	(115)
Vibrio cholerae	*hlyA*	(115)
Parasites		
Enterocytozoon bieneusi	ITS	(115)
Encephalitozoon intestinalis	SSU rRNA	(115)
Cryptosporidium spp *C. hominis* *C. parvum*	*18S rRNA* *LIB13* *LIB13*	(115) (115) (115)
Cyclospora belli	*18S rRNA*	(115)
Entamoeba histolytica	*18S rRNA*	(115)
Giardia *Giardia A/B*	*18S rRNA* *TPI*	(115)
Isospora	*18S rRNA*	(115)
Ancylostoma duodenale	*ITS2*	(115)
Ascaris lumbricoides	*ITS1*	(115)
Necator americanus	*ITS2*	(115)
Strongyloides stercoralis	Dispersed repetitive sequence	(115)
Trichuris	*18S rRNA*	(115)

[a] The assay detected all adenovirus serotypes.

[b] The ipaH gene was targeted for detection of both *Shigella* and EIEC.

The current study only consisted of diarrhoeal cases and there was no enrolment of a healthy control group during the surveillance period. Therefore, it was not possible to compare the

prevalence of pathogens detected in cases and controls. If a pathogen causes diarrhoea, it would be more prevalent in cases than controls, whereas pathogens that do not cause diarrhoea are more likely to be found in both diarrhoea cases and non-diarrhoea controls. Specific enteric pathogen Ct cut-offs have been established from previous studies that enrolled both diarrhoea cases and healthy controls. These cut-offs were used in this analysis to help distinguish between diarrhoea-associated and non-diarrhoea-associated pathogens, in the absence of controls. To detect the prevalence of diarrhoeal cases due to a specific enteric pathogen the Ct cut-offs from the Global Enteric Multicentre (GEMS) (13) study were preferentially used as this study assessed moderate to severe diarrhoea and was a better representation of the current study. The Ct cut-offs from the Malnutrition and Enteric Disease (MAL-ED) (15) study were used in the absence of specific cut-offs from the GEMS study. This study assessed community diarrhoea and was less representative of the current analysis.

Pathogen-associated diarrhoea was described as enteric pathogen detection at strict Ct cut-offs and pathogen non-associated diarrhoea was described as no detection of enteric pathogens at specific Ct cut-off, only at Ct cut-off of 35. Unfortunately, these specific cut-offs were not available for all pathogens tested for in my study. Specific Ct cut-offs from either the GEMS or MAL-ED studies were available for adenovirus, astrovirus, norovirus GII, rotavirus, sapovirus, *Cryptosporidium, C. jejuni/coli, H. pylori*, tEPEC, ST-ETEC (STh only), *Salmonella, and Shigella*/EIEC. The Ct cut-off values from the GEMS and MAL-ED studies are listed in Table 2.2 below.

Table 2.2 Specific Ct cut-off values for enteropathogens extrapolated from the GEMS and MAL-ED studies

Pathogen	GEMS	MAL-ED
Viruses		
Adenovirus 40/41	22.7	24.1
Astrovirus	22.2	23.7
Norovirus GII	23.4	27.2
Rotavirus	32.6	31.7
Sapovirus	N/A	26.1
Bacteria		
Cryptosporidium	24.0	22.0
C. *jejuni/coli*	15.4	21.8
Helicobacter *pylori*	30.8	N/A
Typical EPEC (bfpa)	16.0	17.8
ST-ETEC (STh)	22.8	23.5
Salmonella	30.7	N/A

Shigella/EIEC	27.9	28.8

2.4.3 Co-detection of selected pathogens

Using the specific Ct cut-offs derived from the GEMS and MAL-ED studies, co-infections and single-infections were described for those enteric pathogens with specific Ct cut-offs. The most common rotavirus co-infections were also assessed using the specific Ct cut-offs.

Co-detection: more than one pathogen detected using pathogen-specific cycle threshold (Ct) cut-offs.

For the purposes of analysing mono infections vs co-infections, a variable "co-infection" variable was created where mono-infections were coded "0" and detection of more than one pathogen in a stool was coded "1". The co-infection variable was created for only those pathogens that had specific Ct cut-off values (Objective 2).

2.5 Statistical considerations

All data cleaning and statistical analyses (descriptive, univariable and multivariable) were conducted in STATA version 15.1 (StataCorp, Texas, USA) and figures were generated using Microsoft EXCEL.

2.5.1 Data management and processing

The original data was received in Microsoft excel database format and converted to STATA. The data management process included merging and appending the dataset files received from gatekeepers into one master file, labelling of the variables and generating new categorical variables from existing variables, using STATA. Six observations with missing values for the variable "age" were dropped and excluded from the analysis (Figure 2.1).

Participants who were above 59 months were also excluded from the analyses. Age was originally a continuous variable from which four categories were created, in keeping with the literature: <6 months, 6-11 months, 12-23 months, and 24-59 months. The sex variable consisted of male and female participants, coded "0" for male participants and "1" for females.

Participants were also categorised into those who have: "in-house flushing toilets" or "other" types of toilets, and "running water" or "other" sources of water. The education level of the mother was categorised as "no schooling", "primary" and "high school/tertiary".

Two new variables for seasons were generated: season of birth of the participants and the season they were admitted into the hospital. Seasons were defined as summer (December,

January, February), autumn (March, April, May), winter (June, July, August), and spring (September, October, November). Seasons were also categorised into dry (April, May, June, July, August, September) and wet (October, November, December, January, February, March) seasons (116). The number of people with whom each participant shared a room with was categorised as less than five roommates or five or more roommates.

Children who had received at least one dose of the rotavirus vaccine were coded as "1" and those who had not received any vaccine doses as "0". Participants were also categorised according to the number of doses they had received: no dose, one dose or two doses.
Other variables included race (black vs. other races), surveillance site, method of feeding in the first four months of life (breastfeeding, formula feeding or both), method of feeding beyond four months, use of antibiotics prior to hospitalisation (yes or no), previous hospital admission (yes or no), pre-existing chronic illnesses (asthma, heart problems, kidney problems or cerebral palsy), refusal to feed, fever, vomiting, malnutrition (physician-diagnosed), nursery school attendance (yes or no). The original case report forms are provided in Appendix D.

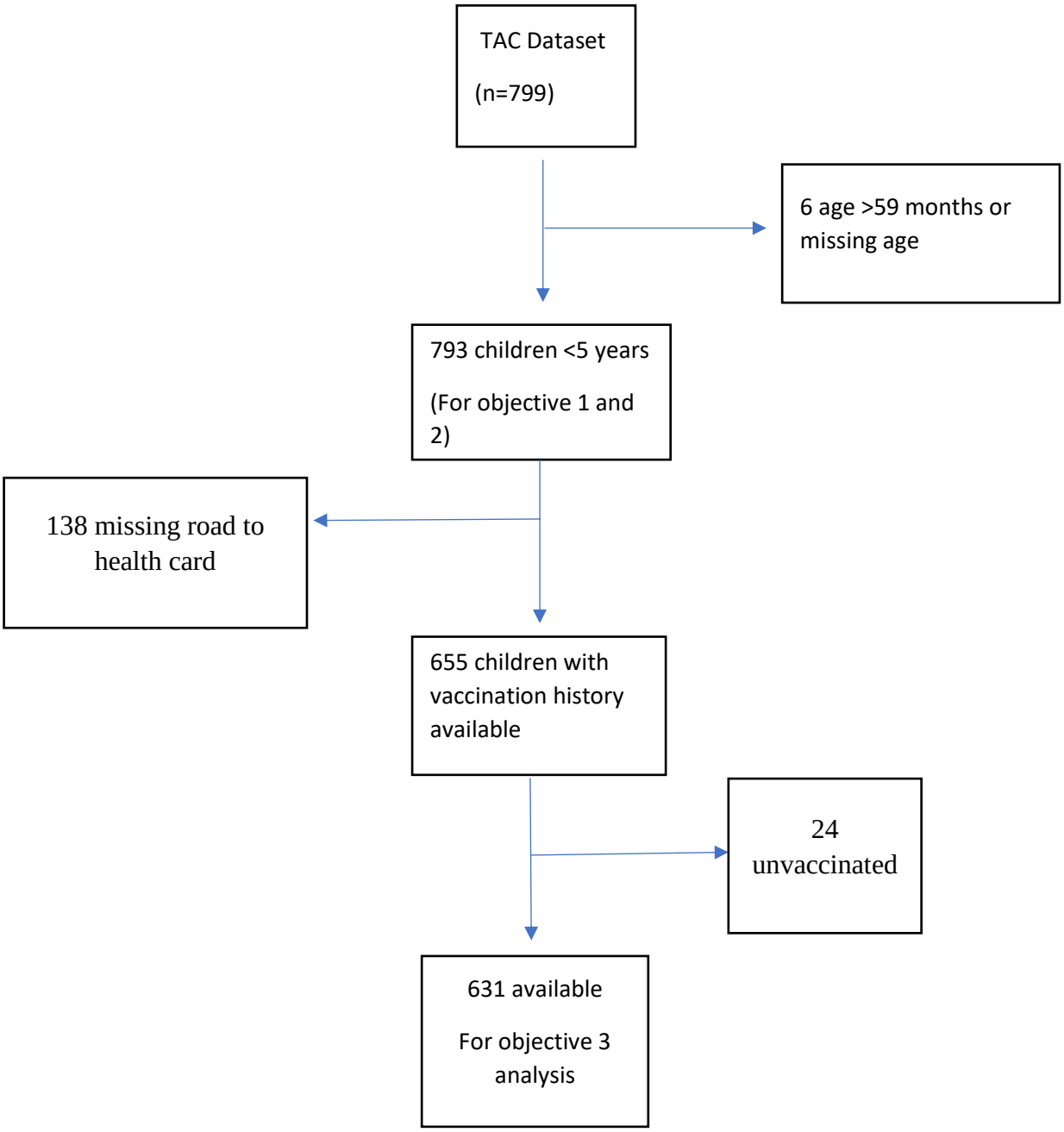


Figure 2.1 Flowchart showing the selection of participants at different stages of the analysis

Objective 1

The focus of objective 1 was to describe the spectrum/aetiology of enteric pathogens among children <5 years hospitalized for acute diarrhoea. For this objective, a descriptive analysis was done, including a description of the study population.

Prevalence of pathogens in the total study population was described using the detection Ct cut-off value of ≤35. The prevalence of all pathogens was described by age group, site, and season

of admission. The frequency of P and G genotypes and strains of rotavirus was assessed among those participants who tested positive for rotavirus. The prevalence of selected enteric pathogens was compared using Ct≤35 and specific Ct cut-offs and a proportions test was used to compare the prevalence of the pathogens at different Ct cut-offs.

Objective 2

The focus of objective 2 was to describe the prevalence of co-infections (more than one pathogen detected using pathogen-specific Ct cut-offs).

For selected pathogens, prevalence using the detection Ct value of ≤35 was compared to the prevalence of the enteric pathogens using specific diarrhoea-associated Ct cut-offs. The proportions significance test was used to assess if the proportions differed when using different Ct cut-offs. Comparison of mono-infection/ no co-infection (detection of only one pathogen) and co-infections (detection of more than one enteric pathogen) of those pathogens with specific Ct cut-offs was done.

Bivariate analysis of pathogens and medical history of the participants and presenting symptoms on admission between 2015 and 2016 was conducted. Medical history included the use of antibiotics prior to hospitalisation, whether the patient had been hospitalised previously, history of diarrhoea illnesses, pre-existing chronic medical conditions and malnutrition. The number of deaths caused by each enteric pathogen was also described. The symptoms that were considered in the analysis included bloody stools, vomiting, and refusal to feed, fever and dehydration. The type method of feeding (breast milk or formula) during the first four months and beyond four months were also analysed. HIV status and birth weight were not analysed as they were not adequately recorded in the dataset and had too many missing variables.

Objective 3

The focus of objective 3 was to analyse the different factors that might influence rotavirus diarrhoea among children who had received at least one dose of the vaccine. Children who had tested positive and negative for rotavirus infection, despite having been vaccinated, were compared. For this analysis, a Ct cut-off value of ≤35 for rotavirus detection was used as there was not much difference in the prevalence of the rotavirus between the use of Ct of ≤35 (22,1%) and the pathogen-specific cut-off of 32.3 (20,7%). Logistic regression was used for objective 3 as the outcome variable (rotavirus positive or rotavirus negative) was binary. Adjusted odds

ratios were calculated for the risk factors. A p-value of <0.05 was used to determine significance. A confidence interval of 95% was used in the analysis.

Firstly, bivariate logistic regression analysis was conducted to assess the association of rotavirus detection and various risk factors such as number of vaccine doses, age group, sex, race, surveillance site, method of feeding in the first four months of life and beyond four months, use of antibiotics prior to hospitalisation, previous hospital admission, pre-existing chronic illnesses, refusal to feed, fever, vomiting, malnutrition, number of people sharing a bedroom, nursery school attendance, education level of the mother, the season of admission, and season of birth with rotavirus diarrhoea without adjusting for other possible confounders.

A multivariable logistic regression analysis was then performed to assess risk factors for rotavirus detection in vaccinated children. The stepwise forward selection was used to create a parsimonious model for assessing risk factors for rotavirus detection. All variables with p-value <0.2 in the bivariate analysis were added to the model. Variables were kept in the final model only if their p-values remained significant in the multivariate regression analysis. The exception were those variables considered important risk factors, based on published literature, they were kept in the final model even if the p-value was not significant.

The likelihood ratio test was used to compare different models. Hosmer and Lemeshow's goodness of fit test was used to check if there was any evidence of a lack of fit in the model.

All the independent variables used in the logistic regression analysis were categorical. For categorical variables, the category with the largest number of observations was used as the reference category in the logistic regression analyses. Exceptions included age where the oldest children were used as a reference to assess how younger age groups who had received the vaccination compared to the older age group. The majority of the participants were black children, however, other races (white, coloured, and Indian/Asian) children were used as the reference category for interpretation purposes Patients who presented with no symptoms (coded as "0") (diarrhoea, chronic illness, refusal to feed, vomiting, and malnutrition) or negative medical history (no previous admission, antibiotics prior to hospitalisation) were used as references in the logistic regression. Odds ratios, 95% confidence intervals and p-values were recorded.

2.6 Ethical considerations

The ethical approval to carry out this secondary data analysis was obtained from the University of the Witwatersrand, Human Research Ethics Committee (Medical); M181171. Permission to use data was obtained from the NICD. Analysis of the data only commenced after the permission letter was received from the gatekeeper. All the parents of the participants who took part in the original study were presented with and signed informed consent.

3. Chapter 3 - Results

3.1 Objective 1- The aetiology of acute diarrhoeal hospitalisations

3.1.1 Description of the study population

Six of the 799 participants in the dataset were excluded as age was not known or >59 months. The analysis, thus, included a total of 793 participants from 2014 (n=2), 2015 (n=140) and 2016 (n=653). Most children were black (88,4%), with the remainder of coloured (11,6%), Indian (<1%), and white (<1%) race. Of 778 participants with sex available, 409 (51,6%) were male and 369 (46,5%) were female.

The participants were stratified into four age groups, <6 months (n=195; 24,6%), 6-11 months (n=241; 30,4%), 12-23 months (n=228; 28,8%), and 24-59 (n=129; 16,3%) months old. The median age of the study participants was 11 months (interquartile range= 6 to 18 months).

The participants were admitted at the following sites: CHBAH (n=208; 26,2%), M/M Hospitals (n=113; 14,3%), Edendale Hospital (n=73; 9,2%), RCCH (n=44; 5,6%), Kimberley Hospital (n=80; 10,1%), Polokwane Hospital (n=12; 1,5%), Pelonomi Hospital (n=180; 22,7%), Dora Nginza Hospital (n=49; 6,2%), and Klerksdorp Hospital (n=34; 4,3%). No samples from Dr George Mukhari Hospital were available for this analysis.

3.1.2 Detection of enteric pathogens

The prevalence of the enteric pathogens detected in stool was initially analysed using a cycle threshold (Ct) cut-off value of ≤35. In 2015 and 2016, rotavirus was still a prominent enteric pathogen in children who were hospitalised for diarrhoea; it was detected in 22,1% (n=175) of the stool samples. Other prominent enteric pathogens detected included EAEC (n=263; 33,2%), adenovirus (n=227; 28,6%), *Shigella*/EIEC (n=191; 24,1%), *Cryptosporidium* (n=187; 23,6%), EPEC (n=149; 18,8%), and enterovirus (n=146; 18,4%) (Figure 4.1).

Other detected pathogens included *Campylobacter* spp (n=100; 12,6%), *Giardia* (n=95; 12,0%), ETEC (10,3%), norovirus (n=76; 10%), sapovirus (n=62; 7,8%), B. *fragilis* (n=58; 7,3%), C. *jejuni*/*coli* (n=58; 7,3%), H. *pylori* (n=43; 5,4%), astrovirus (n=27; 3,4%), C. *difficile* (n=24; 3,0%), *Salmonella* (n=17; 2,1%), E. *bieneusi* (n=10; 1,3%), *Aeromonas* (n=10; 1,2%), and STEC (n=4; 0.5%). There were several parasites that were not detected in any of the children's stools. These included *Ancylostoma, Ascaris, Encephalitozoon intestinalis,*

Entamoeba histolytica, Enterocytozoon bieneusi, Cyclospora, Necator, Strongyloides, and Trichuris.

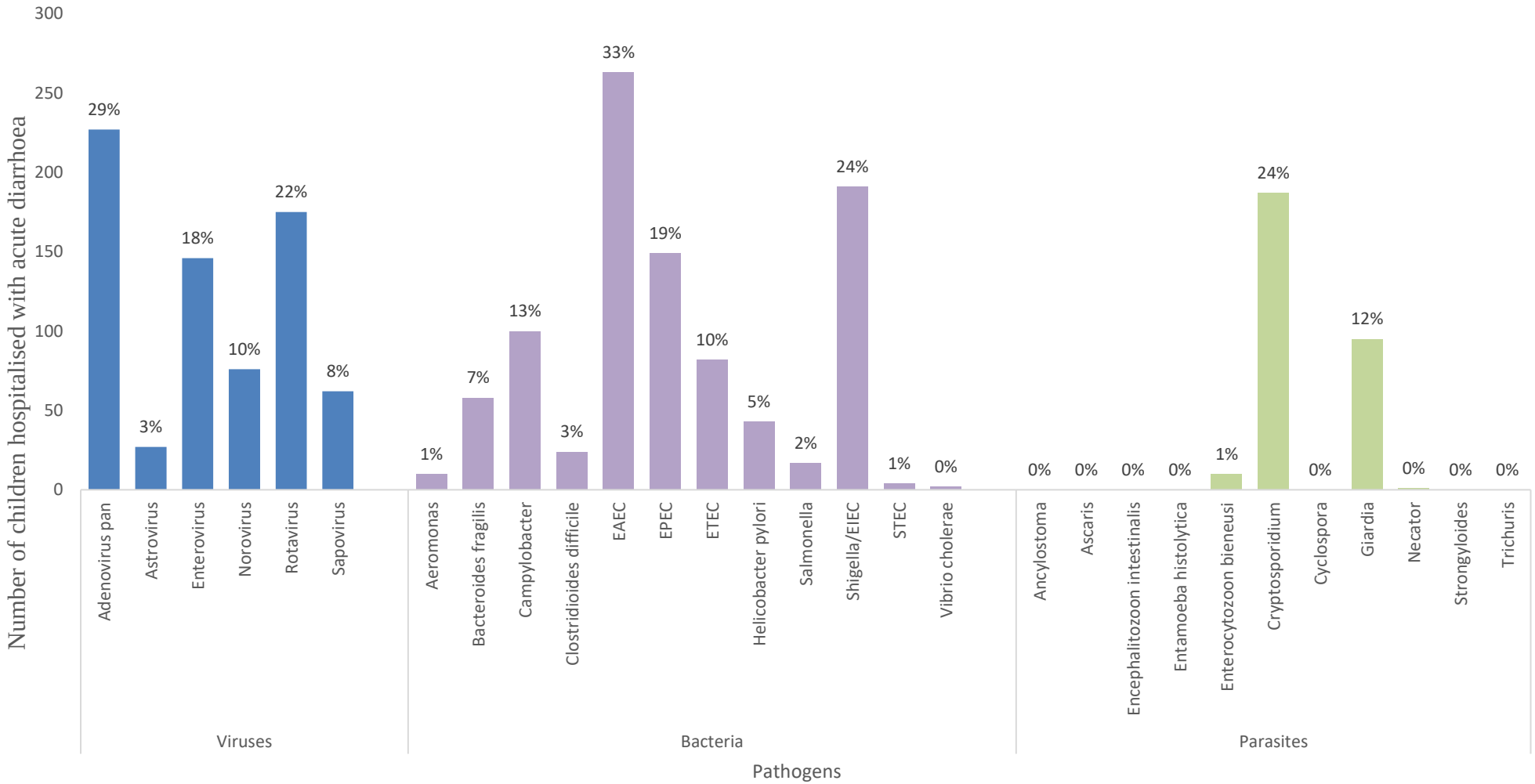


Figure 3.1 The prevalence of enteric pathogens (Ct≤35) detected in stool samples of children ≤5 years from 2015 to 2016. Norovirus (Norovirus GI (n=15; 2%) and Norovirus GII (n=62; 8%). *Campylobacter* (*Campylobacter* pan (n=97; 12%) and C. *jejuni/coli* n=58; 7%).

Figure 3.2 shows the prevalence of enteric pathogens stratified by age. In children less than 6 months old (n=195; 25%), the most prominent enteric pathogens were EAEC (n=60; 30,8%), adenovirus (n=45; 23,0), EPEC (n=35, 18,0%), *Cryptosporidium* (n=31; 15,9%), rotavirus (n=28; 14,4%), and enterovirus (n=28; 14,4%). *Shigella*/EIEC (n=21; 10,8%), norovirus (n=20; 10,3%) and *Campylobacter* (n=18; 9,2%) were less prevalent in children under 6 months. In those aged 6-11 months, EAEC (n=87; 36,1%), rotavirus (n=85; 35,3%), adenovirus (n=70; 29,0%), *Cryptosporidium* (n=64; 26,6%), enterovirus (n=49; 20%) and EPEC (n=43; 17,8%) had the highest prevalence. Other enteric pathogens detected in this age group included, *Campylobacter* (n=34; 14,1%), *Shigella/*EIEC (n=26; 10,8%), and norovirus (n=26; 10,8%).

EAEC (n=84; 36,8%), adenovirus (n=81; 35,5%), and *Cryptosporidium* (n=68, 29,8%) remained leading diarrhoeal pathogens detected in children 12-23 months of age (n=228; 29%). However, the prevalence of both *Shigella*/EIEC (n=75; 32,9%) and *Giardia* (n=43; 18,9%) increased in this age group. Enterovirus (n=46; 20%), rotavirus (n=21; 19,3%), EPEC (n=41; 18,0%), *Campylobacter* (n=35; 15,4%), ETEC (n=29; 12,7%), and sapovirus (n=24; 10,5%) were detected in ≥10% of stool samples in children in the second year of life. In children 24-59 (n=129; 16%), *Shigella*/EIEC (n=69; 53,5%) had the highest prevalence, detected in over half of the children. EAEC, (n=32; 24,8%), adenovirus (n=31; 24%), EPEC (n=30; 23,3%), *Giardia* (n=29; 22,5%), *Cryptosporidium* (n=24; 18,6%), enterovirus (n=23; 18%), rotavirus (n=18; 14,0%), ETEC (n=16; 12,4%), B. *fragilis* (n=15; 11,6%), *Campylobacter* (n=13; 10,1%), were also detected in this age group.

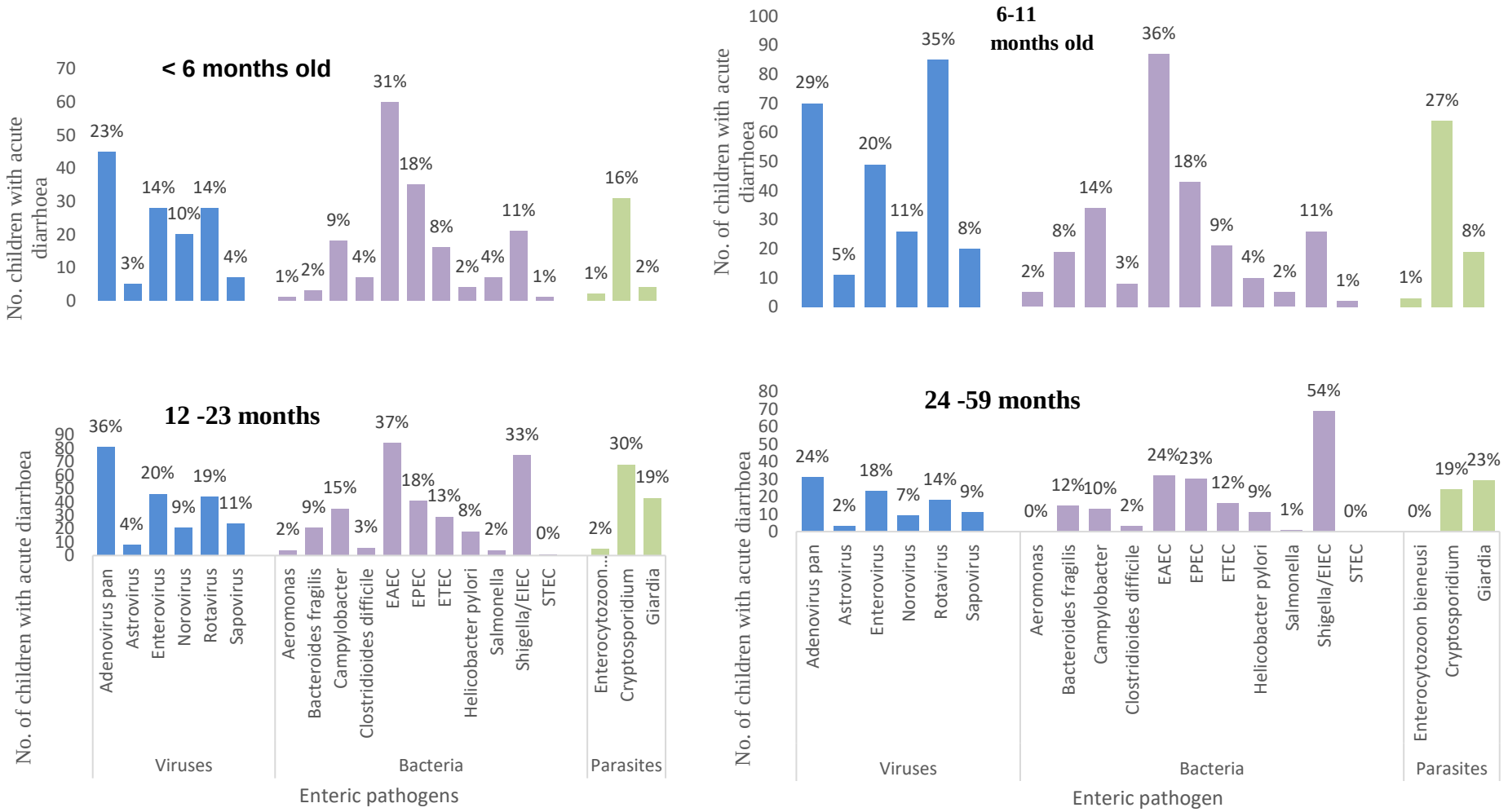
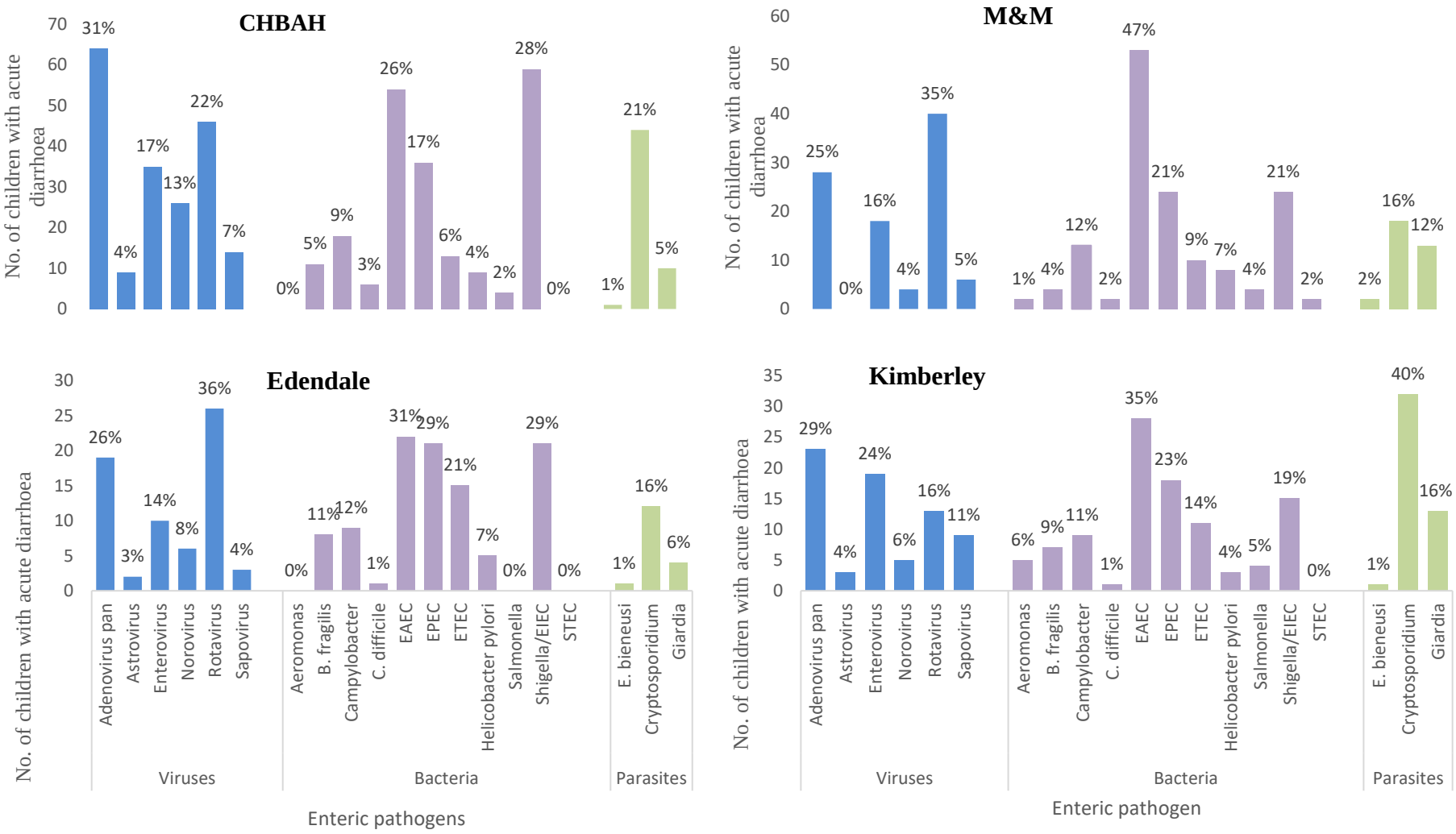


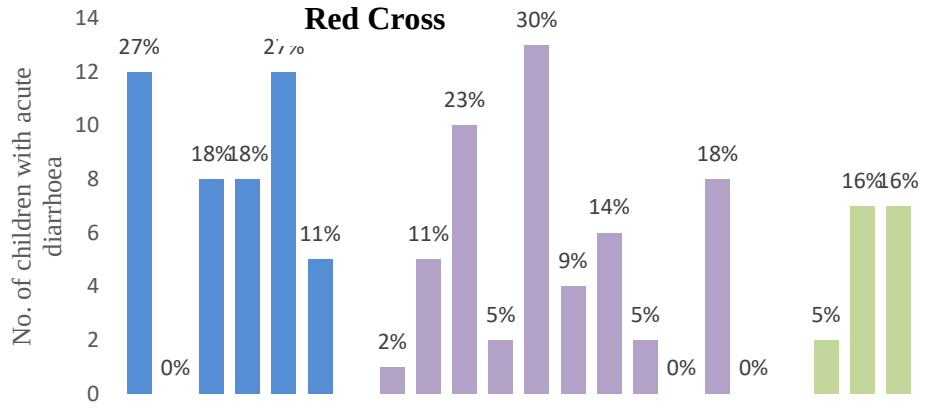
Figure 3.2 Enteric pathogens stratified by age group in children ≤5 years from 2015 to 2016 : >6 months old, 6 to less than 12 months, 12 to less than 24 months, 24 to less than 60 months old

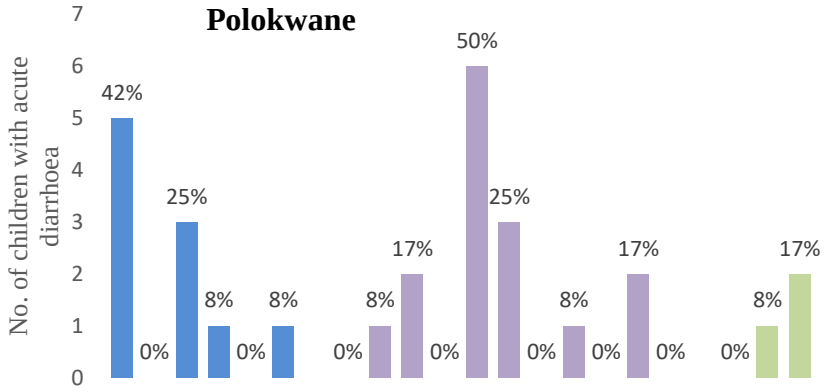
Figure 3.3 shows the prevalence of enteric pathogens across diarrhoea surveillance sites in South Africa. EAEC was frequently detected in participants from all surveillance sites including Polokwane (n=6; 50,0%), M/M (n=53; 46,9%), Pelonomi (n=66; 36,7%) Kimberley (n=28; 35%), Edendale (n=22; 30,1%), RCCH (n=13; 29,6%), Klerksdorp (n=10; 29,4%), CHBAH (n=54; 26,0%), and Dora Nginza (n=11; 22,5%). Adenovirus was also a prominent diarrhoea pathogen in all surveillance sites and was most frequently detected in Polokwane (n=5; 41,2%), CHBAH (n=64; 30,8%), Dora Nginza (n=15; 30,6%), Kimberley (n=23; 28,8%), Edendale (n=19; 26,0%), and RCCH (n=12; 27,3%).
Rotavirus was one of the leading enteropathogens in participants from Edendale (n=26; 35,6%), M/M (n=40; 35,4%), and RCCH (n=12; 27,3%). However, rotavirus was among the least frequently detected pathogens in Klerksdorp (n=3; 8,8%) Pelonomi (n=25; 13,9%), and Kimberley (n=13; 16,3%), and was not detected at all in participants from Polokwane (n=0; 0%).

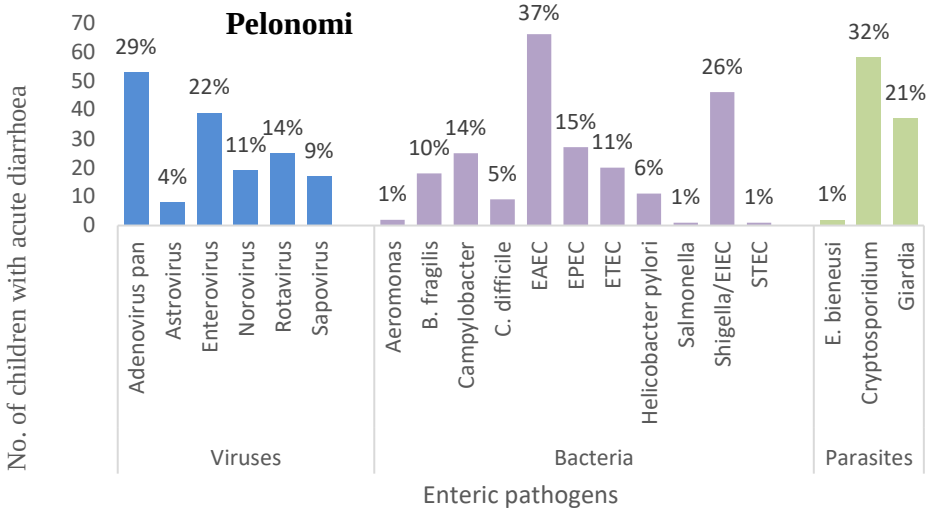
Most Shi*gella*/EIEC detections were in Edendale (n=21; 28,8%) followed by Klerksdorp (n=9; 26,5%), Pelonomi (n=46; 25,6%), CHBAH (n=24; 21,2%), M/M (n=24; 21,2%), Kimberley (n=15; 18,8%), and RCCH (n=8; 18,2%). EPEC was also frequently detected in Edendale (n=21; 28,8%), Dora Nginza (n=14; 28,6%), Kimberley (n=18; 22,5%), and M/M (n=24; 21,2%). Surveillance Hospitals that did not have any prominent EPEC detections were RCCH (n=4; 9,1%) and Klerksdorp (n=2; 5,9%). *Cryptosporidium* was a dominant pathogen in participants from Kimberley (n=32; 40,0%), Pelonomi (n=58; 32,2%), CHBAH (n=44; 21,2%). Polokwane (n=1; 8,3%) had the lowest *Cryptosporidium* detections. Enterovirus was most frequently detected only in Kimberley (n=19; 23,8%), CHBAH (n=35; 16,8%), and M/M (n=18; 15,9%).

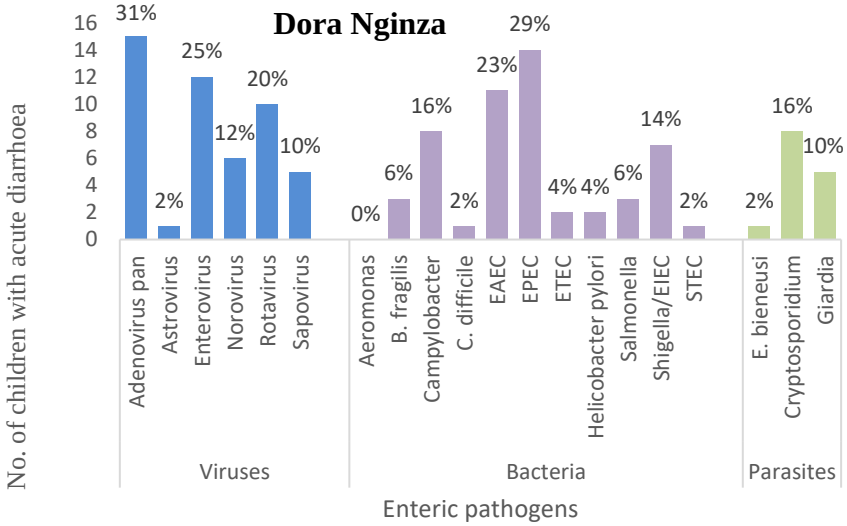
Norovirus, astrovirus, sapovirus, B. *fragilis*, *Campylobacter*, *Salmonella*, and *Giardia*, although detected in some surveillance sites, was often in low frequencies. STEC was detected in less than 1% of the participants from CHBAH, Edendale, Kimberley, RCCH, Polokwane, and Klerksdorp.


CHBAH
No. of children with acute diarrhoea
70
60
50
40
30
20
10
0
31%
4%
17%
13%
22%
7%
0%
5%
9%
3%
26%
17%
6%
4%
2%
28%
0%
1%
21%
5%
M&M
No. of children with acute diarrhoea
60
50
40
30
20
10
0
25%
0%
16%
4%
35%
5%
1%
4%
12%
2%
47%
21%
9%
7%
4%
21%
2%
2%
16%
12%
Edendale
No. of children with acute diarrhoea
30
25
20
15
10
5
0
26%
3%
14%
8%
36%
4%
0%
11%
12%
1%
31%
29%
21%
7%
0%
29%
0%
1%
16%
6%
Adenovirus pan
Astrovirus
Enterovirus
Norovirus
Rotavirus
Sapovirus
Aeromonas
B. fragilis
Campylobacter
C. difficile
EAEC
EPEC
ETEC
Helicobacter pylori
Salmonella
Shigella/EIEC
STEC
E. bieneusi
Cryptosporidium
Giardia
Viruses
Bacteria
Parasites
Enteric pathogens
Kimberley
No. of children with acute diarrhoea
35
30
25
20
15
10
5
0
29%
4%
24%
6%
16%
11%
6%
9%
11%
1%
35%
23%
14%
4%
5%
19%
0%
1%
40%
16%
Adenovirus pan
Astrovirus
Enterovirus
Norovirus
Rotavirus
Sapovirus
Aeromonas
B. fragilis
Campylobacter
C. difficile
EAEC
EPEC
ETEC
Helicobacter pylori
Salmonella
Shigella/EIEC
STEC
E. bieneusi
Cryptosporidium
Giardia
Viruses
Bacteria
Parasites
Enteric pathogen


Red Cross
No. of children with acute diarrhoea
0
2
4
6
8
10
12
14
27%
0%
18%
18%
27%
11%
2%
11%
23%
5%
30%
9%
14%
5%
0%
18%
0%
5%
16%
16%


Polokwane
No. of children with acute diarrhoea
0
1
2
3
4
5
6
7
42%
0%
25%
8%
0%
8%
0%
8%
17%
0%
50%
25%
0%
8%
0%
17%
0%
0%
8%
17%


Pelonomi
No. of children with acute diarrhoea
0
10
20
30
40
50
60
70
29%
4%
22%
11%
14%
9%
1%
10%
14%
5%
37%
15%
11%
6%
1%
26%
1%
1%
32%
21%
Adenovirus pan
Astrovirus
Enterovirus
Norovirus
Rotavirus
Sapovirus
Aeromonas
B. fragilis
Campylobacter
C. difficile
EAEC
EPEC
ETEC
Helicobacter pylori
Salmonella
Shigella/EIEC
STEC
E. bieneusi
Cryptosporidium
Giardia
Viruses
Bacteria
Parasites
Enteric pathogens


Dora Nginza
No. of children with acute diarrhoea
0
2
4
6
8
10
12
14
16
31%
2%
25%
12%
20%
10%
0%
6%
16%
2%
23%
29%
4%
4%
6%
14%
2%
2%
16%
10%
Adenovirus pan
Astrovirus
Enterovirus
Norovirus
Rotavirus
Sapovirus
Aeromonas
B. fragilis
Campylobacter
C. difficile
EAEC
EPEC
ETEC
Helicobacter pylori
Salmonella
Shigella/EIEC
STEC
E. bieneusi
Cryptosporidium
Giardia
Viruses
Bacteria
Parasites
Enteric pathogens

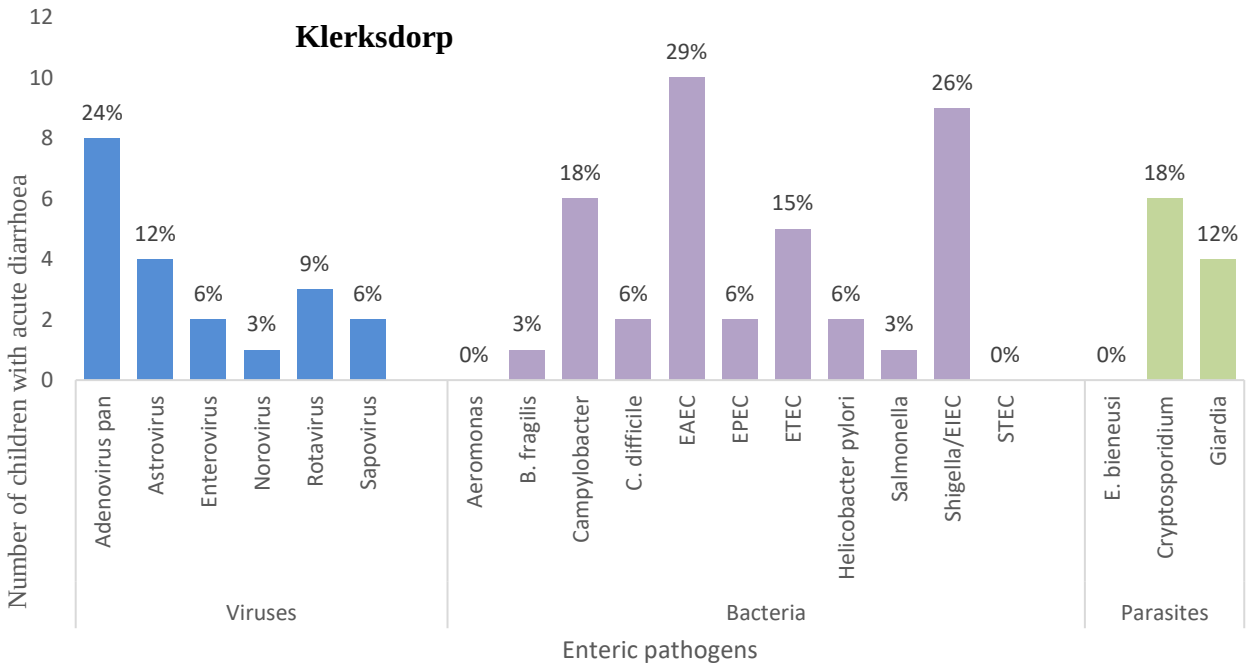


Figure 3.3 is a depiction of the prevalence of enteric pathogens across diarrhoea surveillance sites in South Africa from 2015 to 2016. Enteric pathogen prevalence at each surveillance site was calculated using the frequency at each site divided by the number of samples from that particular surveillance site.

Figure 3.4 shows the prevalence of enteric pathogens stratified by season. The most prominent enteric pathogens in the summer of 2015-2016 were EAEC (n=74; 36,1%), adenovirus (n=71; 34,6%), *Cryptosporidium* (n=58; 28,3%), *Shigella*/EIEC (n=50; 24,4%), enterovirus (n=39; 19,0%), EPEC (n=38; 18,5%), *Campylobacter* (n=26; 12,7%), *Giardia* (n=24; 11,7%), ETEC (n=23; 11,2%), and norovirus (n=22; 10,7%).

In autumn, EAEC (n=89; 38,5%), *Cryptosporidium* (n=86; 37,2%), *Shigella*/EIEC (n=71; 30,7%), EPEC (n=66; 28,6%), enterovirus (n=65; 28,1%), ETEC (n=39; 16,9%), and *Campylobacter* (n=32; 13,9%), showed an increase in prevalence compared to the warmer summer months.

Rotavirus (n=112; 55%) was detected in over half of the samples collected during the winter periods of 2015 and 2016, while it was only detected in approximately 5% of the samples in summer and 3% in autumn. The prevalence of EAEC (n=57; 27,8%), adenovirus (n=42; 20,5%), *Shigella*/EIEC (n=35; 17,1%), *Cryptosporidium* (n=30; 14,6%), EPEC (n=25; 12,2%), and *Campylobacter* (n=22; 10,7%) decreased in winter.

There was a decrease in the prevalence of rotavirus diarrhoea in spring (n=45; 20%) compared to winter (n=112; 55%). However, pathogens such as EAEC (n=43; 28,3%), adenovirus (n=39; 25,7%), *Shigella*/EIEC (n=35; 23,0%), *Campylobacter* (n=20; 13,2%), and EPEC (n=20; 13,2% showed a slight increase compared to winter.

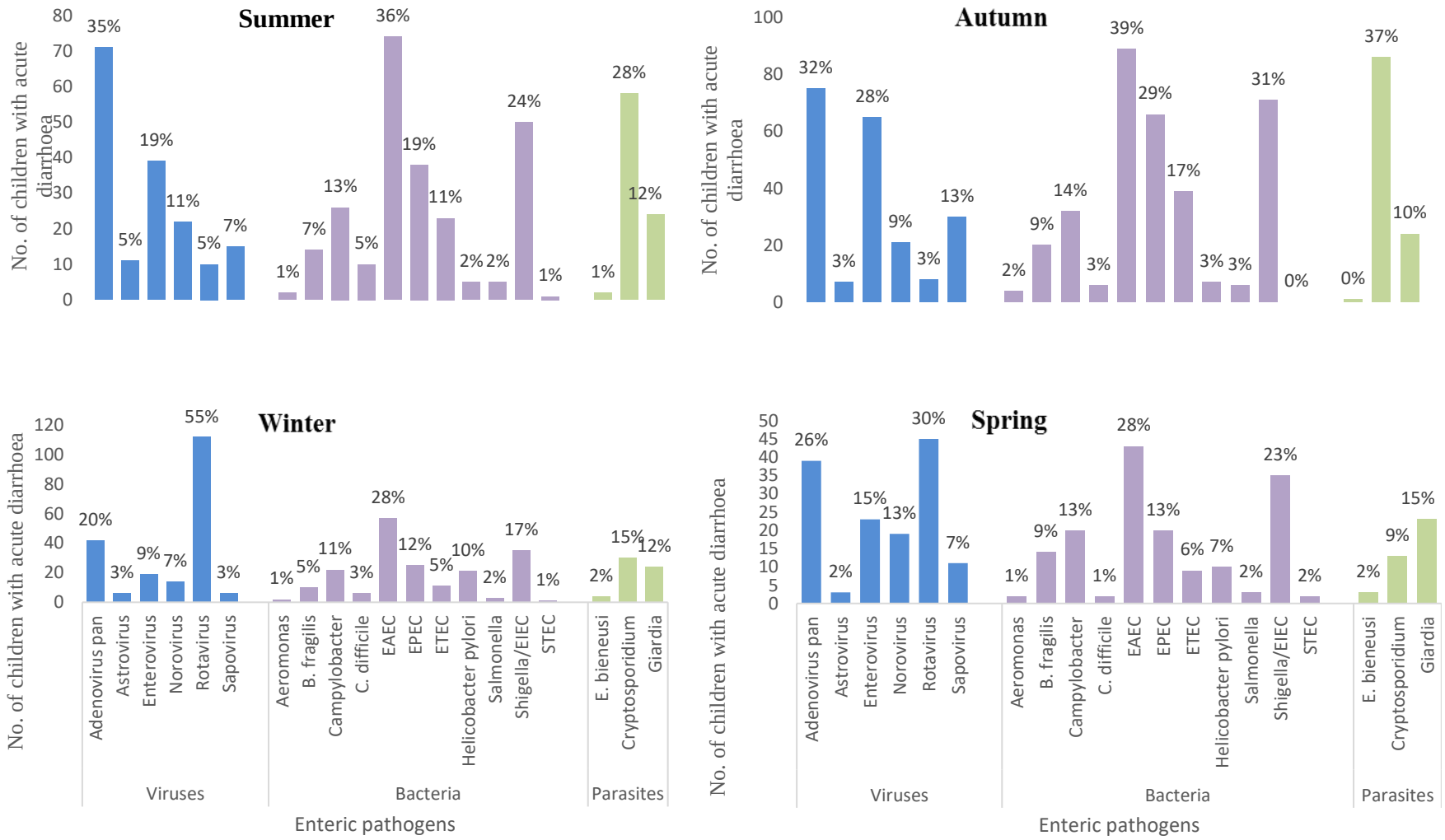


Figure 3.4a The prevalence of diarrhoeal pathogens during different South African seasons, summer: December, January, February; Autumn: March, April, May; Winter: June, July, August; Spring: September, October, November.

Figure 3.4b shows the prevalence of the pathogens in wet and dry South African seasons. The most common pathogens during the wet seasons of 2015 and 2016 included adenovirus (46.3%), EAEC (36.8%), *Cryptosporidium* (27.2%), *Shigella*/EIEC (26.0%), enterovirus (24.6%), and EPEC (22.2%). Other prominent pathogens in the wet seasons were *Giardia* (12.7%), *Campylobacter* (12.4%), ETEC (12.2%), norovirus (11.5%), sapovirus (10.0%). Rotavirus (6.7%), B. *fragilis* (7.2%), astrovirus (4.1%), C. *difficile* (3.8%), H. *pylori* (3.8%), *Salmonella* (2.6%), *Aeromonas* (2.0%), STEC (1.0%), and E. *bieneusi* (1.0%) had very low prevalence in the wet seasons of 2015 and 2016.

In the dry season adenovirus (41.8%) and rotavirus (39.3%) had the highest prevalence among children with diarrhoea, followed by EAEC (29.1%), *Shigella*/EIEC (21.9%), and *Cryptosporidium* (19.5%). EPEC (15.0%), enterovirus (11.5%), *Campylobacter* (12.8%), Giardia (11.2%) were also prominent in the dry seasons. Norovirus (7.5%), B. *fragilis* (7.5%), ETEC (8.3%), H. *pylori* (7.2%), sapovirus (5.4%) were not common in the dry seasons. Other pathogens that had a low prevalence during this time included astrovirus (2.7%), C. *difficile* (2.1%), *Salmonella* (1.6%), E. *bieneusi* (1.6%), *Aeromonas* (1.0%), and STEC (1.0%).

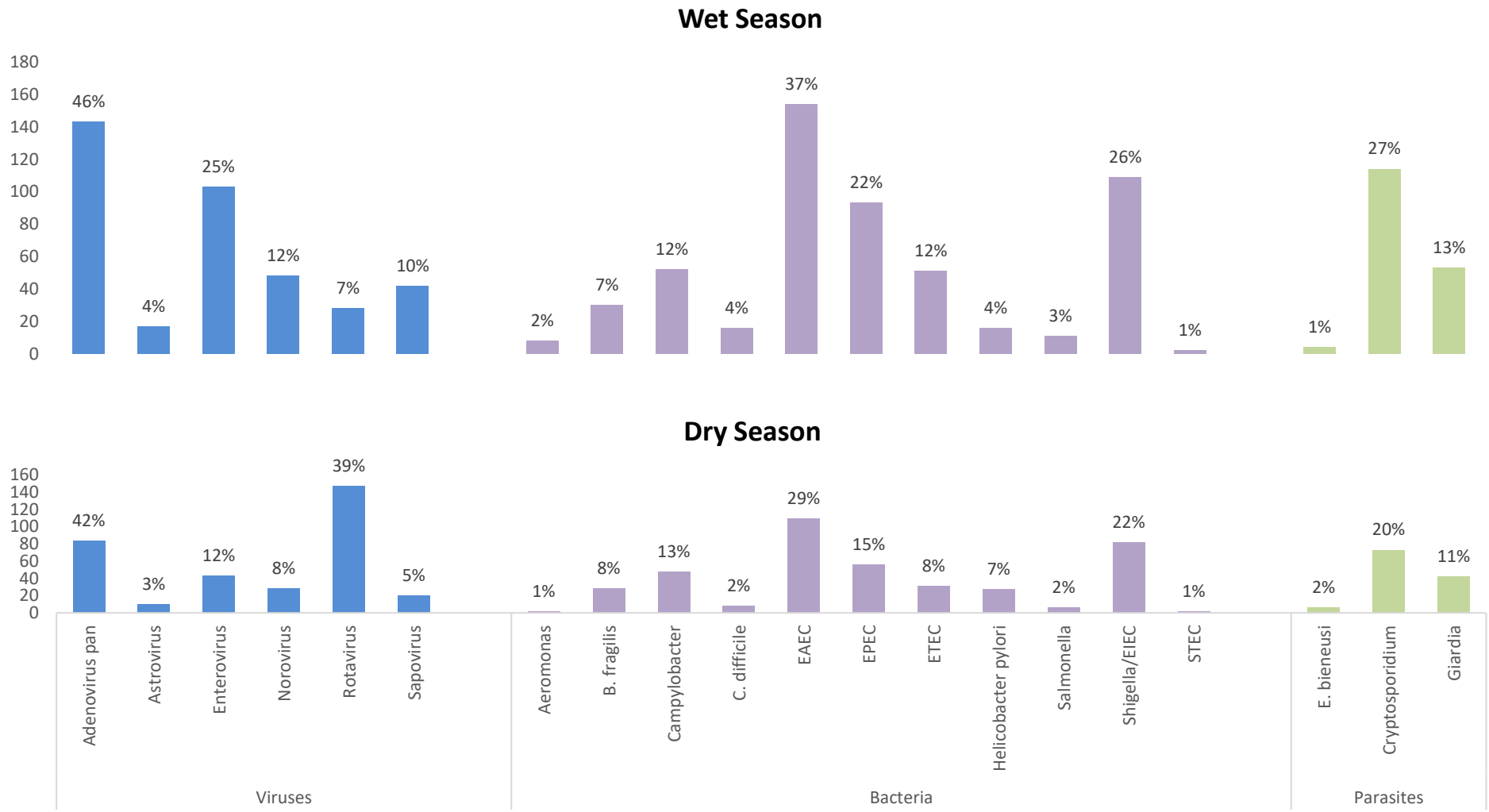


Figure 3.4b The prevalence of diarrhoeal pathogens during South African wet and dry seasons, wet season: October, November, December, January, February, March; dry season: April, May, June, July, August, September.

Among rotavirus-positive samples (ct≤35, n=175), we found that strain P[8] (n=79; 45,1%) and G3 (n=75; 42,9%) were the most prominent strains. Strains that were detected in less than 1% of the participants were G4, G10, P[9], P[10], and P[11]. Genotype G3P[8] (n=32; 18,3%), G9P[8] (n=26; 14,9%), and G3P[4] (n=23; 13,4%) had the highest detection frequencies. Other rotavirus genotypes that were detected in 5% and above of the rotavirus positive participants included G3P[4]P[8] (n=15; 8,6%), G2P[4] (n=13; 7,4%), and G2P[4]P[8] (n=10; 5,7%) (Figure 3.5a and 3.5b).

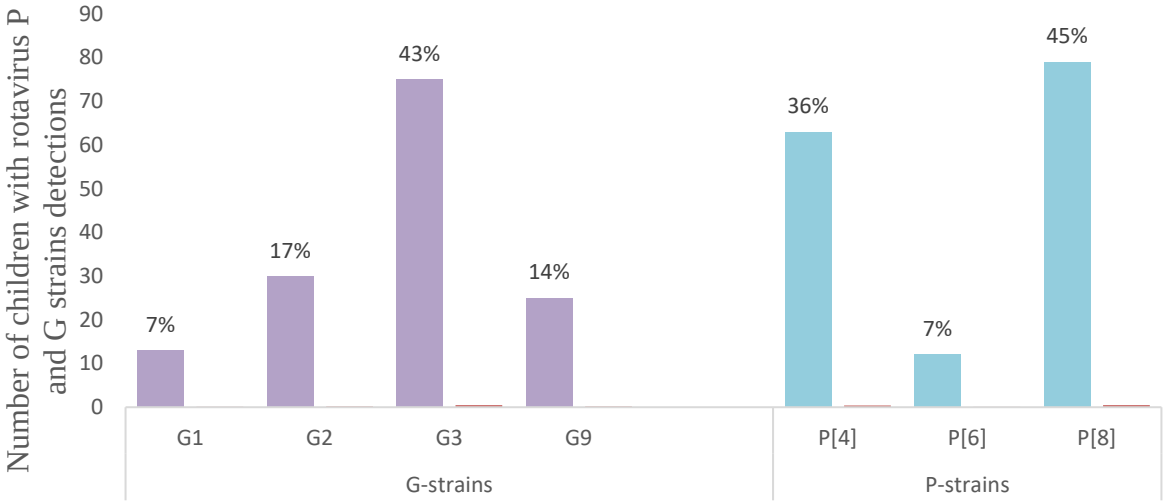


Figure 3.5a Detected rotavirus G and P strains amongst participants who had positive stool results for rotavirus. Only those strains that had a prevalence >5% were included in the figure.

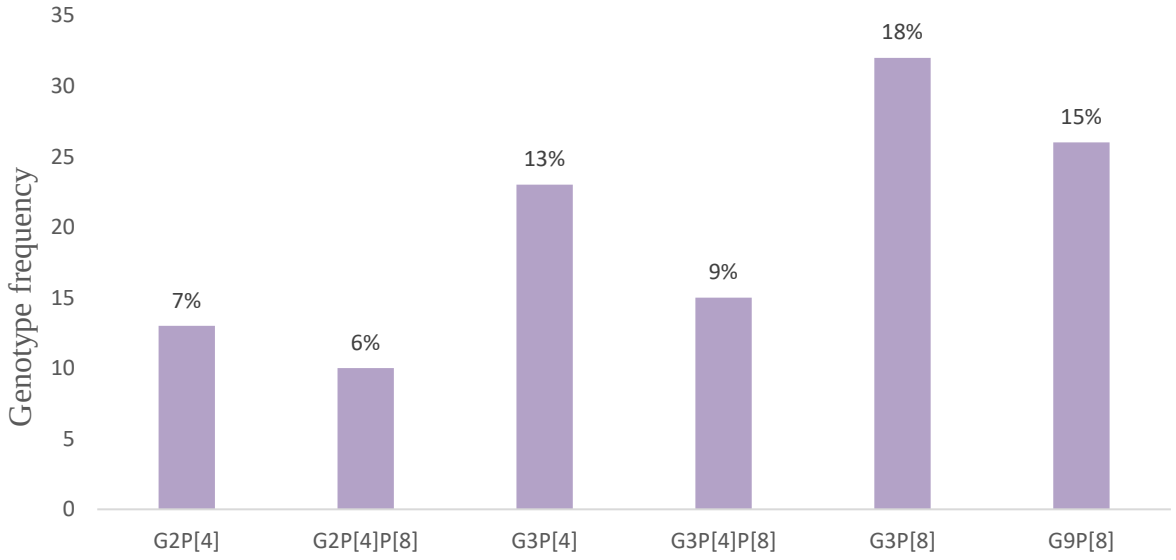


Figure 3.5b shows the prevalence of rotavirus P genotypes amongst patients whose stools rotavirus was detected using the Taqman array card. Only those genotypes that had a prevalence >5% were included in the figure.

Table 3.1 shows the differences in the prevalence of enteric pathogens detected amongst children under the age of five years using ≤35 Ct cut-off and pathogen-specific Ct cut-offs, for selected pathogens. There was a significant decrease in the prevalence of all pathogen proportions with the exception of two: rotavirus (p=0.4967) and *Salmonella* (0.2178) when using the specific Ct cut-offs. No C. *jejuni/coli* was detected when using the specific cut-off of 15,4.

Table 3.1 Comparison of selected enteric pathogen prevalence using different PCR cycle threshold cut-offs[1]

Pathogen	Using 35 Ct cut-off n/793 (%)	Using specific Ct cut-offs n/793 (%)	P-value*
Viruses			
Adenovirus	227 (28,6)	55 (6,9)	<0.0001
Astrovirus	27 (3,4)	6 (0,8)	0.0003
Norovirus GII	62 (7,8)	11 (1,4)	<0.0001
Rotavirus	175 (22,1)	164 (20,7)	0.4967
Sapovirus	62 (7,8)	19 (2,4)	<0.0001
Bacteria			
C. *jejuni/coli*	58 (7,3)	0	-
H. *pylori*	43 (5,4)	16 (2,0)	0.0003
tEPEC	49 (6,2)	2 (0,3)	<0.0001
ST-ETEC	50 (6,3)	19 (2,4)	0.0001
Shigella/EIEC	191 (24,1)	122 (15,4)	<0.0001
Parasites			
Cryptosporidium	186 (23,5)	103 (13,0)	<0.0001
Salmonella	17 (2,1)	10 (1,3)	0.2178

*Proportions test was used to get the p-value

Figure 3.6 below shows pathogen-associated diarrhoeal cases using specific cut-offs for the selected enteric pathogens. Rotavirus had the highest diarrhoea-associated prevalence (n=164/175; 93,7%) followed by *Shigella*/EIEC (n=122/191; 63,9%), *Salmonella* (n=10/17; 58,8%), and *Cryptosporidium* (n=103/186; 55,3%). Lower diarrhoea-associated prevalence

[1] Specific Ct cut-off values. GEMS: Adenovirus 40/41(22.7), astrovirus (22.2), norovirus GII (23.4), rotavirus (32.6), sapovirus (N/A), *Cryptosporidium* (24.0), C. *jejuni/coli (15.4),* Helicobacter *pylori (30.8),* typical EPEC (bfpa) (16.0), ST-ETEC (STh) (22.8), *Salmonella (30.7), Shigella*/EIEC (27.9).

MAL-ED: Adenovirus 40/41(24.1), astrovirus (23.7), norovirus GII (27.2), rotavirus (31.7), sapovirus (26.1), *Cryptosporidium* (22.0), C. *jejuni/coli (21.8),* Helicobacter *pylori (N/A),* typical EPEC (bfpa) (17.8), ST-ETEC (STh) (23.5), *Salmonella (N/A), Shigella*/EIEC (28.8).

was shown for ST-ETEC (n=19/50; 38,0%), H. *pylori* (n=16/43; 37,2%), sapovirus (n=19/62; 30,6%), adenovirus (n=55/227; 24,2%), astrovirus (n=6/27; 22,2%), norovirus GII (n=11/64; 17,2%), and tEPEC (n=2/49; 4,1%).

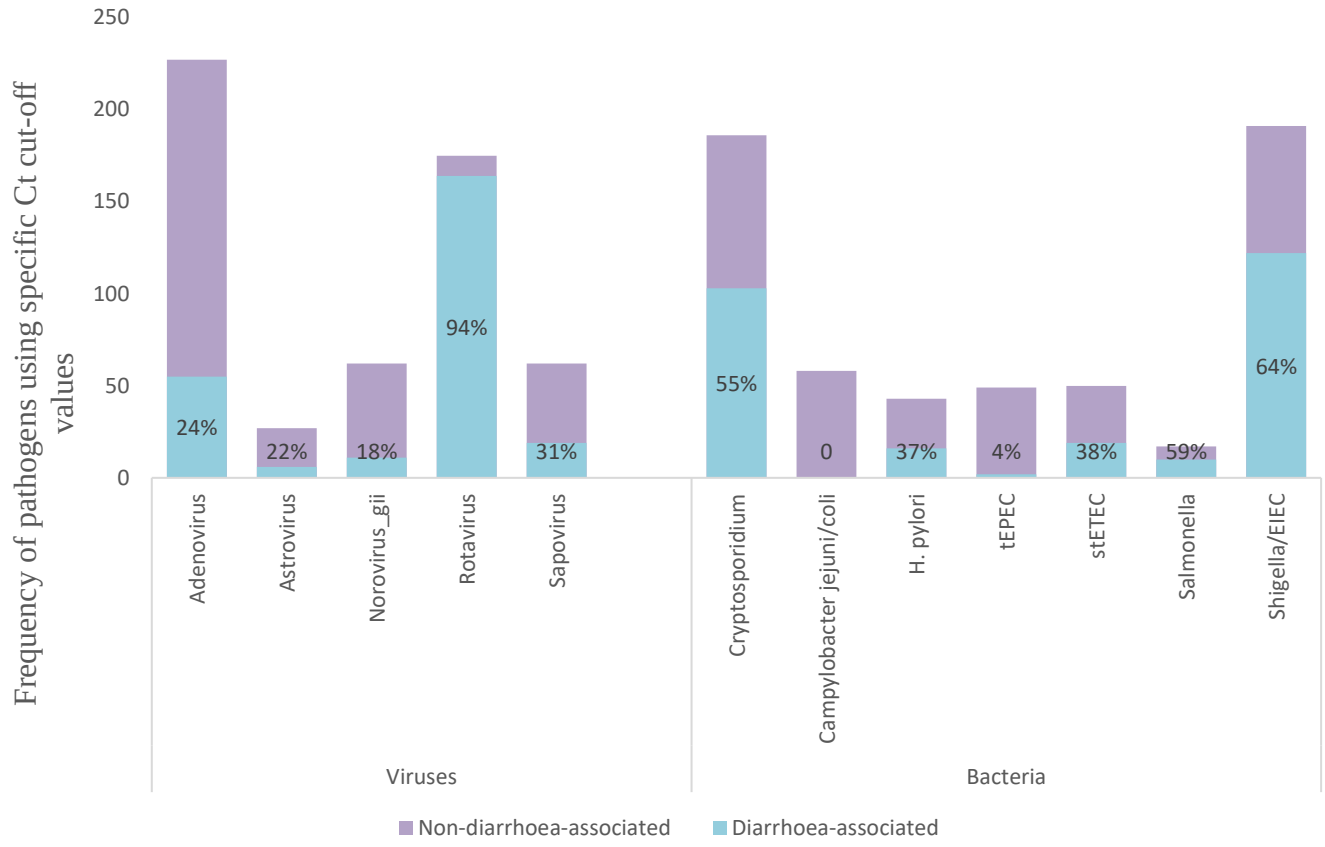


Figure 3.6 Detection of enteric pathogens using diarrhoea associated cut-offs for selected pathogens. The figure shows the percentage of pathogen-associated diarrhoea compared to non-associated diarrhoea.

3.2 Objective 2- Enteric co-infections among children <5 years of age hospitalised with acute diarrhoea

Figure 3.7 below shows the comparison between enteric pathogen mono-infections/ no co-infection (detection of only one pathogen) and co-infections (detection of more than one enteric pathogen) using pathogen-specific Ct cut-off values. Enteric pathogens that had more co-infections than single infections included H. pylori (n=13/16; 81,3%) and tEPEC (n=2/2; 100%). Most enteric pathogen infection using specific Ct cut-offs were single infections including rotavirus (n=147/164; 89,6%), *Cryptosporidium* (n=81/103; 78,6%), *Shigella*/EIEC (n=85/122; 69,7%), adenovirus (n=37/55; 67,3%), astrovirus (n=6/6; 100%), norovirus GII

(n=9/11; 81,8%), sapovirus (n=10/19; 52,6%), ST-ETEC (n=10/19; 52,6%), and *Salmonella* (n=6/10; 60,0%).

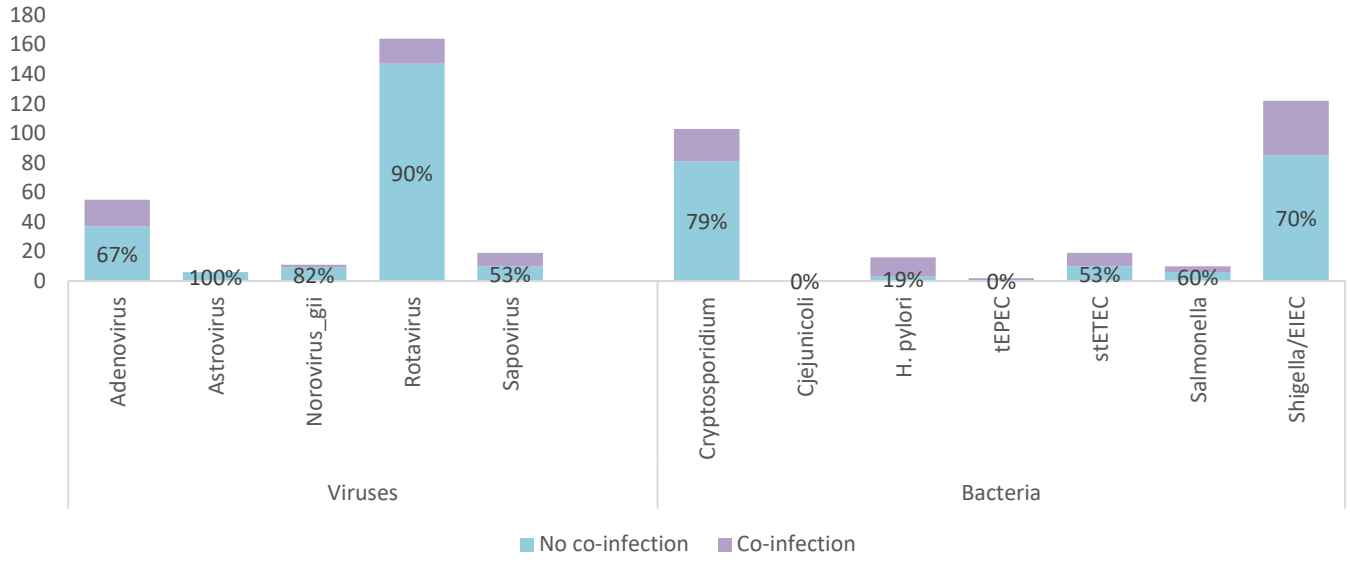


Figure 3.7 Co-infection and mono infections for enteric pathogens using specific PCR cycle thresholds

Table 3.2 shows the prevalence of rotavirus detections with other enteric pathogens found among South African children <5 years old. The most common rotavirus co-infections were H. pylori (n=8/164; 4,9%), *Shigella*/EIEC (n=6/164; 3,7%), and *Salmonella* (n=3/164; 1,8%). There were no observed co-detections of rotavirus and pathogens such as astrovirus, sapovirus, tEPEC, and ST-ETEC.

Table 3.2 Enteric pathogens co-detected in rotavirus-positive stool samples

Pathogens	**Rotavirus-positive (N=164)**
Rotavirus-only	147 (90)
Rotavirus co-detections	17 (10)
Viruses	
Adenovirus	2 (1, 2%)
Astrovirus	0 (0%)
Sapovirus	0 (0%)

Bacteria	
Cryptosporidium	1 (0.6%)
C. *jejuni/coli*	-
H. *pylori*	8 (4, 9%)
tEPEC	0 (0%)
ST-ETEC	0 (0%)
Salmonella	3 (1, 8%)
Shigella/EIEC	6 (3, 7%)

Table 3.3 below shows the prevalence of 11 factors including clinical symptoms, medical history conditions and outcome of infection for each enteric pathogen (defined as infection with a single pathogen using the specific Ct cut-off value). Vomiting was the common presenting symptom for most pathogens except *Salmonella* (n=2; 33%) and *Shigella*/EIEC (n=37; 44%). Bloody stool was found in just over a third of *Shigella*/EIEC infections (n=26; 30,6, %), but was not a common presenting symptom for other pathogens. Most patients who had sapovirus (n=5; 50,0%), H. *pylori* (n=3; 100%), and ST-ETEC (n=6; 60,0%), presented with dehydration. The overall mortality was low (n=11/394; 2,8%).

Table 3.3 Symptoms observed in children with a single enteric pathogen detected in stool (stool detection of only one enteric pathogen using specific quantification cycle cut-offs)

Symptom/ Outcome	**Adenovirus n/37 (%)**	**Astrovirus n/6 (%)**	**Norovirus GII n/9 (%)**	**Rotavirus n/147 (%)**	**Sapovirus n/10 (%)**	***Cryptosporidium* n/81 (%)**	**H. *pylori* n/3 (%)**	**ST-ETEC n/10 (%)**	***Salmonella* n/6 (%)**	***Shigella*/EIEC n/85 (%)**
Fever	19 (51,4)	1 (16,7)	4 (44,4)	68 (46,3)	3 (30,0)	41 (50,6)	3(100,0)	1 (10,0)	4 (66,7)	55 (64,7)
Refusal to feed	19 (51,4)	2 (33,3)	5 (55,6)	60 (40,8)	50(50,0)	35 (43,2)	1 (33,3)	5 (50,0)	1 (16,7)	38 (44,7)
Vomiting	24 (64,9)	4 (66,7)	7 (77,8)	106 (72,1)	6 (60,0)	56 (69,1)	2 (66,7)	7 (70,0)	2 (33,3)	37 (43,5)
Bloody stool	2 (5,4)	-	-	3 (2,0)	1 (10,0)	2 (2,5)	1 (33,3)	-	1 (16,7)	26 (30,6)
Dehydration	24 (64,9)	5 (83,3)	4 (44,4)	93 (63,3)	6 (60,0)	61 (75,3)	3 (100,0)	9 (90,0)	4 (66,7)	51 (60,0)
Previous antibiotics	2 (5,4)	-	3 (33,3)	24 (16,3)	1 (10,0)	18 (22,2)	-	1 (10,0)	-	12 (14,1)
Previous admission	6 (16,2)	1 (16,7)	3 (33,3)	19 (12,9)	1 (10,0)	12 (14,8)	-	3 (30,0)	-	15 (17,6)
Previous diarrhoea	1 (2,7)	1 (16,7)	1 (11,1)	4 (2,7)	-	-	-	-	-	1 (1,2)
Chronic disease	2 (5,4)	1 (16,7)	1 (11,1)	8 (5,4)	1 (10,0)	5 (6,2)	-	1 (10,0)	-	4 (4,7)
Malnutrition	3 (8,1)	-	1 (11,1)	12 (8,2)	1 (10,0)	14 (17,3)	-	3 (30,0)	1 (16,7)	4 (4,7)
Died	1 (2,7)	-	-	3 (2,0)	1 (10,0)	4 (4,9)	-	-	1 (16,7)	1 (1,2)

3.3 Objective 3- Risk factors associated with rotavirus-positivity on stool testing among children <5 years of age who received rotavirus vaccination

Of 793 participants, 655 (82,6%) had the road to health card (RTHC) available which contains information about the vaccine status of each participant (Appendices D-G). Of these, 631 (96,3%) had received at least one dose of the rotavirus vaccine and were included in the analysis. None of the 12 children from Polokwane hospital was vaccinated and this site was thus excluded from the analysis. Forty-seven percent (n=297/618) were female and 50,9% (n=321/618) were male, 13 participants had missing values for sex. Age distribution of these children was: 144 were <6 months (22,8%); 201 were 6-11 months (31,9%); 183 were 12-23 months (29,0%); and 103 were 24-59 months (16,3%). One hundred and forty-three (22,7%) tested positive for rotavirus diarrhoea (Ct≤35), and could potentially be considered "vaccine failures".

The results from the bivariate logistic regression analysis showed that medical history factors such as receiving antibiotics prior to hospitalisation, pre-existing chronic illnesses, and previous diarrhoea were not significant risk factors for rotavirus diarrhoea (Table 4.4). Symptoms such as refusing to feed, fever, and malnutrition were also not significant risk factors for rotavirus diarrhoea. Other factors that were not significantly associated with rotavirus in the bivariate logistic regression analysis included the number of people sleeping in the same room, nursery attendance, education level of the mother, access to electricity, flushing toilet, running water and season of birth.

The odds of detecting rotavirus despite vaccination in children who were 6-11 months old were 3.10 (95% CI: 1.692 – 5.683) times greater than those who were 24-59 months old. The odds of rotavirus diarrhoea in children from M/M hospitals (OR= 2.32; 95% CI: 1.302 – 4.122) and Edendale Hospital (OR=2.13; CI: 1.013 – 4.491) were greater than those of children in CHBAH Hospital. Children who were fed "other" types of food (OR=0.28; 95% CI: 0.146 – 0.540); beyond 4 months of age were at less risk of infection than those who were only fed formula. The results also showed that children who were vomiting (OR=2.51; 95% CI: 1.569 – 4.022) had greater odds of rotavirus diarrhoea than children who did not vomit. Children admitted in winter (OR= 19.88; 95%CI: 9.769 – 40.432) and spring (OR=6.87; 95%CI: 3.264 – 14.443) were also more likely to have rotavirus diarrhoea than those admitted in summer.

Table 3.4 Risk factors associated with rotavirus diarrhoea amongst children who received at least one dose of the Rotarix® vaccine between 2015 and 2016 (unadjusted analysis)

Risk factor	**Rotavirus (N=143) n/N (%)**	**No rotavirus (N=488) n/N (%)**	Crude OR (95% CI)	**p-value**
Number of doses				
1 (n=47)	15 (10,5)	32 (6,6)	Reference	
2 (n=584)	124 (89,5)	456 (93,4)	0.60 (0.314 – 1.140)	0.119
Age				
Less than 6 months (n=144)	20 (14,0)	124 (25,4)	0.88 (0.430 – 1.788)	0.718
6 to 11 months (n=201)	73 (51,1)	128 (26,2)	3.10 (1.692 – 5.683)	<0.0001
12 to 23 months (n=183)	34 (23,8)	149 (30,5)	1.24 (0.647 – 2.378)	0.516
24 to <60 months (n=103)	16 (11,1)	87 (17,8)	Reference	-
Sex				
Female (n=297)	63/140 (45,0)	234/478 (49,0%)	0.85 (0.584 – 1.245)	0.410
Site				
CHBAH (n=150)	31 (21,7)	119 (24,4)	Reference	-
M/M (n=93)	35 (24,5)	58 (11,9)	2.32 (1.302 – 4.122)	0.004
Edendale (n=42)	15 (10,5)	27 (5,5)	2.13 (1.013 – 4.491)	0.046
RCCH (n=42)	12 (8,4)	30 (6,2)	1.54 0.706 – 3.341)	0.280
Kimberley (n=72)	13 (9,1)	59 (12,1)	0.85 (0.412 – 1.736)	0.648
Pelonomi (n=164)	25 (17,5)	139 (28,5)	0.69 (0.386 – 1.234)	0.211
Dora Nginza (n=37)	9 (6,3)	28 (5,7)	1.23 (0.528 – 2.883)	0.627
Klerksdorp (n=31)	3 (2,1)	28 (5,7)	0.41 (0.117 – 1.442)	0.165
Black (n=550)	127/141 (90,1)	423/483 (87,6)	1.29 (0.696 – 2.379)	0.421
Method of feeding (first 4 months)				
Formula (n=130)	36/141 (25,5)	94/469 (20,0)	Reference	-
Mixed (n=102)	27 (19,2)	75 (16,0)	0.94 (0.524 – 1.686)	0.835
Breast (n=378)	78 (55,3)	300 (64,0)	0.69 (0.429 – 1.073)	0.097
Method of feeding (beyond 4 months)				

Formula (n=264)	72/127 (56,7)	192/407 (47,2)	Reference	-
Mixed (n=33)	10 (7,9)	23 (5,7)	1.15 (0.526 – 2.555)	0.714
Breast (n=111)	33 (26,0)	78 (19,2)	1.13 (0.692 – 1.840)	0.629
Solids (with/without milk) (n=126)	12 (9,5)	114 (28,0)	0.28 (0.146 – 0.540)	<0.001
Symptoms and medical history				
Antibiotics prior to hospitalisation (n=105)	27/114 (23,7)	78/413 (18,9)	1.33 (0.811 – 2.191)	0.257
Previous admission (n=107)	23/142 (16,2)	84/478 (17,5)	0.91 (0.547 – 1.502)	0.703
Previous diarrhoea (n=20)	5/142 (3,5)	15/478 (3,1)	1.12 (0.402 – 3.155)	0.845
Chronic medical condition (n=38)	9/139 (6,5)	29/465 (6,2)	1.04 (0.480 – 2.255)	0.919
Refusal to feed (n=281)	66/140 (47,1)	215/481 (44,7)	1.10 (0.757 – 1.609)	0.609
Fever (n=353)	74/139 (53,2)	279/481 (58,0)	0.82 (0.564 – 1.204)	0.318
Vomit (n=430)	116/141 (82,3)	314/484 (64,8)	2.51 (1.569 – 4.022)	<0.001
Malnutrition (n=69)	15/143 (10,5)	54/483 (11,2)	0.93 (0.508 – 1.705)	0.817
Education level of the mother				
No schooling (n=21)	5/141 (3,6)	16/475 (3,4)	1.02 (0.369 – 2.856)	0.961
Primary school (n=51)	9 (6,4)	42 (8,8)	0.70 (0.333 – 1.485)	0.356
High school/Tertiary (n=544)	127 (90,1)	417 (87,8)	Reference	-
Socio-economic factors				
More than 5 roommates (n=32)	5 (3,5)	27 (5,5)	0.62 (0.234 – 1.637)	0.333
Nursery attendance	22 /142(15,5)	94/485 (19,3)	0.76 (0.459 – 1.266)	0.295
Electricity (n=571)	133 (93,0)	438 (89,8)	1.52 (0.749 – 3.076)	0.246
Flushing toilet (n=402)	88/142 (62,0)	314 (65,0)	0.88 (0.596 – 1.292)	0.507
Running water (n=267)	69/138 (50,0)	198/481 (41,2)	1.43 (0.978 – 2.090)	0.065
Brick house (n=484)	114/142 (80,3)	370/485 (76,3)	1.27 (0.796 – 2.011)	0.319
Season of admission				
Summer (n=169)	10/143 (7,0)	159/488 (32,5)	Reference	-
Autumn (n=174)	5 (3,5)	169 (34,6)	0.47 (0.157 – 1.406)	0.177
Winter (n=162)	90 (62,9)	72 (14,8)	19.88 (9.769 – 40.432)	<0.001
Spring (126)	38 (26,6)	88 (18,0)	6.87 (3.264 – 14.443)	<0.001
Season of birth				
Summer (n=167)	42/143 (29,4)	125/488 (25,5)	Reference	
Autumn (n=166)	33 (23,1)	133 (27,3)	0.73 (0.440 – 1.239)	0.251

Winter (n=131)	24 (16,8)	107 (21,9)	0.67 (0.379 – 1.173)	0.160
Spring (n=167)	44 (30,8)	123 (25,2)	1.06 (0.652 – 1.739)	0.802

Results from the final model showed that the odds of having rotavirus diarrhoea in children who were 6 -11 months old were 4.27 (95% CI: 2.484 – 10.143) times that of children who were 24 to 59 months old, after adjusting for the number of rotavirus doses, site and season of admission. Patients from RCCH (aOR=2.92; 95% CI: 1.139 – 7.511), were more likely to get rotavirus diarrhoea despite being vaccinated compared to patients from CHBAH after adjusting for other factors in the model. Patients from Klerksdorp Hospital (aOR=0.15; 95% CI: 0.040 – 0.611) were less likely to get an infection compared to patients from CHBAH. The odds of infection in children who were admitted in winter were 29.57 (95% CI: 13.212 – 66.164) greater than those of children admitted in summer, after adjusting for age, the site and the number of rotavirus doses. Those admitted in spring (aOR=7.05; 95% CI: 3.214 – 15.483) also had higher odds of infection than those admitted in summer.

Table 3.5 Model-based predictor factors of rotavirus diarrhoea amongst children who received at least one dose of the Rotarix® vaccine between 2015 and 2016.

	aOR (95%CI)	**p-value**
Age		
<6 months	0.79 (0.338 – 1.861)	0.595
6-11 months	4.27 (2.022 – 9.020)	<0.001
12-23 months	1.43 (0.658 – 3.095)	0.369
24-59 months	Reference	-
Site		
Chris Hani Baragwanath Hospital	Reference	-
Mapulaneng& Matikwana	1.98 (0.962 – 4.055)	0.064
Edendale Hospital	1.42 (0.498 – 4.049)	0.512
Red Cross Children's Hospital	2.92 (1.139 – 7.511)	0.026
Kimberley Hospital	1.68 (0.652 – 4.330)	0.283
Pelonomi Hospital	0.85 (0.415 – 1.749)	0.663
Dora Nginza Hospital	1.07 (0.358 – 3.207)	0.901
Klerksdorp Hospital	0.15 (0.040 – 0.611)	0.008
Season of admission		
Summer	Reference	-
Autumn	0.50 (0.163 – 1.551)	0.231
Winter	29.57 (13.212 – 66.164)	<0.001
Spring	7.05 (3.214 – 15.483)	<0.001
Number of doses		
1 dose	Reference	
2 doses	0.89 (0.380 – 2.125)	0.807

4. Discussion

This study described the aetiology of enteric pathogens amongst children hospitalised with diarrhoea under the age of five years old from nine South African diarrhoea surveillance sites after the introduction of rotavirus vaccination. The study also compared the prevalence of enteric pathogens found in these children using different Ct cut-offs and described co-infections using these pathogen-specific cut-offs. We also assessed risk factors for hospitalisation for rotavirus diarrhoea amongst children who had received at least one rotavirus vaccine dose.

PCR amplification is a novel diagnosis for pathogens using DNA or RNA (26). PCR amplification can be performed in either uniplex or multiplex mode using gel electrophoresis, probe hybridization, or real-time fluorescence (112). This novel method of diagnosis is usually preferred over conventional methods such as pathogen culturing, gram stain, and enzyme immunoassay because of how laborious and time-consuming these traditional methods can be (112). On the other hand, real-time PCR has proven to be more sensitive than most conventional methods and has decreased results turnaround time which usually took 2-3 days to run the tests and almost a week to get back results using traditional methods (26,117). PCR results are usually available within hours of running the test (118). Use of the Taqman Array Card, a quantitative PCR platform developed by the University of Virginia, enabled detection of multiple enteric pathogens from stool samples collected through the Rotavirus Surveillance Programme in 2015-2016.

Using the standard ≤35 Ct cut-off for defining detection of an enteric pathogen, our results showed that in the years 2015 and 2016, the following pathogens had the highest prevalence in South African children hospitalised for diarrhoea under the age of 5 years old: EAEC (33,2%), adenovirus (28,6%), *Shigella*/EIEC (24,1%), *Cryptosporidium (23,6%)*, and rotavirus (22,1%). Other pathogens that showed high prevalence at the ≤35 Ct cut-off were EPEC (18,8%), enterovirus (18,4%).

Other detected pathogens were *Campylobacter* (12,6%), *Giardia* (12,0), ETEC (10,3%), norovirus (10,0%), sapovirus (n=62; 7,8%), B. *fragilis* (n=58; 7,3%), C. *jejuni*/*coli* (n=58; 7,3%), H. *pylori* (n=43; 5,4%), astrovirus (n=27; 3,4%), C. *difficile* (n=24; 3,0%), *Salmonella* (n=17; 2,1%), E. *bieneusi* (n=10; 1,3%), *Aeromonas* (n=10; 1,2%), and STEC (n=4; 0.5%).

Pathogens that were not detected at all using the Ct ≤35 cut-off included the parasites *Ancylostoma, Ascaris, Encephalitozoon intestinalis, Entamoeba histolytica, Enterocytozoon bieneusi, Cyclospora, Necator, Strongyloides, and Trichuris*. This may be because helminths, particularly soil-transmitted helminths (STH), are more prevalent in children 5 to 14 years of age as one study suggested by one study (119).

Detection of enteric pathogens using quantitative PCR can pose some difficulties in interpretation of the results. Pathogens that do not contribute to the disease might also be detected. This can be overcome by comparing the frequency of pathogens identified in diarrhoeal cases to that in non-diarrhoea controls and calculating an attributable fraction. Our study only enrolled diarrhoeal cases, and no comparator group of non-diarrhoeal controls was available. With the absence of controls in our study, we could not conclude that all pathogens detected in stool samples were the cause of the acute diarrhoea in the children. Our results showed that EAEC (Ct ≤35) had the highest frequency of detection in children hospitalised with acute diarrhoea. However, a study conducted in Malawi post rotavirus vaccine introduction showed that EAEC was frequently detected in both hospitalised diarrhoea cases (51,8%) and asymptomatic community controls (47,8%) (32). The Malawian study results suggest that EAEC is likely to be a bystander rather than a causative agent of diarrhoea. *Giardia* was detected in 12% of stool samples in our study. However, this pathogen was not significantly positively associated with MSD in the GEMS study. To the contrary, in both GEMS and the Malawi study, *Giardia* was more frequently detected in non-diarrhoea controls than in diarrhoea cases (13,32). Adenovirus was also amongst the pathogens with the highest prevalence in our study. While adenovirus 40/41 has been significantly associated with hospitalisation for diarrhoea in other studies, we measured all adenovirus serotypes, not only enteric adenovirus 40/41. This might have caused an overestimation of the pathogen's prevalence.

Rotavirus, *Shigella*/EIEC and *Cryptosporidium* were detected in 22%-24% of the stool samples in 2015 and 2016. These enteric pathogens were more frequently detected in diarrhoeal cases than non-diarrhoea controls and have been significantly associated with diarrhoeal hospitalisations in case-control studies conducted in Africa. In the GEMS study, most diarrhoea cases were attributed to four pathogens: rotavirus, *Cryptosporidium*, ST-ETEC and *Shigella*. This is in keeping with our results, except for ST-ETEC – we only detected this pathogen in 6% of stool samples.

In order to overcome the limitation of not having a non-diarrhoea comparison group, we used specific Ct cut-offs for selected pathogens described in published studies that had enrolled and analysed pathogens for both cases and controls, namely the GEMS and MAL-ED studies (13,15). These studies were large, multi-centre case-control studies that enrolled hospitalised and community diarrhoea cases, respectively, as well as healthy non-diarrhoea controls. Using the pathogen-specific Ct cut-offs described in the GEMS and MAL-ED studies there were differences in the prevalence of some of the pathogens amongst children hospitalised with diarrhoea compared to those attained from using the ≤35 Ct cut-off in our study. For example, using the specific cut-off of 22.7, adenovirus was only detected in 24,2% of the participants who tested positive for adenovirus using the ≤35 cut-off. Almost all pathogens for which a pathogen-specific Ct cut-off was available had a significantly lower prevalence of detection when using the specific cut-off compared to the standard cut-off of ≤35. The only exceptions were rotavirus and *Salmonella*. Using the pathogen-specific Ct cut-offs of ≤32.6 for rotavirus, there was no significant reduction in the prevalence of rotavirus: 23% prevalence using Ct≤35 vs. 21% prevalence using Ct≤32.6. Using the pathogen-specific Ct cut-off values we found that rotavirus (20,7%), *Shigella*/EIEC (15,4%), and *Cryptosporidium* (13,0%) had the highest prevalence. Unfortunately, there were not published pathogen-specific Ct cut-offs for all the pathogens in the PCR panel and so we were only able to assess some of the pathogens in this way.

In both, the GEMS study and the study conducted in Malawi post-rotavirus-vaccination introduction rotavirus was the leading diarrhoea-causing pathogen (13,32). In the MAL-ED study, rotavirus was the second leading cause of diarrhoea in children <1 year and the third leading cause in children in the second year of life (15). In South Africa, prior to rotavirus vaccine introduction, rotavirus was associated with approximately 25% of diarrhoeal hospitalisations, with the greatest burden of disease (75%) in children <12 months of age (61). Although there has been an observed decline in all-cause and rotavirus-specific diarrhoea hospitalisations in South Africa since the introduction of the rotavirus vaccine (43), our results show that rotavirus was still a prominent cause of diarrhoeal hospitalisations post-vaccine introduction (22%), especially in children aged 6-11 months (35,3%). Rotavirus prevalence was lower in the youngest (<6 months old) and oldest age group (24-59 months old) of the cohort; 14,4% and 14,0%, respectively. Low prevalence in children <6 months old could be due to protection from maternal antibodies (44). In our final logistic regression model, we

showed that age was a significant risk factor for rotavirus hospitalisation after adjusting for site, the number of rotavirus doses and season of hospitalisation, with children aged 6-11 months having increased odds of hospitalisation due to rotavirus than children aged 24-59 months.

Rotarix® contains one rotavirus strain, G1P[8], and was designed to prevent rotavirus diarrhoea caused by G1 and non-G1 types (G3, G4, and G9) when administered as a two-dose series in infants and children (120). Although the monovalent rotavirus vaccine has shown cross-protection against the commonly circulating strains, there are some concerns that the vaccine may not provide adequate prevention of infection from newly circulating strains that are not included in the vaccine. Therefore, it is important to continue monitoring rotavirus strains among hospitalised children after the introduction of the vaccine (40).

In our analysis of the diarrhoeal surveillance data from 2015 and 2016, the highest detected G serotype was G3 (42,9%), followed by G2 (17,1%), G9 (14,3%), and G1 (7,4%) and the highest detected P serotypes were P[8] (45,1%), P[4] (36%), and P[6] (6,8%). We also analysed the prevalence of rotavirus strains, and those whose detection was over 5% included: G3P[8], G9P[8], G3P[4], G3P[4]P[8], G2P[4]P[8], G2P[4], and G2P[4]P[8]. Mapaseka et al. found that between 2010 and 2015, G1P[8] was the most predominant rotavirus strain in the East and South regions of Africa (53). On the contrary, G1P[8] was one of the least dominant strains in our study (2015-2016), only detected in approximately 3,7% participants.

In addition to age, site and season of admission were also significant risk factors in our logistic regression model for rotavirus hospitalisation amongst children who had received at least one dose of Rotarix®. Rotavirus detection was highest in winter, with the virus being detected in over half (54,6%) of the participants. Other studies have also shown that rotavirus diarrhoea cases were more prevalent during the colder seasons (autumn and winter) in South Africa (18,61,71). Although lower than winter detections, rotavirus detections were still high in children hospitalised with diarrhoea in spring. Our logistic model showed that the odds of rotavirus detection in children hospitalised during winter and spring were significantly high than those hospitalised during the summer months.

Shigella/EIEC was the most frequently detected pathogen in older children (24-59 months old). Our results correspond with those from the MAL-ED study where *Shigella* had the highest AF in children in their second year of life (15). In the GEMS study, *Shigella* was also a prominent causative agent for MSD, especially after the second year of life. The adjusted AF of *Shigella* increased with increasing age in all the sites investigated in the GEMS study (13). In the Malawian study, *Shigella*/EIEC was also among pathogens that were frequently detected in children with severe diarrhoea (32). Our results also showed that *Shigella* was a leading cause of dysentery (30,6%), as observed in other studies (13,15,60). *Shigella* is an important cause of MSD therefore; measures to prevent child infection are a priority. There are currently no licensed *Shigella* vaccines, however; trials are underway (121).

Since the introduction of the rotavirus vaccine, countries such as England and the USA have found norovirus to be the leading causative agent of diarrhoea in children (30,122–126). In this study, norovirus (GI and GII) was found in approximately 9,5% (Ct ≤35) of the 793 participants and norovirus GII was only detected in 7,8% (Ct ≤35) of the participants. Using the specific Ct cut-off value of 23,4, norovirus GII was only detected in 1,4% of the participants. We can, therefore, conclude that norovirus was not a major cause for gastroenteritis in hospitalised children <5 years in 2015 and 2016. However, it is possible that the burden of norovirus disease is higher among children with diarrhoea in the community than among children hospitalised with diarrhoea. In the MAL-ED study, norovirus GII was the leading cause of diarrhoea in children in their first year of life and the second leading cause in children in their second year of life (15). Therefore, even though norovirus is currently not among the leading diarrhoea-causing pathogens in the country, we cannot ignore that it is an important diarrhoeal agent on the continent.

There were some differences in the detection of enteric pathogens between sites. From the final logistic regression model, we also found that patients from RCCH, had higher odds of rotavirus hospitalisation despite vaccination (aOR=2.92; CI: 1.139 – 7.511), compared to patients from CHBAH, and patients from Klerksdorp Hospital (aOR=0.15; CI: 0.040 – 0.611) were less likely to develop rotavirus diarrhoea compared to CHBAH patients. We stratified the prevalence of enteropathogens by site and found that rotavirus was highly prevalent in children admitted at RCCH (27,3%) and was only detected in approximately 8,82% of the participants from Klerksdorp. Enrolment numbers were different at the sites with some sites enrolling a limited number of children during 2015-2016. This may have affected our results.

Our final model also showed that two doses of rotavirus vaccine were more protective in preventing rotavirus infections in children <5 years old compared to a single dose, however, the difference in protection was not significant (OR=0.89; CI: 0.380 – 2.125). Our results warrant further investigation of the effect of the number of doses on rotavirus vaccine effectiveness. Other studies have shown that more than one dose was more effective than a single dose, while other studies showed no difference in the effectiveness of a single dose compared to multiple doses (68–70).

Co-detection of pathogens in a single stool sample is common when using a PCR platform which detects multiple pathogens. As some of these pathogens may not be associated with diarrhoea, it may be very difficult to discern which are meaningful co-infections. In an attempt to facilitate the interpretation of co-infections, we only described co-infections for pathogens that had a pathogen-specific Ct cut-off available. The majority of rotavirus detections (90%) identified rotavirus as the only pathogen, and only 10% of rotavirus detections were in combination with another enteric pathogen. H. *pylori* had the highest co-infection prevalence with rotavirus (4,9%). We found rotavirus-*Shigella*/EIEC co-infections in 3,7% of the participants in the current study. This was the second-highest rotavirus co-infection. For *Shigella*/EIEC detections, 30% had detection of another pathogen; and 21% of those positive for *Cryptosporidium* had another pathogen detected. Pathogen-specific Ct cut-offs were not available for all the enteric pathogens tested by means of TAC, and thus we could not investigate all possible co-infections.

Fever (17-100%), refusal to feed (33-56%), and vomiting (33-78%) were commonly occurring symptoms in almost all enteric pathogen single infections. Dehydration (33-100%) was also a commonly occurring symptom in patients infected with any of the pathogens tested for, with the exception of adenovirus where only 6% of the patients infected with adenovirus suffered dehydration. Bloody stools were present in just under a third of children with *Shigella*/EIEC detected in their stool. Our logistic regression model also assessed presenting symptoms at admission as predictors for rotavirus infection. “Other type” of feed beyond four months of age and vomiting were significant risk factors for rotavirus hospitalisation in the bivariate analysis but were not significantly associated with rotavirus hospitalisation in the multivariable analysis. Amongst the participants who had rotavirus detection despite vaccination, there were few who had pre-existing medical conditions (6,8%), had previously taken antibiotics (24,1%),

were previously hospitalised (16,3%), were malnourished (10,3%) or previously had another case of severe diarrhoea (6,8%).

Limitations

Secondary data was used for the analyses of the current study and, therefore, other possible risk factors of diarrhoea hospitalisation in children such as the HIV status of the mother and child could not be included in the analyses, as they were not adequately recorded for the purposes of the current study. We also faced a challenge in defining "co-infection" as we could not be certain if the detection of a pathogen meant that the pathogen actually contributed to the illness of the patient. Therefore, risk factors of co-infection could not be analysed. Due to the high rotavirus vaccine coverage among children testing rotavirus-positive and rotavirus-negative (90% and 93%, respectively), vaccine effectiveness could not be calculated due to insufficient power.

No controls were available for the analyses, making it difficult to draw conclusions on the causes of diarrhoea amongst the participants. However, the use of specific Ct cut-offs from the GEMS and MAL-ED studies were used to assist in estimating which enteric pathogens were probable causes of diarrhoea in the participants. Another limitation was the overestimation of the prevalence of adenovirus since the target used was for all adenovirus types (not only adenovirus 40/41).

Conclusion

Using the standard Ct cut-off of ≤35, EAEC and adenovirus had the highest prevalence in children with acute diarrhoea. However, with the use of specific pathogen Ct cut-offs from the GEMS and MAL-ED study, rotavirus, *Shigella*/EIEC, *Cryptosporidium*, and adenovirus had the highest detection frequencies. Therefore, after the introduction of the oral rotavirus vaccine, rotavirus remains a leading cause of diarrhoea in South Africa. Rotavirus prevalence was highest in children 6 -11 months old hospitalised with diarrhoea and was the most prevalent cause of diarrhoea in the autumn and winter seasons. *Shigella*/EIEC was frequently detected in children 24 to 59 months old, highlighting the importance of this pathogen in older children. Assessing only those pathogens with specific Ct cut-offs, we found that even though there were notable co-infections, most of the detections were singular. Lastly, age, the season of admission

and the surveillance hospital where the child was admitted were significant risk factors for rotavirus infection in children who had received at least one dose of the rotavirus vaccine.

Recommendations

Diarrhoea-causing pathogens that need to be targeted include rotavirus, *Shigella*/EIEC (especially in older children), and *Cryptosporidium*. Reducing diarrhoea caused by these pathogens could have a major impact on lowering the overall prevalence of acute diarrhoeal hospitalisations in South African children <5 years old.

Vaccines should be a priority in developing countries as other measures such as access to clean water and proper sanitation are often lacking and difficult to improve over a short period of time. So far, vaccines have proven to be the most effective strategy against severe diarrhoea in children. Improvements in the effectiveness of the currently licenced rotavirus vaccines, development of new rotavirus vaccines and new vaccine schedules, for example, combination oral and parenteral vaccines are needed to further reduce the burden of rotavirus diarrhoea. Vaccines against *Shigella* are urgently needed to reduce the burden of diarrhoeal disease in older children. In our study, ETEC was not frequently detected and thus vaccines against ETEC may be a lower priority in our country compared to other African countries.

Our study highlights the need to enrol controls at diarrhoea surveillance sites, as this will help in estimating the contribution of each pathogen to the burden of diarrhoea in the country.

Use of the Taqman array card enabled us to investigate a wider range of enteric pathogens than conventional methods allowed. Future use of this platform in an ongoing Diarrhoeal Surveillance Programme in South Africa would broaden the scope of the surveillance and allow us to monitor the prevalence of multiple pathogens. Results of the current study provided valuable information in tailoring a Taqman array card to the South African context. We found that the prevalence of the helminths was quite low in the study population and these targets could potentially be replaced by targets assessing antimicrobial resistance (AMR) or *Shigella* subtypes.

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Appendix A: Plagiarism Declaration

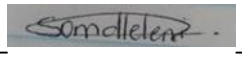
PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

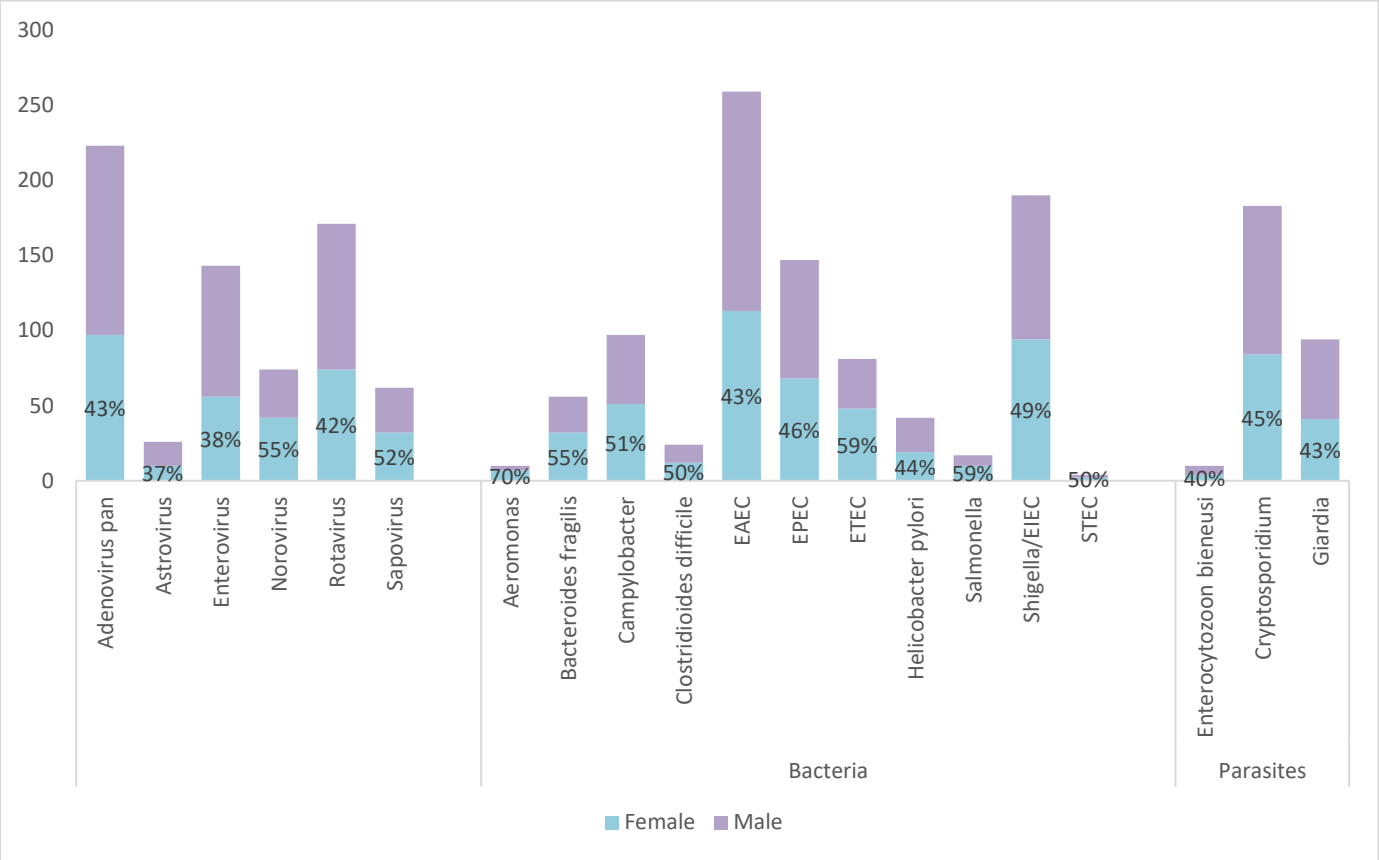
I _Simbulele Onesimo Mdleleni_ (Student number: 1405529_) am a student registered for the degree of _Epidemiology__ in the academic year __2018__.

I hereby declare the following:

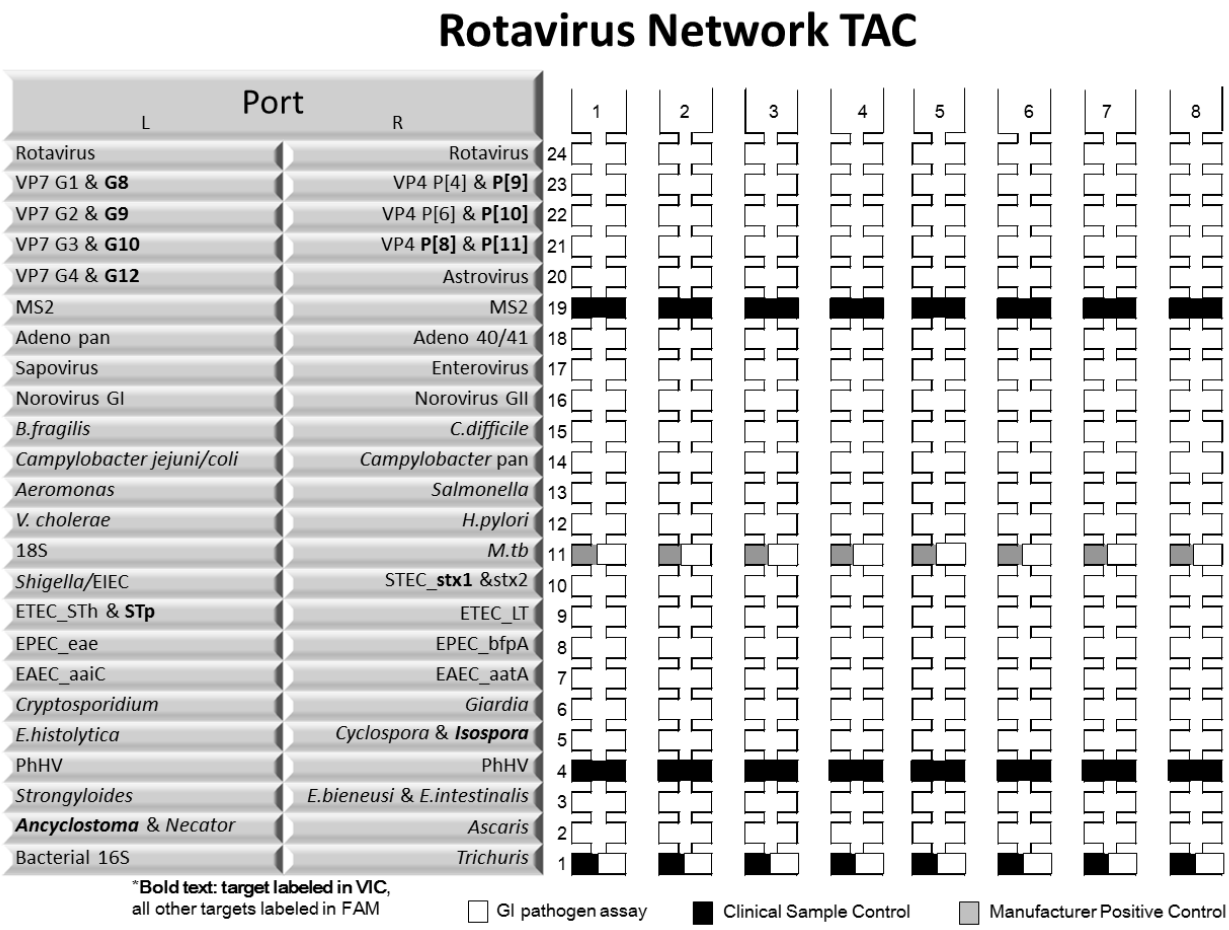
- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.

Signature: ____  ___ Date: 13 October 2020

Appendix B- Prevalence of enteric pathogens stratifies by sex



Appendix C- TaqMan Array Card used in the Rotavirus Network



The TAC card used by the NICD for the Rotavirus Sentinel Surveillance Programme samples in 2015 and 2016 was a modification of the TAC card used for the African Rotavirus Network.

APPENDIX D: Rotavirus surveillance – Parent interview form A

All potential risk factors that will be used in the analysis are highlighted in the table below.

Rotavirus study number: __ __ - __ __ __ __ __ OR Sticker

1.	During the first 4 months of life, how was your child fed?	☐ Breast milk ☐ Formula (never breastfed)	☐ Breast milk and formula ☐ Unknown
2.	If older than 4 months: how is your child being fed at present? (*In addition to solids*)	☐ Breast milk ☐ Formula ☐ Unknown	☐ Breast milk and formula ☐ Other: _____ ☐ Not applicable
3.	What is your house made of?	☐ Bricks ☐ Tin/iron sheeting ☐ Unknown	☐ Mud/traditional ☐ Other: _____
4.	How many people sleep in the same room as the child *(not counting the child)?*	_____ people	☐ Unknown
5.	Do you use any of the following for heating and/or cooking in the household? (*check all that apply*)	☐ Electricity ☐ Coal ☐ Wood ☐ Unknown	☐ Gas ☐ Paraffin ☐ Other: _____
6.	What is the main source of water in the household?	☐ In-door tap water ☐ River water ☐ Other: _____	☐ Borehole ☐ Outdoor/communal tap ☐ Unknown
7.	What type of toilet do you have at the house?	☐ Flush toilet ☐ Bucket system ☐ Other: _____	☐ Pit latrine ☐ None/ Outdoors ☐ Unknown
8.	Does your child attend a nursery or crèche?*(at least 2 other children for at least 4 hours per day, 3 days per week)*	☐ Yes ☐ No	☐ Unknown
9.	What is the highest level of education the mother has completed?	☐ No School ☐ Higher education ☐ Unknown	☐ Schooling: highest grade completed ___ ___
10.	Did your child have any of the following symptoms during the current illness?		
10.1	Fever	☐ Yes ☐ No	☐ Unknown
10.1.1	*If yes:* Duration	_____ days	☐ Unknown
10.2	Refusal to feed	☐ Yes ☐ No	☐ Unknown
10.2.1	*If yes:* Duration	_____ days	☐ Unknown
10.3	Vomiting	☐ Yes ☐ No	☐ Unknown
10.3.1	*If yes:* Duration	_____ days	☐ Unknown
10.3.2	Maximum number of episodes during any day	_____	☐ Unknown
10.4	Diarrhoea	☐ Yes ☐ No	☐ Unknown
10.4.1	*If yes:* Duration	_____ days	☐ Unknown
10.4.2	Maximum number of episodes during any day	_____	☐ Unknown
10.4.3	Did you notice blood in any of the stools?	☐ Yes ☐ No	☐ Unknown

APPENDIX E: Rotavirus surveillance – Parent interview form B

Rotavirus study number: __ __ - __ __ __ __ __ OR Sticker

14. 14.1	Did the child receive antibiotic before coming to the hospital? *If Yes,* number of days on antibiotics?	☐ Yes, oral antibiotic ☐ Unknown ☐ Yes, injection ☐ No _____ days ☐ Unknown	*(skip to 15 if No or Unknown)*
15. 15.1	Does your child take cotrimoxazole (bactrim)? *If Yes,* about how long has your child been taking it?	☐ Yes ☐ No ☐ Unknown _____ months or ____ weeks ☐ Unknown	*(skip to 16 if No or Unknown)*
16. 16.3	Has the child ever been admitted to hospital? *(Before this admission)* *If Yes,* Were any of these previous admissions for diarrhoea?	☐ Yes ☐ No ☐ Unknown ☐ Yes ☐ No ☐ Unknown	*(skip to 17 if No or Unknown)*
17.	Has your child ever been diagnosed with one of the following chronic medical conditions? *(before this admission)*	☐ Malnutrition	
18. 18.1	Did the child's mother have an HIV test during pregnancy? *If Yes,* what was the result of the latest test?	☐ Yes ☐ No ☐ Unknown ☐ Positive ☐ Negative ☐ Unknown	*(skip to 19 if No or Unknown)*
19. 19.1	Has the child ever been tested for HIV? *If Yes,* What was the result?	☐ Yes ☐ No ☐ Unknown ☐ Positive ☐ Negative ☐ Unknown	*(skip to end if No / Unknown)* *(skip to end if Neg / Unknown)*

APPENDIX F: Rotavirus surveillance – RTHC / Vaccination history

Rotavirus study number: __ __ - __ __ __ __ __ OR Sticker

Copy the following information from the card, if no RTHC available, ask person completing interview

1.	Date of Birth (dd/mm/yy) If DOB unknown, enter age in months:		__ __ / __ __ / 20__ __ _____	☐ Unknown
2.	Birth weight		___ . ___ ___ kg	☐ Unknown
3.	Gestational age If gestational age unknown		___ ___ weeks ☐ Term ☐ Premature (<37 weeks)	☐ Unknown ☐ Unknown
	ROTAVIRUS	1 2	__ __ / __ __ / 2 0 __ __ __ __ / __ __ / 2 0 __ __	☐ Not given ☐ Not given

APPENDIX G: Rotavirus surveillance – Medical records

1.	Sex	☐ Male ☐ Female
2.	Date of admission	___ ___ / ___ ___ / 2 0 ___ ___ (dd/mm/yyyy)
3.	Race	☐ Asian/Indian ☐ Black ☐ White ☐ Coloured
4.	Admission weight	__ __ . __ kg
5.	Temperature	__ __ . __ °C
6.	Level of dehydration as assessed by the clinician on admission	☐ Not dehydrated ☐ 1-5% (mild) ☐ ≥ 6% (moderate/severe) ☐ Not recorded
7.	Presence of oedema	☐ Yes ☐ No ☐ Not recorded
8.	Did the child receive: IV fluids for rehydration Antibiotics	 ☐ Yes ☐ No ☐ Yes ☐ No

Outcome Summary – to be obtained on all participants when discharged.

9.	Outcome of child	☐ Discharged ☐ Died ☐ Refused Hospital Treatment ☐ Transferred to another hospital
10.	Date of discharge/ death/ transfer	___ ___ / ___ ___ / 2 0 ___ ___ (dd/mm/yyyy)

APPENDIX H: List of Variables

Parent Interview Form A

1. Feeding at four months: Breast milk, Breast milk and formula, formula (never breast feed), Unknown
2. Feeding at >4months: Breast milk, Breast milk and formula, formula (never breast feed), Unknown, Other, Not applicable
3. House material: Bricks, Tin/Iron sheets/ Mud/traditional, Unknown, Other
4. Number of people sharing bedroom with child: number of people, Unknown
5. Heating technique: Electricity, Gas, Coal, Paraffin, Wood, Unknown, Other
6. Source of water: Indoor tap water, Borehole, River water, outdoor/communal tap, Unknown, Other
7. Type of toilet: Flush toilet, Pit latrine, Bucket system, None/Outdoors, Unknown, Other
8. Creche attendance: Yes, No, Unknown
9. Mother level of education: No schooling, Schooling (highest grade completed), Higher education, Unknown
10. Symptoms experienced by child:
11. Fever: Yes, No, Unknown
12. If yes, duration?
13. Refusal to feed: Yes, No, Unknown
14. If yes, duration?
15. Vomiting: Yes, No, Unknown
16. If yes, duration?
17. Diarrhoea: Yes, No, Unknown
18. If yes, duration?
19. Season of birth
20. Season of admission

Rotavirus Surveillance –Parent Interview Form B

21. Antibiotic before hospitalisation: Yes, oral antibiotic, Yes, injection, Unknown, No
22. Child admitted to hospital before: Yes, No, Unknown
23. Has child been ever diagnosed of malnutrition

Rotavirus Surveillance – RTHC/ Vaccination History

24. Age
25. Number of rotavirus doses received by child: 0, 1, 2

Rotavirus Surveillance – Medical Records

26. Sex: Male, Female
27. Admission date: (dd/mm/yyyy)
28. Race: Asian/Indian, Black, White, Coloured
29. Level of dehydration: Not hydrated, 1-5% (mild), ≥ 6 (moderate/severe), not recorded
30. Outcome of child: Date of discharge/death/transfer

APPENDIX I: Permission to use data for secondary analysis-

Protocol Title: Rotavirus Sentinel Surveillance Programme (RSSP)

NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES
Division of the National Health Laboratory Service

Centre for Enteric Diseases – Virology Division
National Institute for Communicable Diseases (NICD)
1 Modderfontein Road, Sandringham, 2031
Tel: +27 (0)11 555 0370 |: +27 (0)11 555 0502

19 October 2018

To whom it may concern

Re: Permission to use data for secondary analysis

Protocol: RSSP
Protocol Title: Rotavirus Sentinel Surveillance Programme

HREC Ref No: M091018

As gatekeeper of the above-mentioned data, I hereby give Miss Simbulele Mdleleni (student number 1405529) permission to use the RSSP dataset for a secondary data analysis in fulfillment of the research component of her MSc degree. The necessary variables will be made available to her as one or more Stata files.

Please do not hesitate to contact me should you require any additional information.

Yours sincerely

Prof Nicola Page
Principal Investigator
+27 82 4472745
nicolap@nicd.ac.za

APPENDIX J: Permission to use data for secondary analysis

Protocol Title: Surveillance on pathogen-specific causes of pneumonia and diarrhea hospitalization in children (Pneumogastro surveillance)

11th Floor, West wing, Nurses residence
Chris Hani Baragwanath Hospital
P.O. Box 90753, Bertsham, 2013
Tel: +27 11 983 4265

RMPRU
respiratory & meningeal pathogens research unit

22 October 2018

To whom it may concern

Re: Permission to use data for secondary analysis

Protocol: Pneumogastro surveillance
Protocol Title: Surveillance on pathogen-specific causes of pneumonia and diarrhoea hospitalization in children.

HREC Ref No: 131109

As gatekeeper of the above-mentioned data, I hereby give Miss Simbulele Mdleleni, student number 1405529, permission to use the above-mentioned dataset for a secondary data analysis in fulfilment of the research component of her MSc degree. The necessary variables will be made available to her as one or more Stata files.

Please do not hesitate to contact me should you require any additional information.

Yours sincerely

Dr Michelle Groome
Co-investigator +27 82 374 7345
groomem@rmpru.co.za

APPENDIX K: Final Ethics Approval- Rotavirus Sentinel Surveillance Programme Clearance Certificate No: M091018

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Michelle Groome

CLEARANCE CERTIFICATE **Protocol M091018**

PROJECT Rotavirus Sentinel Surveillance Progrmme
(Rotaprotocol 2032013 V4.0)
(Previously Dr C Cohen)

INVESTIGATORS Dr Michelle Groome.

DEPARTMENT DST/NRF Vaccine Preventable Disease Unit

DATE CONSIDERED 09.01.30

DECISION OF THE COMMITTEE* Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 10/06/2013 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor :

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX L: Current Study Ethics Clearance-

Clearance Certificate No: M181171

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Miss Simbulele Onesimo Mdleleni

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M181171

NAME: (Principal Investigator)	Miss Simbulele Onesimo Mdleleni
DEPARTMENT:	Public Health
PROJECT TITLE:	Aetiology and pathogen specific risk factors for diarrhoea among children under the age of 5 years
DATE CONSIDERED:	30/11/2018
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Michelle Groome
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	16/01/2019

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

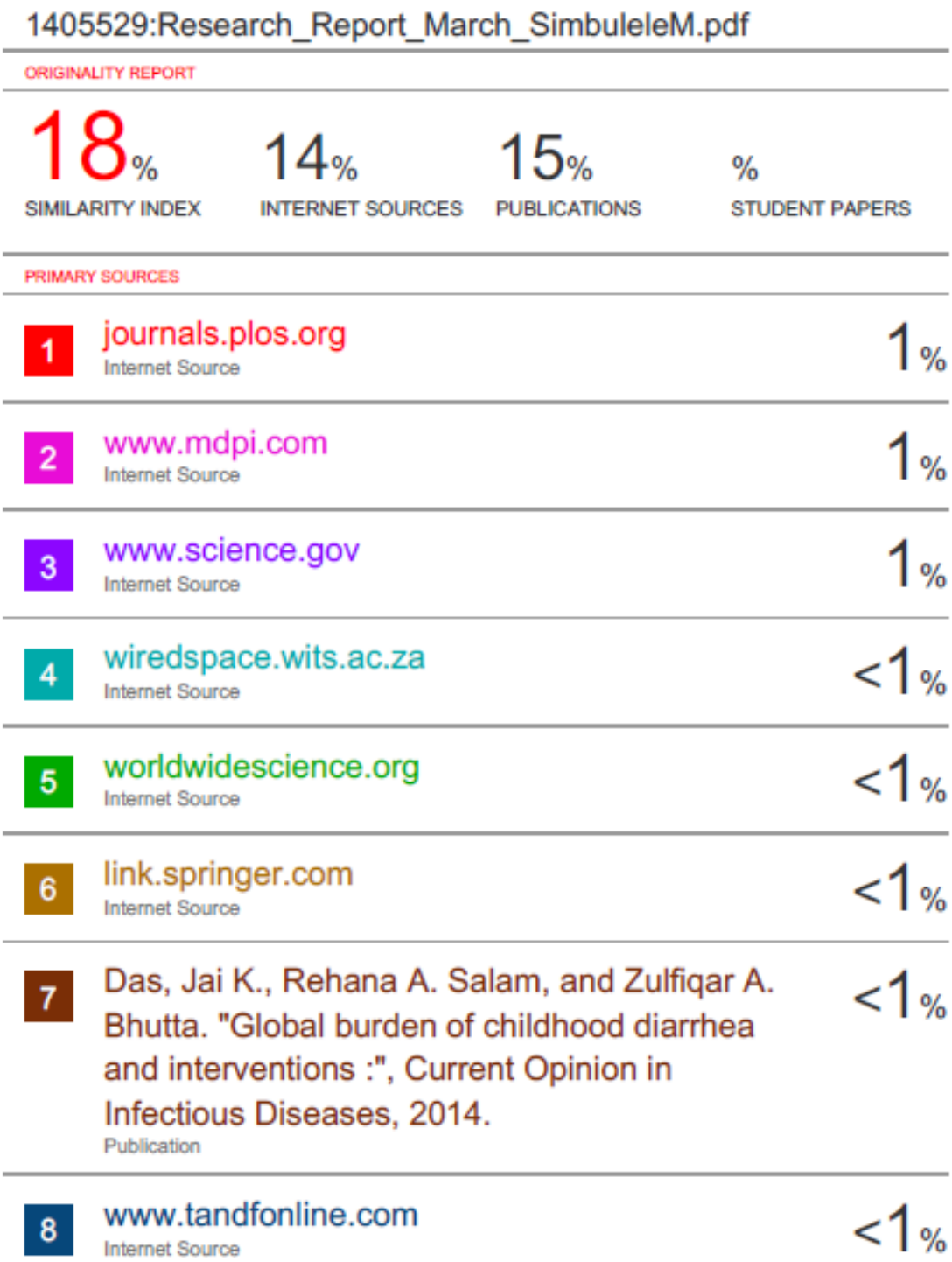
To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Philip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **November** and will therefore be due in the month of **November** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 18 January 2019

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix M: Report rom TurnItIn



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