CHARACTERISATION OF DIARRHOEAGENIC *ESCHERICHIA COLI* AND HUMAN NOROVIRUS FROM DAYCARE CENTERS IN THE VHEMBE REGION OF THE LIMPOPO PROVINCE, SOUTH AFRICA

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THE MASTERS DEGREE IN

MICROBIOLOGY

TO THE

DEPARTMENT OF MICROBIOLOGY

SCHOOL OF MATHEMATICAL AND NATURAL SCIENCE

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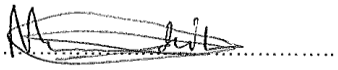
MARCH 2020

Declaration

I, Lutendo Munzhedzi (Student number 11636213), declare that this dissertation is my original work and has not been submitted for any degree at any other university or institution. This dissertation does not contain other persons' writing unless specifically acknowledged and referenced accordingly.

Signed (Student):



Date:

11 June 2020

DEDICATION

I dedicate this work to my Lord and my Redeemer, Jesus Christ.

ACKNOWLEDGEMENTS

A special thanks to my Supervisor Prof N Potgieter, Co-supervisor Prof AN Traore and Dr JP Kabue for giving me the opportunity as well as being patient with me during out the course of this research project.

Also special thanks to Dr M Sigidi, Mr M Magwalivha, Mr S Karambwe, Mr I Nemutanzhela, Mr A Khwathisi, Miss R Khumela, Miss M Munzhedzi and my fellow colleagues for support throughout.

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LIST OF ABBREVIATIONS

%	**:**	percentage
^{o}C	**:**	degree Celsius
µl	**:**	microliter
µm	**:**	micrometer
cm^{-1}	**:**	per centimeter
bp	**:**	base pairs
CDC	**:**	Center for Disease Control and Prevention
DNA	**:**	Deoxyribonucleic Acid
dNTP	**:**	Deoxyribose Nucleotide Triphosphate
E. coli	**:**	*Escherichia coli*
EAEC	**:**	Entero-aggregative *E. coli*
EDTA	**:**	Ethylene Diamine Tetra Acetic acid
EHEC	**:**	Entero-haemorrhagic *E. coli*
EIEC	**:**	Entero-invasive *E. coli*
EPEC	**:**	Entero-pathogenic *E. coli*
ETEC	**:**	Entero-toxigenic *E. coli*
h	**:**	hour
LTs	**:**	Heat-labile toxin
mg	**:**	milligram
mL	**:**	milliliter
m-PCR	**:**	multiplex- Polymerase Chain Reaction
MPN	**:**	Most Probable Number
nm	**:**	nanometer
PCR	**:**	Polymerase Chain Reaction
s	**:**	seconds
STs	**:**	Heat stable toxin
Stx	**:**	Shigella like toxin
SA	**:**	South Africa
DCC	**:**	Daycare center

EMB	**:**	Eosin Methlene Blue
KIA	**:**	Kliglers Iron Agar
NoV GI	**:**	Norovirus Genogroup one
NoV GII	**:**	Norovirus Genogroup two
TC	**:**	Total coliform
UNICEF	**:**	The United Nations Children's Emergency Fund
USA	**:**	United State of America
NoV	**:**	Norovirus
WHO	**:**	World Health Organization

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ABSTRACT

BACKGROUND: Child daycare centers (DCCs) host a variety of pathogenic enteric microorganisms which may be found in various areas within the compound. Enteric pathogens are the most common cause of diarrhoea worldwide in children under the age of 5 years and are a burden in developing countries with high numbers of diarrhoeal diseases leading to a high mortality rate.

OBJECTIVE: To perform a pilot study to characterize diarrhoeagenic *E. coli* and Norovirus strains from child daycare centers in the Vhembe Region in Limpopo Province, South Africa.

MATERIALS AND METHODS: Two daycare centers were randomly selected in the Vhembe District for this study. A total of 83 samples were collected from various areas within the daycare centers. For *E. coli*, the environmental and handwash samples were analysed using the Colilert®/ Quanti-tray® 2000 technique and stool samples were cultured on Eosin Methylene Blue agar: presumptive *E. coli* isolates were confirmed using three biochemical tests (Kliglers Iron Agar test, Indole test and Urease test). Total nucleic acid was extracted from presumptive *E. coli* isolates using a semi-automated method and DNA was used for further m-PCR confirmation. For human norovirus identification, environmental and handwash samples were subjected to membrane filtration and RNA was extracted using the semi-automated system. RNA was extracted from stool samples (clinical samples) using the Allprep® Powerfecal® RNA Kit**.** For detection of Norovirus, RNA extracts were subjected to the Seegene Allplex™ Gastrointestinal Full Panel Assay. Positive samples for Norovirus were amplified using a One-step Ahead RT-PCR followed by Sanger sequencing. Phylogenetic analysis was done using MEGA 7 software.

RESULTS: *E. coli* was isolated from the environmental samples (10%), stool samples (100%), handwash samples of daycare workers (40%) and handwash samples of children (13.6%); several bacterial co-infections among pathotypes were observed. Norovirus prevalence in this study, was detected in 5% of handwash samples of the children, 10% from handwash samples of the workers and 27% were stool specimens. All detected samples were co-infection between bacterial pathotypes (90.4%) as well

as bacterial-viral co-infections (9.6%). Norovirus genotype GII.1 was shown through sequencing of one positive sample.

CONCLUSION: The study findings showed genetic diversity of *E. coli* in these settings. The phylogenetic analysis revealed NoV genotype GII.I capsid sequenced to share a common ancestor with previously reported strains associated with outbreaks of NoV globally. The high prevalence of *E. coli* and NoV detected in this study will aid in setting guidelines for hygiene in the DCC environment.

Key-words: *E. coli*; Daycare centers; Norovirus; Enteric pathogens.

CHAPTER 1: GENERAL INTRODUCTION

1.1 BACKGROUND

In the world of today, children under the age of five years are put into daycare- centers (DCC's) because their parents are working (Holaday et al., 2008). The DCC environment hosts a variety of enteric microorganisms in different areas such as the children's sleep area, play area and diaper changing area (Liu et al., 2015). Enteric pathogens are the most common cause of diarrhoea worldwide in children under the age of five years (Kotloff et al., 2013).

The World Health Organisation (WHO) in 2005 defined diarrhoea as the frequent passage of loose, watery stools three or more times daily. It is the second leading cause of death in children under the age of five (WHO., 2017). Globally about 1.7 billion diarrhoeal cases are reported annually with over half a million deaths among children under the age of five (Navab-Daneshmand et al., 2018; Mattioli et al., 2015; Walker et al., 2013). Generally, diarrhoea is caused by enteric bacteria, viruses and parasites which causes malabsorption of water, electrolytes and nutrients (Miller et al., 2003). Intestinal infections have been associated with young age, hygienic practices, level of schooling of guardians/caregiver, gatherings of institutions such as DCC's, lack of basic sanitation, access to treated water and seasonal changes in temperatures (Castro et al., 2015; Phumpholsup et al., 2015a).

Enteric pathogens are mainly transmitted through fecal-oral exposure, with contaminated hands and fomites playing an important role in the spread of these pathogens from one individual to another (Wang et al., 2017; Nworie et al., 2012). In DCC's, many outbreaks have been associated with child-to-child or caregiver-to-child transmission, but transmission may also be through contaminated environmental surfaces (Li et al., 2014a). *Escherichia coli* (*E. coli*) and human Norovirus (NoV) are among the enteric pathogens which are of epidemiological importance. In developed countries such as Japan, *E. coli* pathotype enterohemorrhagic (EHEC) had 4000 reported cases annually including outbreaks associated with child DCC's and facilities (Kanayama et al., 2015; Sonoda et al., 2008). Among these outbreaks, 35% were transmitted through direct contact (Kanayama et al., 2015).

In developing countries, diarrhoeal disease outbreak and gastrointestinal diseases have been directly associated with high infant morbidity and mortality with 2.5 million deaths annually (Navab-Daneshmand et al., 2018; Castro et al., 2015). Castro et al. (2015) found that the DCC in Brazil was a potential environment for transmission. Several studies done in the Vhembe region of Limpopo Province of South Africa have reported a high prevalence of *E. coli* strains detected in household water storage containers, river water and latrine facilities (Traore et al., 2016; Samie et al., 2012; Potgieter et al., 2009; Obi et al., 2002). The presence of *E. coli* has been associated with fecal contamination and a link has been observed between high levels of *E. coli* and diseases such as gastroenteritis and diarrhoea (Blaustein et al., 2013). Kabue et al. (2016b) reported a high prevalence of NoV among children under the age of five years, showing that there was frequent exposure of young children to enteric pathogens. Studies in Tanzania have shown NoV to be associated with childhood diarrhoea in low-income settings and has been the most common cause of under-five mortality (Elfving et al., 2014; Boschi-Pinto et al., 2008).

1.2 STUDY RATIONALE

With working class women of today, pre-school age children are subject to licensed DCCs, where a noticeable high frequency of infectious diseases in these children is an increasing public health problem (Ibfelt et al., 2015; Holaday et al., 2008). Studies in Denmark reported that young children in DCCs showed increased risk of up to seven times for infectious diseases (Kaarme et al., 2016; Uldall., 1990). There is a high rate of diarrhoea in children attending DCCs compared to children at home care simply due to fecal contamination of the daycare environments (Ibfelt et al., 2015; Holoday et al., 2008; Pickering et al., 1986; Ekanem et al., 1983).

Hands and fomites are known to be important routes for enteric pathogen transmission, causing approximately 297000 diarrhoeal deaths, which could be avoided through improved hygiene in children under-five years (Navab-Daneshmand et al., 2018; Prüss-Ustün et al., 2014). In the DCC setting, these agents are either directly transmitted through person-to-person contact or indirectly through contact between contaminated inanimate objects (toys) (Ledwaba et al., 2019), the environment, food or water (Enserink et al., 2015). Inadequate personal hygiene allows for the spread of diseases, increasing the diversity of enteric pathogens on

hands and by extension on contacted surfaces (Julian., 2016; Ross et al., 2015; Prüss-Ustün et al., 2014).

Enteric pathogens are a burden in developing countries such as South Africa with high numbers of diarrheal diseases leading to deaths. In South Africa, little has been reported in the prevalence of circulating enteric viruses across the country (Kabue et al., 2016a; Platts-Mills et al., 2015; Mans et al., 2010). There is limited data on NoV transmission and contamination of hands in these low-income settings (Mattioli et al., 2015).

The Limpopo Province is in the North East region of South Africa and is a rural region with a very poor economic status (Traore et al., 2016). In the region many communities do not practice good hygiene, and still lack safe drinking water (Ledwaba et al., 2019; Samie et al., 2012; Potgieter et al., 2009; Obi et al., 2002). Despite the hygiene situation in this region, numerous DCCs exist in this region. However, there is limited data on the prevalence of *E. coli* and NoV in the DCC settings in this area. Therefore, this study aimed to characterize selected enteric pathogens in DCCs in the region.

1.3 STUDY OBJECTIVES

1.3.1 PRIMARY OBJECTIVE

- ✓ To perform a pilot study to characterize diarrhoeagenic *E. coli* and Norovirus strains from child daycare centers in the Vhembe Region in Limpopo Province, South Africa.

1.3.2 SECONDARY OBJECTIVES

- ✓ To determine the prevalence of diarrhoeagenic *E. coli* (using m-PCR) and NoV (using RT-PCR) circulating on different surfaces, hands of caregivers and children in DCCs in the Vhembe District.
- ✓ To determine the genetic diversity of diarrhoeagenic *E. coli* (using m-PCR) and NoV strains (partial sequencing) circulating in the DCCs in the Vhembe District.

CHAPTER 2: LITERATURE REVIEW

2.1 BACKGROUND

Enteric pathogens are defined or classified as any microorganism that is a causative agent of enteric disease or diseases (Kolling et al., 2012). Enteric bacteria, viruses and parasites are known to cause gastrointestinal diseases, including diarrhoea (Rosen et al., 2000). Of 75 enteric disease outbreaks reported in childcare settings bacteria and viruses accounted for 93.4 % causative of the agents (Li et al., 2014a; Lee and Greig., 2008). Some examples of these enteric pathogens in the Table 2.1.

Table 2.1- Examples of enteric pathogens (Santamaria et al., 2003)

ENTERIC PATHOGEN TYPE	EXAMPLES
Bacteria	*E. coli* (diarrhoea), *Salmonella* species (salmonellosis), *Vibrio cholera* (Cholera), *Shigella* species (dysentery), and many other infections caused by *Camphylobacter jejuni*, *yersinia* species
Viruses	Human norovirus, astrovirus, adenovirus, rotavirus, enterovirus, sapovirus, hepatitis A and E, poliovirus types 1 and 2, echoviruses and coxsakievirus
Parasites	*Entamoeba histolytica*, *Girdia intestinalis* and *Cryptosporidium parvum*.

2.2 DIARRHOEA

Diarrhoea is the frequent passage of liquid or loose stools three or more times daily, is normally a symptom of gastrointestinal infection and may be caused by a diversity of viral, bacterial and parasitic organisms. Severe diarrhoea in young children and old people can be life threatening, especially those with impaired immunity or are suffering from malnutrition (WHO., 2008). In 2005 Nguyen et al., reported that under-five years children in Africa, Asia and Latin America had estimated deaths of 12, 600 due to diarrhoeal illnesses. However recently, more than 500 000 children under five years of age die annually from diarrhoeal diseases, with a reported 1.7 billion cases annually in low-income settings (Julian et al., 2016; Walker et al., 2013). Diarrhoea is due to pathogenic microorganisms such as rotavirus, enteropathogenic *E. coli*, enterotoxigenic *E. coli* and human NoV (Julian., 2016). In low-income settings, childhood diarrhoea has been associated with human NoV (Kotloff et al., 2013).

Human Norovirus is the leading cause of non-bacterial gastroenteritis, which affects people of all age groups in both developing and developed countries (Yang et al., 2010). Norovirus accounts for approximately 200 000 deaths annually (Phumpholsup et al., 2015a; Patel et al., 2008).

2.3 DAY CARE CENTERS (DCCs)

Many children are cared for at DCCs because of urbanization and working-class woman who effectively participate economically in the working force. About 67% of mothers who have children under the age of five, work outside the home and of these 61% subject their children to DCCs (from 8h00am-13h00pm) (Li et al., 2014b). Day care centers have become a frequent place for children outside their homes and a potential environment for contamination (Castro et al., 2015). Li et al. (2014a) reported a high rate of transmission of respiratory and enteric diseases in DCCs in young children. Globally DCCs are an ideal place for the spread of infantile disease due to the child-to-child interactions and high density of the children (Ibfelt et al., 2015). Studies have shown that children attending day care centers have more sick days than those being cared for elsewhere (Uldall., 1990; Bartlett et al., 1987). For example, gastroenteritis incidence in children at DCCs has been seen to be 2 or 3 times higher than in those who do not attend DCCs (Kaarme et al., 2016; Enserink et al., 2013; Bartlett et al., 1985).

2.4 PATHWAYS OF CONTAMINATION OF ENTERIC PATHOGENS

As children begin to explore, a change in their behaviour leads them to encounter environmental surfaces and objects (Cohen et al., 2005; Pickering et al., 1986). Wang et al., (2017), describes a pathway as any linkage between a source of microorganism and oral ingestion. Enteric pathogens are mainly spread through the fecal-oral-route with many sources playing a role in the transfer from one place to another. Microorganisms are brought from the community into the childcare center (Brady, 2005).

Different pathways of contamination include hand contact with fomites, hand contact with open drains, accidental contact with child's own feces, hand mouthing, eating without washing hands, hands of caregivers and the type of water storage used in the daycare environment (Navab-Daneshmand et al., 2018; Wang et al., 2017).

There are factors which influence the transmission of enteric pathogens in the daycare environment (Brady, 2005). These factors include: poor hygiene from both the care

staff and children; the larger the size of the center, a higher ratio of children to caregivers, also the drooling, coughing, sneezing from the children are common thus allowing contamination from air and inanimate objects such as toys in the day care environment (Brady, 2005).

2.5 ENTERIC BACTERIA

There are many types of enteric bacteria such as *Salmonella*, *Klebsiella*, *Enterobacter*, *Proteus* and *Pseudomonas* but study only focused on *E. coli*.

2.5.1 *ESCHERICHIA COLI (E. coli)*

Escherichia coli belongs to the genus *Escherichia* and is Gram-negative, non-spore forming bacillus, motile by flagella, oxidase negative, ferments lactose and is catalase positive (Delair., 2016; Munshi et al., 2012; Hunter, 2003). Also *E. coli* is a species of bacteria that is a subgroup of total coliforms and faecal coliforms. Generally, *E. coli* can be found in humans and warm blooded-animal's intestinal tracks and are used to indicate the presence of faecal contamination of water (USEPA, 2006).

The *E. coli* strains that cause diarrhoea and are associated with intestinal infections are called diarrhoeagenic *E. coli* (DEC) (Delair, 2016; Bando et al., 2009). Differentiation of normal flora (non-DEC) and DEC is based on virulence factors in relation to their infectious dose and mechanisms of pathogenicity (Kimata et al., 2005; Moon et al., 2005)

Diarrhoeagenic *E. coli* have been classified into pathovars/pathotypes by adhesion patterns to epithelial cells or by virulence genes (Chao et al., 2017; Kaper., 1998). *Escherichia coli* pathotypes include, enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic (ETEC), Shiga-toxin producing *E. coli* (STEC) and enteroinvasive *E. coli* (EIEC). For these pathotypes there is clear molecular, epidemiological and clinical data to prove their role as enteropathogens (Chao et al., 2017; Nataro et al., 2006; Cohen et al., 2005).

2.5.2 DIARRHOEAGENIC *E. coli* PATHOTYPES

- **Enteroinvasive *E. coli* (EIEC)**

Enteroinvasive *E. coli* is non-motile, negative for lactose fermentation and lysine decarboxylase tests (Brady et al., 2010; Rolland et al., 1998). Infection with EIEC is reported mainly in low-income-settings; however, it has also been reported in developed countries with the primary reservoir being travellers (Hsu et al., 2010). This

pathotype is known to frequently occur less in children under the age of one year (Podewils et al., 2004). EIEC consists of genes *ip*A, *ip*B, *ip*C, and *ip*D which code for signalling of epithelial cells forming a cytoskeletal arrangement, allowing entry of the bacteria into the host. It also has *Ics*A gene which enables localization of outer membrane allowing the bacteria to bind to the host (Ledwaba et al., 2018; Steiner et al., 2011). Most strains of EIEC are genetically, biochemically and pathogenically much related to *Shigella*.

Mainly, EIEC causes bloody diarrhoea that is clinically indistinguishable from that caused by *Shigella* (Nataro and Kaper, 1998). The expression of virulence genes is transcriptionally regulated by temperature; bacteria which are invasive when grown at 37°C become non-invasive when grown at 30°C (Sooka et al., 2004). This study targeted gene *ial* and *Ip*AH for the detection of EIEC (Omar and Barnard, 2014).

- **Enteropathogenic *E. coli* (EPEC)**

Enteropathogenic *E. coli* is a common cause of infantile diarrhoea in low-income settings and is known to cause sporadic diarrhoea in industrialized countries (Dean et al., 2013; Chandra et al., 2012; Abba et al., 2009; Alikhani et al., 2006; Clarke et al., 2003). EPEC is commonly known to infect infants younger than 12 months old. This pathotype is prevalent in hospital settings and in the community (Liu, 2015; Ochoa and Contreras, 2011).

EPEC induces profuse watery, sometimes bloody diarrhoea with mucus, fever and dehydration. Bloody diarrhoea is associated with the attachment of the EPEC strain to the epithelial cells of the intestines, leading to its physical alteration by the formation of attaching and effacing lesions, which interfere with the host cell signal transduction (Rivas et al., 2015). Virulence genes are located on the EPEC adherence factor (EAF) and enable the localised adherence of bacteria to intestinal walls with no toxins produced (Todar, 2009). The destruction of the epithelial cells leads to subsequent diarrhoea and it is more common in children residing in developing countries (Prescott et al., 2005). In this study the presence of EPEC was detected by m-PCR targeting *eaeA* and *bfp* genes (Omar and Barnard, 2014).

- **Enteroaggregative *E. coli* (EAEC)**

EAEC is a known emerging enteric pathogen associated with persistent diarrhoea in children under the age of two years, both in developed and low-income settings (Delair, 2016; Boisen et al., 2015; Cordeiro et al., 2008; Bhardwaj et al., 2006; Cerna

et al., 2003). In the elderly, EAEC is isolated in both healthy and sick persons (Ruitller et al., 2006).

EAEC are characterized by aggressive adherence (AA) to Hep-2 cells in a stacked-brick pattern (Nataro et al, 1987).The EAEC produces an enteroaggregative heat-stable toxin, which is similar to the heat-stable enterotoxin produced by ETEC and it is encoded on a plasmid by the *astA* genes and this toxin is thought to be responsible for the symptoms of infection (Sekse et al., 2009). Many of the EAEC strains attach to Hep-2 cells in an aggregative adherence pattern, which lead to cytotoxic effects on the intestinal mucosa. The detection of EAEC is mediated by the presence of *AggR* activator gene (Nataro and Kaper, 1998). For this pathotype, the *eagg* and *Asta* genes were targeted in this study (Omar and Barnard, 2014).

- **Enterohaemorrhagic *E. coli* (EHEC)**

Enterohaemorrhagic E*. coli* was identified in 1982 as a dangerous foodborne pathogen responsible for the hemorrhagic colitis outbreak after consumption of contaminated hamburgers from a fast food restaurant in the USA (Su and Brand, 1995). Sporadic infections are known to occur with EHEC, with reported outbreaks in DCCs, nursing homes and other closed settings (Cohen., 1996). Infections are mostly associated with children under the age of five years (Dennehy, 2005). Boyce et al. (1995) reported EHEC to causing 10 000 to 20 000 infections with 250 deaths annually.

EHEC produces a shiga-like toxin (*Stx*). Virulence of EHEC have two subunits which are *stx1* and *stx2* which are integrated in the plasmid (Liu, 2015). *Stx1* is neutralized by antiserum against shiga toxin of *S. dysenterial*; however, *Stx2* is not neutralized by the anti-shiga toxin. EHEC has been associated with foodborne outbreaks in industrialized countries worldwide, causing deaths every year (Gould et al., 2013). The genes *stx1*, *stx2* and *eaeA* were used to detect EHEC (Omar and Barnard, 2014).

- **Enterotoxigenic (ETEC)**

In developing countries, ETEC has been associated with childhood diarrhoea with over 400 million reported cases, half resulted to death (Torres et al., 2015). This pathotype in developed countries is known to be a leading cause of traveller's diarrhoea among areas that have poor hygienic conditions such as Africa and Asia (Ennis et al., 2012; Ram et al., 2010; Galbadge et al., 2009; Quadri et al., 2005; Kaper et al., 2004).

Diarrhoea is caused through plasmid-mediated enterotoxins (Delair., 2016; Rajkhowa et al., 2009). Virulence genes *lt-1* and *st-a* were used in this study to detect ETEC (Omar and Barnard, 2014).

2.5.3 GENERAL SYMPTOMS OF DISEASES CAUSED BY *E. COLI* PATHOTYPES

The general symptoms of disease caused by *E. coli* pathotypes are diarrhoea as well as bloody diarrhoea, and in the case of enterohemorrhagic strains (e.g., *E. coli* O157:H7), haemolytic uremic syndrome (Anderson et al., 1997).

2.5.4 LABORATORY DIAGNOSIS OF *E. COLI*

Escherichia coli detection can be done using culture methods. However biochemical tests can be done to identify the presumptive *E. coli* isolates. Molecular methods can also be used in detection of *E. coli* such as multiplex PCR.

Culture methods are used as the gold standard for pathogen detection and identification. Traditionally methods include membrane filtration, multiple-tube fermentation and more recently used the colilert® quanti-tray® 2000 (Maheux et al., 2015; Bain et al., 2012).

The Analytical Profiling Index (API) is a method of identification of non-fastidious, negative rods in which has 20 reduced biochemical tests and uses a database for identification of the Gram-negative, Enterobacteriaceae or non-Enterobacteriaceae. It contains 20 micro tubes in one strip which comprise of dehydrated substrates. The suspension of bacteria that re-forms the media are inoculated with the 20 reduced biochemical tests. After the incubation period, a variety of colours, which are either spontaneous or shown by the addition of reagents, occur as a result of metabolism. The interpretation table allows the readings of the reactions and identification by referring to the identification profile (Bio Merieux® SA).

The Multiplex Polymerase reaction (m-PCR) allows the amplification of different DNA sequences simultaneously using polymerase chain reaction (PCR). DNA in samples is amplified using multiple primers and a temperature mediated DNA polymerase in a thermal cycler. The design of primers should be optimized for all primers so that all primers can work at the same annealing temperature during the PCR (SA Biosciences and Qiagen).

2.6 ENTERIC VIRUSES

Some enteric viruses include human astrovirus, adenovirus, rotavirus, enterovirus, sapovirus, hepatitis A and E, poliovirus types 1 and 2, echoviruses and coxsakievirus. However, this study focused only on human norovirus.

2.6.1 A BRIEF HISTORY OF HUMAN NOROVIRUS (NoV)

NoV illness was first described as hyperemesis hemis or winter vomiting disease by Zahorsky in 1929. The illness was characterized by diarrhoea and self-limiting vomiting that typically peaked in the winter months/season (Zahorsky., 1929). In 1972, the virus was first detected by immune microscopic examination of stool samples from volunteer students and teachers from Norwalk Ohio, who were affected by the gastroenteritis outbreak; thus, the virus was named as the Norwalk virus (Kapikian et al., 1972).

2.6.2 CLASSIFICATION OF HUMAN NOROVIRUS

NoV are non-enveloped, single-stranded, and positive-sensed RNA viruses, have a genome size of 7.3 to 7.4 kbs in length, are approximately 38 nm in diameter and have 3 Open Reading Frames (Figure 2.1) (ORF's), (Park et al., 2012). ORF1 is made of six structural proteins (p48, NTPase, p22, VPg, 3CL and RNA dependant RNA polymerase (RdRp)) that are involved in viral replication (Kroneman et al., 2013; Hardy, 2005). ORF2 translates the viral protein 1 (VP1) which consists of N-terminal domain, the shell domain (S) and the protruding 1(P1) and protruding 2 (P2) units (Tan et al., 2003). ORF3 encodes a small virion-associated protein (Kabue et al., 2016b; Zheng et al., 2006; Hardy 2005)

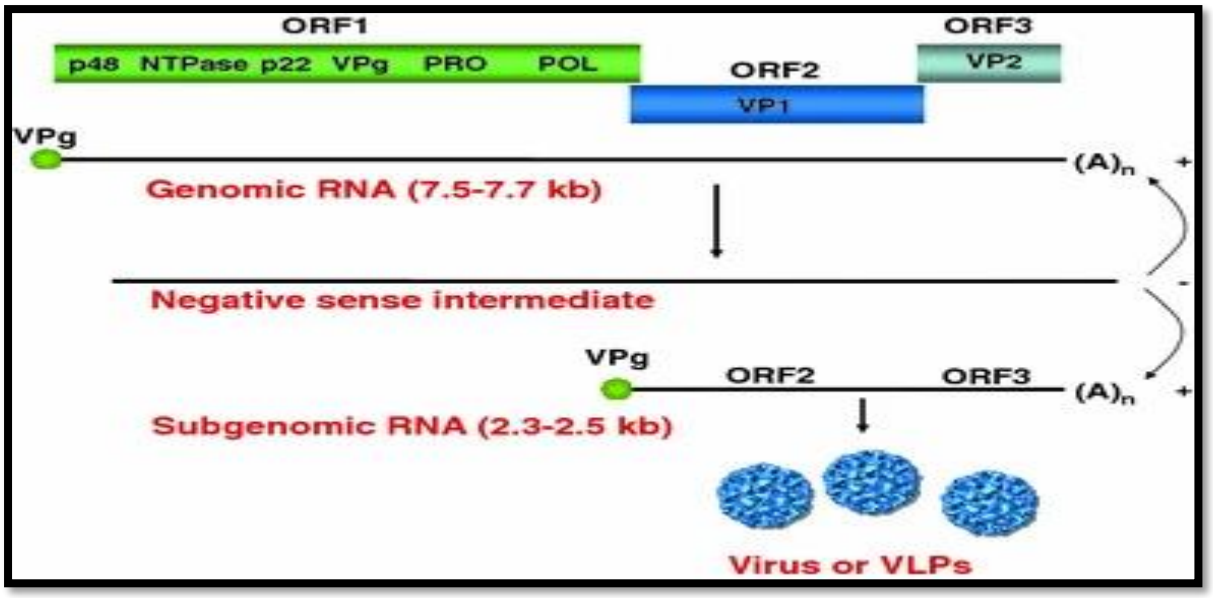

Figure 2.1: NoV genome organization, showing 3 ORF's adapted from Atmar., (2010).

Based on analysis of their sequences, human NoVs have been divided into seven genogroups (Figure 2.2) (G), GI, GII, GIV; GIII and GV are only found in animals (Kroneman et al., 2013). Genotype I (GI) has 14 GI and GII has 29 GII capsid genotypes, which are predominant in human NoV infections. These genotypes are highly associated with gastroenteritis epidemics globally (Patel et al., 2008).

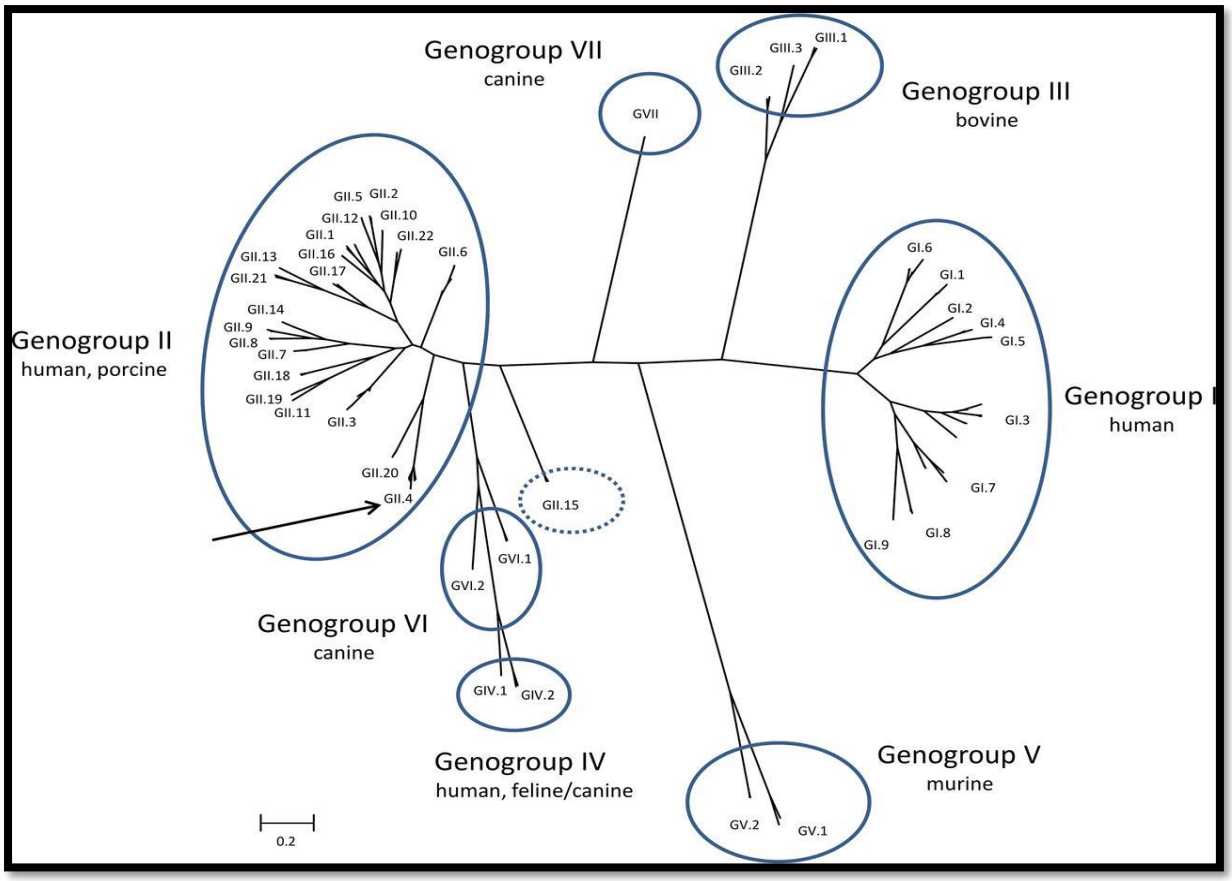

Figure 2.2: NoV classification into seven genogroups based on sequence of amino acid diversity in complete VP1 capsid protein. Adapted from Vinje, (2015).

Previously, NoVs was known to only contain seven genogroups (Figure 2.2), which infected humans, porcine, feminine, murine, canine and bovine. However, according to Chhabra et al., (2019), the genogroups have expanded from seven to now having ten genogroups as several new unclassified NoVs have been identified in harbour porpoises, sea lions and bats.

2.6.3 PATHOGENESIS OF HUMAN NOROVIRUS

Due to difficulties in NoV in vitro cultivation and animal models, most pathogenesis and immunological data relating to NoV infection have been obtained from human volunteer studies (Ettayebi et al., 2016). Up to date, knowledge on pathogenesis of NoV infection has been based on histological, physical and biochemical studies of infected human volunteers (Kabue et al., 2016b, Karst., 2010).

According to Karst (2010), NoV pathogenesis is firstly characterised by, histological alterations of the intestines, secondly physical and biochemical manifestation, and lastly systemic infection.

Through ingestion, the virus enters the jejunum and duodenum, thus, infecting the enterocytes in the pilli, leading to atrophy and crypt hyperplasia. Infection then leads to diarrhoea with malabsorption and increased secretion (Ledwaba et al., 2018).

2.6.4 EPIDEMIOLOGY OF HUMAN NOROVIRUS

Noroviruses affect individuals of all age groups but have been seen predominantly in under-five years old children with the highest norovirus gastroenteritis rates (Lopman et al., 2016). On average, a person may experience approximately three to eight episodes of NoV illness in their lifetime, of which one will occur by five years of age (Phillips et al., 2010). The incidence of NoV disease in under five children is approximately five times higher (21% per year) in comparison to the whole population (4.5% per year) in high-income countries. However, in low-income settings, cohort studies have pointed to a higher disease incidence in children under five years of age (Lopman., 2015; Saito et al., 2013; Phillips et al., 2010).

2.6.5 TRANSMISSION OF HUMAN NOROVIRUS

Human NoV is mainly transmitted through the faecal-oral-route, through person-to-person contact, ingestion of contaminated water or food, contact with fomites as well as inhalation of airborne aerosol (Figure 2.1) (Sabria et al., 2014; Matthews et al., 2012).

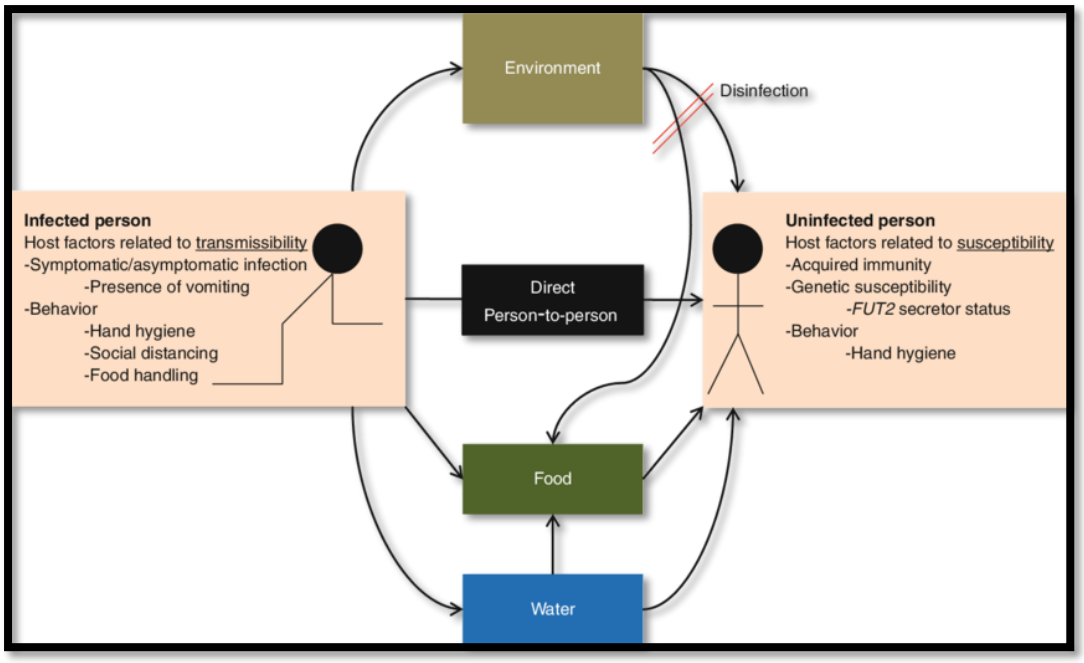

Figure 2.3: Transmission of NoV (Lopman et al., 2012)

2.6.6 SYMPTOMS OF HUMAN NOROVIRUS INFECTION

Typical symptoms include nausea, vomiting, and appearance of diarrhoea within 12 hours of virus ingestion; this may usually last for 1-3 days. In immunocompromised and the elderly, NoV infection is fatal and may last for days (Mathijs et al., 2012; Harris et al., 2011).

2.6.7 LABORATORY DIAGNOSIS OF HUMAN NOROVIRUS

Human NoV cannot be cultured and there is no suitable animal model for infection with this virus, so PCR is the main detection technique used for NoV in water, environmental and food samples (Loisy et al., 2005; Kageyama et al., 2003). Norovirus RNA can be analysed by sequencing the nucleotides in determining the genotypes and assisting in phylogenetic analysis, which is important in epidemiological studies (Mans et al., 2010; Moyo et al., 2007; Vinje et al., 2003).

2.6.8 PREVENTION OF HUMAN NOROVIRUS INFECTION

NoV prevention is mainly based on the awareness of the transmission modes, hand hygiene, taking precautionary measures against fomites, thorough control and cleaning of contaminated food and water (Kabue et al., 2016b; Patel et al., 2009). However, during NoV outbreaks, the Center of Disease and Control (CDC) has proposed guidelines in healthcare settings that include, the use of personal protective

equipment, hand hygiene, leaving policies for staff, staff cohorting, the isolation of symptomatic patients, ward closure, education, visitor policies, surveillance and environmental disinfection (CDC, 2011).

2.7 SUMMARY OF LITERATURE REVIEW

Daycare centers have become a frequent place for children outside their homes and a potential environment for contamination with infectious microorganisms (Castro et al., 2015). Enteric diseases are caused by enteric pathogens and among others include gastroenteritis and diarrhoea. Human Norovirus is the leading causative agent of non-bacterial gastroenteritis which mostly affects all age groups in both developing and developed countries and accounts for approximately 200 000 deaths annually (Phumpholsup et al., 2015a; Yang et al., 2010; Patel et al., 2008). Moreover, *E. coli* is used as an indicator organism, there are subgroups related to pathogenic strains such as enteropathogenic (EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), and enterohemorrhagic (EHEC).

Enteric pathogens are mainly spread through the fecal-oral-route, with many sources playing a role in the transfer from one place to another. Different pathways of contamination include hand contact with fomites, hand contact with open drains, accidental contact with child's own feces, hand mouthing, eating without washing hands, hands of caregivers and the type of water storage used in the daycare environment (Navab-Daneshmand et al., 2018; Wang et al., 2017). There are factors that influence the transmission and these include: poor hygiene from both the care staff and children, larger size of the center (the greater the risk of introduction of microbes), a higher ratio of children to caregivers reduces the individual hygiene supervision of each child and drooling, coughing, sneezing are common thus allowing contamination from air and inanimate objects such as toys in the day care environment (Brady, 2005).

In the Vhembe region, previous studies reported high prevalence of *E. coli* and human NoV; however, there is limited data in daycare settings and children attending daycare centers.

CHAPTER 3: METHODOLOGY

3.1 ETHICAL CLEARANCE AND CONSENT

Ethical clearance was obtained from the University of Venda Research Ethics Committee (SMNS/19/MBY/04/2204: Appendix 1). Before sample collection, signed consent forms with their respective questionnaires were obtained from the participating Daycare center principal (profile A), daycare worker (profile B), parents/ guardians of the participating children (profile C) and stool sample participants (profile D) (Appendix 2).

3.2 STUDY SITE

Vhembe (Figure 3.1) is one of the five districts of Limpopo Province of South Africa. It is in the northern region of the country and is mainly a rural, poverty-stricken community with poor sanitation and hygienic practices (Traore et al., 2016). It should be noted that Daycare center (DCC) B was recruited in a peri-urban area around Thohoyandou (Figure 3.1) and DCC A was recruited to be part of the study in Ha-Lambani (Figure 3.1) which is a rural area.

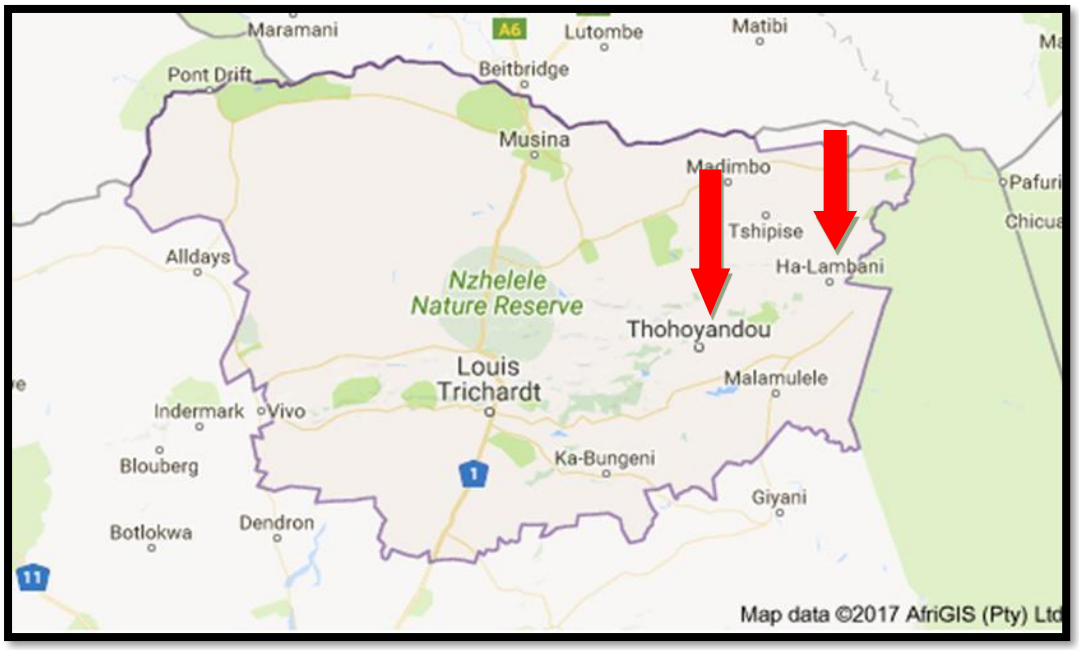

Figure 3.1: Map of Vhembe District indicating the two daycare (https://www.google.com/search?q=map+of+vhembe+district).

3.3 SAMPLE COLLECTION

Figure 3.2 provides an outlay of the methodology followed in this study.

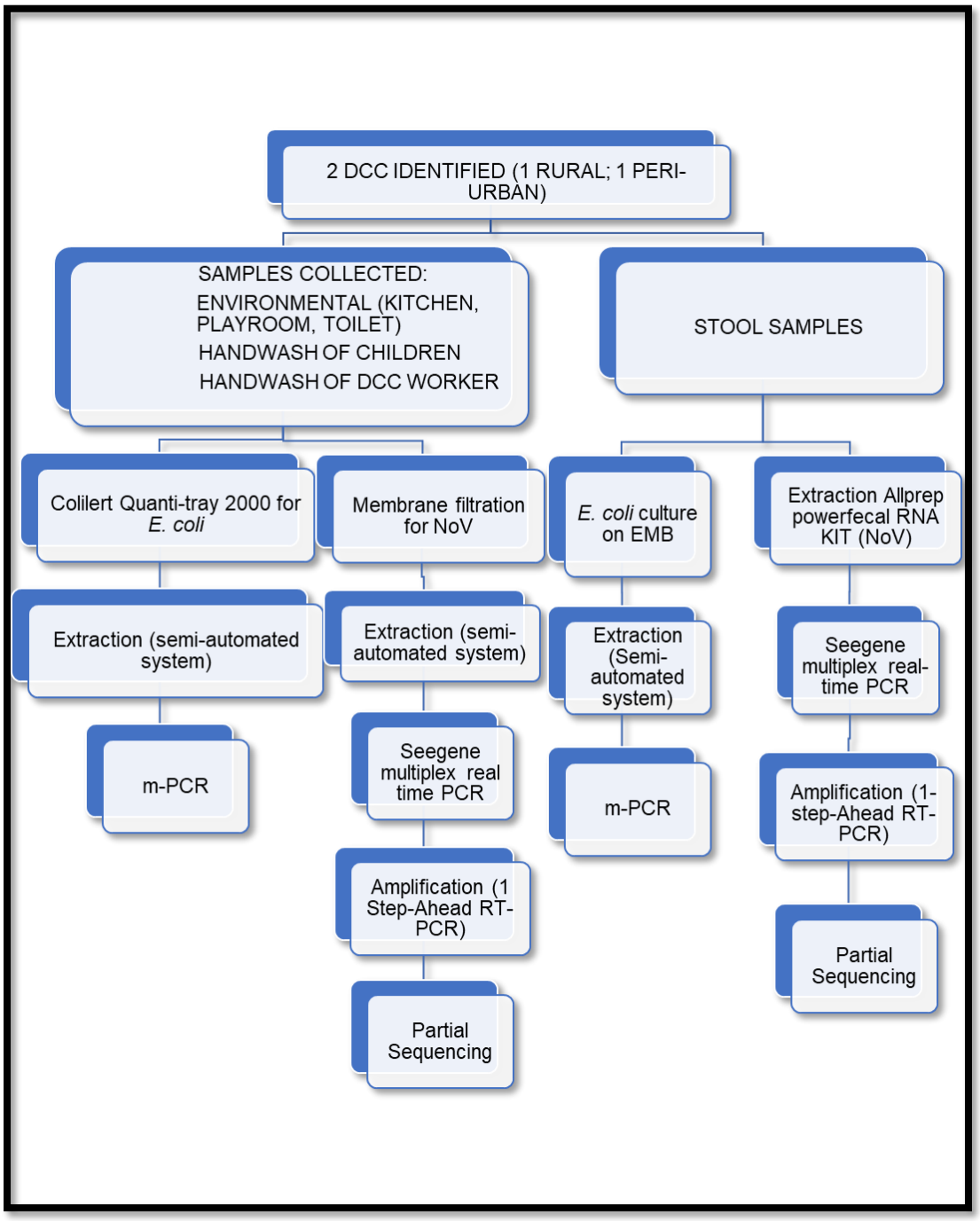

Figure 3.2: A schematic illustration of the methodology followed in this study.

In addition, questionnaires were used to collect demographic data on water, sanitation, the daycare profile, daycare worker and child profiles.

A total of 83 samples (from August to November 2019) were collected once from two (one rural and one peri-urban) randomly selected DCCs in the Vhembe region in

Limpopo Province. In each DCC, handwash samples were taken from the Daycare worker's hands and hands of child in a sterile bag containing 350 ml of PBS using the method described by Mattioli et al., (2015). Stool samples were collected from different diapers collected at each DCC. Inside the daycare center, swab samples were taken from the different areas as described in Table 3.1. It should be noted that not all sampling areas were found, especially in the rural DCC.

Table 3.1: Different sampling points in the DCC in the Vhembe District, Limpopo Province South Africa.

LOCATION IN THE DAY CARE CENTRE	PLACE IN LOCATION
ENVIRONMENTAL SAMPLES	
Kitchen (N=5)	➢ Kitchen table ➢ Kitchen sink ➢ Refrigerator door ➢ Kitchen floor ➢ Storage of drinking water
Playroom (N=5)	➢ Table ➢ Plastic toys ➢ Wooden toys ➢ Food toys ➢ Pillow toys
Toilet (N=5)	➢ Toilet seat ➢ Toilet floor ➢ Diaper changing area/table ➢ Sink ➢ Diaper pail lid
HANDWASH SAMPLES	
Handwash (N=20)	➢ Handwash of day care workers (5) ➢ Handwash of children (0-1 years x5) ➢ Handwash of children (1-3 years x5) ➢ Handwash of children (3-5 years x5)
STOOL SAMPLES	
Stool samples from children's diapers	➢ 22 stool samples

During transportation (30 minutes) to the laboratory of the University of Venda, the samples were stored on ice in a cooler box and analyzed immediately.

3.4 BACTERIAL ANALYSIS

3.4.1 DETECTION AND ENUMERATION OF *E. COLI*

The Colilert®/Quanti-tray®/2000 (IDEXX, Maine) system was used to enumerate viable *E. coli* cells from the 100 ml of handwash samples and 100 ml of swab samples (dilution of 1:40) according to the manufacturer's instructions. The Quanti-trays® were incubated at 35ºC for 18 hours. The Quanti-trays® were examined under UV light of wavelength 366 nm and the well which fluorescenced were identified as *E. coli* positive (Figure 3.3), thus their number recorded per manufacturers results table provided (Omar et al., 2010).

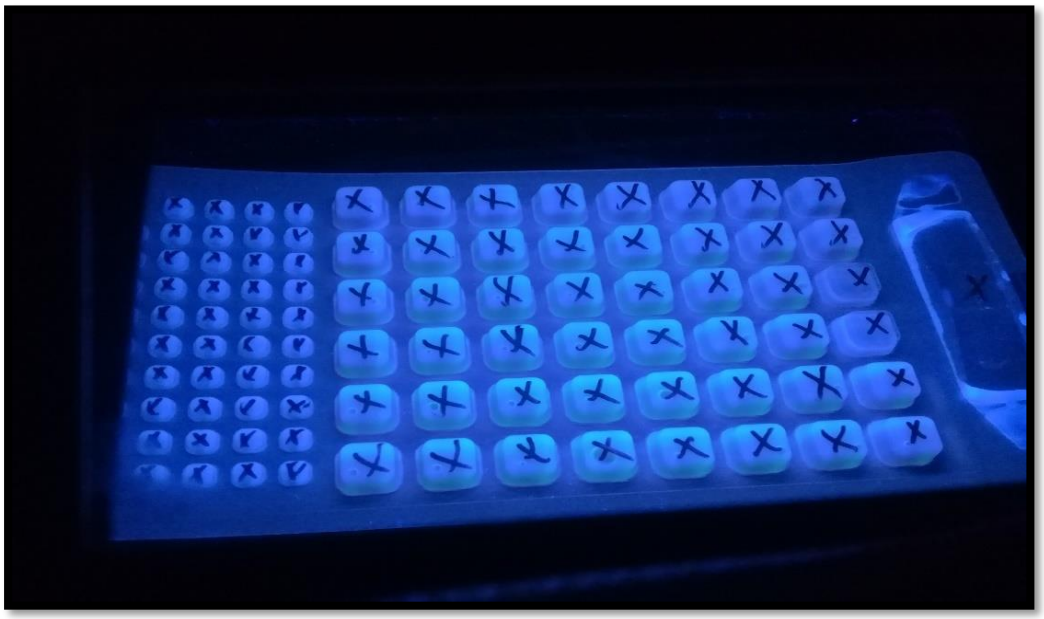

Figure 3.3: Quanti-tray® 2000 under UV light showing fluorescent wells positive for *E. coli*, marked with a X.

3.4.2 CULTURING OF *E. COLI* FROM STOOLS SAMPLES

Upon arrival at the laboratory, stool samples were cultured in Eosin Methylene Blue Agar (EMB) (Davies Diagnostics) (Appendix 3) and incubated at 37ºC for 24 hours. EMB is a selective media for isolation of Gram-negative bacteria that contains dyes (Eosin and Methylene blue) which are toxic to Gram-positive bacteria. *E. coli* ferments lactose, thus, showing nucleated colonies with dark centers (metallic green shinning) (Heidary et al., 2014).

Presumptive *E. coli* positive samples from EMB were further tested using three biochemical tests, namely, Kligler's Iron Agar (KIA), Indole and Urease test. *E. coli*

produces indole through an enzyme, tryptophanase, encoded by the *tnaA* gene reducing tryptophan to pyruvate, ammonia, and indole in a reversible reaction (Li and Young., 2013; Hu et al., 2010). *E. coli* is urease-negative, as it cannot produce the urease enzyme for the degradation of urea (Konieczna et al., 2012). The KIA is a differential mixture that tests for an organism's ability to ferment glucose and lactose plus gas products. *E. coli* ferments lactose, producing a yellow slant and yellow butt with bubbles (Johnson et al., 1966).

3.4.3 DNA EXTRACTION USING SEMI-AUTOMATED SYSTEM

Ten (10) positive E. coli wells were randomly selected. Two milliliters of each isolate was collected using 1 ml disposable syringe, aliquoted into 2 ml sterile Eppendorf tubes (AEC Amersham, SA) and stored at ±4ºC. The DX universal Liquid sample DNA extraction protocol use 4 steps; firstly, lysis of the cells, secondly binding of the nucleic acids to silica membranes, thirdly, washing away of contaminants and a final fourth step of elution of purified nucleic acids from the membrane.

Briefly, 2 ml of sample was loaded on the 2 ml plate then centrifuged for 15 mins at 1800 rpm. 100 ul of Dx liquid digest +10% of DX digest enzyme was added. A further 400 ul of Dx binding, with additive, was added and this was transferred from the lysis plate to plate B. 200 ul of binding buffer (DXB) and 600 ul wash buffer (DXW) was added. The wash step was repeated and 600 ul final wash (DXF) was added. 150 ul of elution buffer was added and incubated for 5 mins at room temperature. Eluted total DNA was stored at -20ºC for further analysis (Delair., 2016).

3.4.4 MULTIPLEX POLYMERASE CHAIN REACTION (M-PCR)

All PCR assays were performed in a Biorad Thermal cycler (Hercules, California U.S.A). A multiplex PCR kit (Qiagen, Cat No./ID: 206143) was used for the m-PCR protocol as developed by Omar et al., (2010). Primers that were used are listed in Table 3.3. Briefly, cycling parameters included an initial activation step at 95ºC for 15 minutes, 35 cycles which consisted of denaturation at 95ºC for 45 seconds, extension at 68ºC for 2 minutes and a final elongation at 72ºC for 5 minutes (Omar and Barnard, 2014). Amplicons were separated by electrophoresis on a 2.5% (w/v) agarose gel stained with ethidium bromide and specific sized bands visualized using a UV light transilluminator. To avoid contamination, all steps of sample processing were done in separate rooms following good laboratory practice.

Table 3.2: List of *E. coli* specific primers for m-PCR used in this study (Omar et al., 2010)

Pathogen	Primer	Sequence (50-30)	Size (base pair)	Reference
E. coli	Mdh (F) Mdh (R)	GGT ATG GAT CGT TCC GAC CT GGC AGA ATG GTA ACA CCA GAG T	300	Tarr et al., (2002)
EIEC	lal (F) lal (R)	GGTATGATGATGATGAGTGGC GGAGGCCAACAATTATTTCC	630	Paton & Paton (1998)
EHEC/EPEC	EaeA (F) EaeA (R)	CTG AAC GGC GAT TAC GCG AA GAC GAT ACG ATC CAG	917	Aranda et al., (2007)
EAEC	Eagg (F) Eagg (R)	AGA CTC TGG CGA AAG ACT GTA TC ATG GCT GTC TT AAT AGA TGA GAA C	194	Kong et al., (2002)
EHEC	Stx1 (F) Stx1 (R) Stx2 (F) Stx2 (R)	ACA CTG GAT GAT CTC AGT GG CTG AAT CCC CCT CCA TTA TG CCA TGA CAA CGG ACA GCA GTT CCT GTC AAC TGA GCA CTT TG	614 779 	Moses et al., (2006) Moses et al., (2006)
ETEC	LT (F) LT (R) ST (F) ST (R)	GGC GAC AGA TTA TAC CGT GC CGG TCT CTA TAT TCC CTG TT TTT CCC CTC TTT TAG TCA GTC AAC TG GGC AGG ATT ACA ACA AAG TTC ACA	330 160 	Pass et al., (2000) Pass et al., (2000)

3.5 VIRUS ANALYSIS

3.5.1 MEMBRANE FILTRATION OF NOROVIRUS

Handwash samples and swab (dilution of 1:40) samples were processed for Human Norovirus using membrane filtration by passing 100 ml of sample through 47 mm, 0.45 µm pore size nitro-cellulose filters (HA-type filters; Millipore, Billerica, MA). The virus-bound membrane filter was adsorbed using 25 ml of 0.5 mM of MgCl2 (Rochelle Chemicals, RSA) and the membrane filter rinsed with 350 ml of 0.5 mM of H2SO4 (Rochelle Chemicals, RSA) to elute cations and fix viral particles unto the membrane. The filter was placed in a petri-dish containing 12 ml of 1 mM NaOH (Rochelle Chemicals, RSA), the petri dish was shaken for ten minutes at room temperature to release the membrane bound viruses. To stabilize the solution, 50 µl of 0.5 mM H2SO4 and 1X TE buffer was added. The eluted sample was then ultrafiltered at 1500 x g for 10 minutes at room temperature to a final volume of 2 ml which was stored at -20ºC for further analysis (Victoria et al., 2009). It should be noted that a modification was made on the ultrafiltration step at room temperature instead of at +4ºC.

3.5.2 NUCLEIC ACID EXTRACTION USING ALLPREP® POWERFECAL® RNA KIT (QIAGEN)

The AllPrep® PowerFecal® DNA/RNA Kit (Cat No./ID: 80244) is designed to purify microbial DNA and RNA simultaneously from the same stool sample, while separating the DNA and RNA into separate eluate fractions. The AllPrep® PowerFecal® DNA/RNA Kit integrates QIAGEN's technology for selective binding of double-stranded DNA with well-established RNeasy technology in addition to incorporating Inhibition Removal Technology (IRT) during initial sample lysis (www.qiagen.com).

The recommended starting material is 0.1–0.2 g of stool. Each sample was homogenized in a 2 ml bead beating tube containing a mixture of lysis beads. Lysis of host and microbial cells was facilitated by both mechanical collisions between beads and chemical disruption of cell membranes. IRT was then used to remove common substances in stool samples that interfere with PCR and other downstream applications. The lysate was then passed through an AllPrep DNA MinElute Spin Column. This column, in combination with a high-salt buffer, allows selective and efficient binding of genomic DNA. DNA was then washed and eluted and was ready for PCR analysis.

Ethanol was added to the flow-through from the AllPrep DNA MinElute Spin Column to provide appropriate binding conditions for RNA. The sample was then transferred on a RNeasy Mini Spin Column, where total RNA binds to the membrane. RNA was then washed and eluted in RNase-Free Water. RNA was then stored at -20°C and used for further analysis.

3.5.3 DETECTION USING SEEGENE ALLPLEX™ GASTROINTESTINAL FULL PANEL ASSAY

Stored RNA was used for the detection of Nov using the Allplex™ Gastrointestinal Panel Assays (AGPA) (Seegene, Seoul, South Korea, Catalog Code: GI9701X) which is a multiplex real-time PCR assay that detects 13 bacteria (*Aeromonas* spp, *Campylobacter* spp , *Clostridium difficile*, *Salmonella* spp, *Shigella* spp, *Vibrio cholerae*, *Yersinia enterocolitica*, EAEC (aggR), EPEC (eaeA), *Escherichia coli* O157, ETEC (lt/st), STEC (stx1/2), EIEC (ipaH)), 6 viruses (*Adenovirus* 40/41, *Norovirus* GI/GII, *Rotavirus*, *Astrovirus*, *Sapovirus*), and 6 parasites (*Cryptosporidium* spp, *Entamoeba histolytica*, *Giardia lamblia*, *Blastocystis hominis*, *Dientamoeba fragilis*,

Cyclospora cayetanensis) in 4 multiplex PCR reactions (two bacterial, one viral and one parasitic).

The Microlab Nimbus IVD system automatically performed the nucleic acid processing and PCR setup. Fluorescence was detected at 2 temperatures (60°C and 72°C), and a positive test result was defined as a well-defined exponential fluorescence curve that crossed the crossing threshold at a value of less than 42 for individual targets (Yoo et al., 2019). To avoid contamination, all steps of sample processing were done in separate rooms following good laboratory practice.

3.5.4 AMPLIFICATION OF NOROVIRUS USING ONE-STEP AHEAD RT-PCR

Extracts that tested positive for norovirus by Allplex™ Gastrointestinal Panel Assay were then subjected to RT-PCR amplification for the purpose of nucleotides sequencing. Primers which were used for RT-PCR are given in Table 3.2. The specific oligonucleotide primer pair G1SKF/G1SKR to amplify 330 bp of the capsid region of NoV genogroup I, G2SKF/G2SKR to amplify 344 bp of the capsid region and JV12/JV13 to amplify 326 bp of the polymerase gene of NoV genogroups II were used to performed One-step Ahead RT-PCR (QIAGEN, GmbH, Germany) as previously described by Kojima et al (2002). For the amplification of the genome of NoV strains, the cDNA was synthesized from 10% of diluted 5µl of RNA extracts for 10 mins at 50 °C followed by heating at 95°C for 5 min to inactivate the enzyme and then amplification using the supplied 0.5 µM of each oligonucleotide primer in 25 µl reaction mixture. Thermocycling conditions for PCR were carried out for 40 cycles as follows: denaturation at 95°C for 10 seconds, annealing at 50°C for 10 seconds (GISK primers) or 56°C (GII SK primers), extension at 72°C for 10 seconds and final extension at 72°C for 10 minutes (Jonckheere et al., 2017). To avoid contamination, all steps of sample processing were done in separate rooms following good laboratory practice.

3.5.5 SEQUENCING AND GENOTYPING OF NOROVIRUS

PCR products were then analysed using a 2.0 % (w/v) agarose gel in TAE buffer stained with ethidium bromine (Fermentas, Waltham, Massachusetts, United States). The RT-PCR products of the amplified fragments were directly purified with a master mix of ExoSAP (Nucleics, Australia). The Sanger sequencing was performed on the ABI 3500XL Genetic Analyzer POP7™ (Thermo-Scientific, Waltham, Massachusetts, United States) using the same specific primers. The raw sequence reads were edited with Finch TV v1.4 (Geospiza, Seattle, USA). The nucleotide sequences obtained from

the selected NoV strains were put on the Noronet typing tool (available at http://www.rivm.nlm/norovirus/typingtool) to get the genotype. To search similar sequences in the NCBI genetic database, BLAST tool was used (available at: http://www.ncbi.nlm.nih.gov/) (Kroneman et al., 2011). The reference strains from Genbank were randomly selected among the Blast hits with >80% similarities on the query sequence of the NoV strains identified from this study (Kabue et al., 2016a).

3.5.6 PHYLOGENETIC ANALYSIS OF NOROVIRUS

Phylogenetic trees were constructed by the neighbor-joining method (Saitou et al., 1987; Tamura et al., 2004) using MEGA 10 software, with 1,000 bootstrap replicates for each gene (Kumar et al., 2018).

Table 3.3: List of NoV specific primers used in this study (Kojima et al., 2002).

Primers	Sequence (5′- 3′)	Polarity	Genotype	Target Size (bp)	Nucleotide	Position	Reference
G1SKF	CTGCCCGAATTYGTAAATGA	F	GI NoV	Capsid	330 bp	5342	Kojima et al., 2002
G1SKR	CCAACCCARCCATTRTACA	R	GI NoV	Capsid		5671	Kojima et al., 2002
G2SKF	CNTGGGAGGGCGATCGCAA	F	GII NoV	Capsid	344 bp	5058	Kojima et al., 2002
G2SKR	CCRCCNGCATRHCCRTTRTACAT	R	GII NoV	Capsid		5401	Kojima et al., 2002
JV12	ATA CCA CTA TGA TGC AGA TTA	F	GII NoV	Polymerase	326 bp	4552-4572	Vinje and Koopmans, 1996
JV13	TCA TCA TCA CCA TAG AAA GAG	R	GII NoV	Polymerase		4878-4858	Vinje and Koopmans., 1996

CHAPTER 4: RESULTS AND DISCUSSION

The aim of this study was to characterize diarrhoeagenic *E. coli* and human NoV from surfaces swabs, handwash samples of children and daycare workers in two (one peri-urban and one rural) daycare centers in the Vhembe region of the Limpopo Province, South Africa. The prevalence and genetic diversity of these enteric pathogens were determined. This chapter reports the study findings and discusses them in relation to the study aims and objectives as outlined in Chapter 1.

4.1 ANALYSIS OF QUESTIONNAIRES FROM THE DAYCARE CENTERS (DCCs)

PROFILE A

Water, hygiene and sanitation demographics of the two DCCs are shown in Table 4.1. The age distribution of DCC A ranged from 0-4.5 years and DCC B was from 0-5 years. DCC B had a higher number of daycare workers of 24 compared to 8 of DCC A. The number of children attending DCC B was higher by a difference of 125 compared to DCC A. Both DCC A and DCC B used a tap as a water source. DCC A used both pit and flushing toilets while DCC B only used flushing toilets. In both DCCs the children's hands are regularly washed, waste was collected daily, the DCC environment was cleaned daily, kitchen utensils and the floor were washed and cleaned.

Table 4.1: Descriptive water, hygiene and sanitation demographics for the study DCC in the Vhembe region, Limpopo Province, South Africa.

VARIABLE	DCC A (rural)	DCC B (peri-urban)
Number of staff employed at DCC	8	24
Number of children at DCC	127	230
Age distribution at DCC	0-4.5 Years	0-5 Years
Hygienic activities practiced by staff	Hand washing & kitchen utensils	Hand washing, floor cleaning & kitchen utensils
Daily activities of the DCC	Teaching, cleaning & protection	Teaching, protection & childcare

Handling of waste	waste collection daily	Municipal waste collection from dustbins
How often the DCC is cleaned?	Daily	10X daily
Type of toilet used at DCC	Pit and flush toilet	Flush toilet
Water source	Tap	Tap

PROFILE B

Study demographics of the handwash samples of the daycare workers (Table 4.2) showed that in DCC A, all the 5 workers did not wash their hands before receiving the kids, whereas in DCC B, all 5 workers washed their hands. The utensils were reported to be cleaned with running water and soap in DCC B; however, in DCC A they were cleaned with soap in a dish. In both DCCs the children did not use one cup to drink water. The nappy changing differed in both DCCs as in DCC A, the nappies were changed by putting the child on a sponge and changing the nappies. In DCC B, the children were put on a mattress with a towel on top and were changed.

Table 4.2: Descriptive demographics of DCC Workers (Profile B) handwash samples in the Vhembe region, Limpopo Province, South Africa.

VARIABLE	**DCC A (rural) (n=5)**	**DCC B (peri-urban) (n=5)**
Do you wash your hands before receiving the kids?	NO: 5/5 YES: 0	NO: 0 YES: 5\5
Where are the children taken after they are received?	Chairs and sponges.	Play area.
With what are the utensils cleaned?	5/5 With soap and water in a dish.	5/5 Running water and soap.

Do all the kids use one cup to drink water?	NO: 5/5 YES: 0	NO:5/5 YES: 0
Where are nappies changed?	5/5 Changed on sponge.	1/5 Changed on mattress with towel on top.

PROFILE C

Study demographics for the children's hand wash samples (Table 4.3) shows that DCC B had a higher number of males (64%) than DCC A (58%). However, DCC A had a higher number of females (42%) comparing to DCC B which had females (36%). The age distribution in DCC A ranged from 1-4 years with DCC B ranging from 1-5 years. The use of diapers was recorded in DCC A (42%) and DCC B (27%) of the participants in which the handwash samples were taken. In DCC B (45%) of the study participants reported symptoms of diarrhoea while in DCC A only 25% showed these symptoms.

Table 4.3: Demographics of children participating in the study (Profile C) for hand wash samples in the Vhembe region, Limpopo Province; South Africa.

VARIABLE	**DCC A (rural) (n=12)**	**DCC B (peri-urban) (n=11)**
GENDER Total n=23: Males: 14 Females: 9	Male: 7/12 (58%) Females: 5/12 (42%)	Male: 7/11 (64%) Female: 4/11 (36%)

AGE Total n=23: 1 year: 4 2 years: 4 3 years: 7 4 years: 5 5 years: 3	1 year: 3/12 (25%) 2 years: 2/12 (17%) 3 years: 4/12 (33%) 4 years: 3/12 (25%)	1 year: 1/11 (9%) 2 years: 2/11 (18%) 3 years: 3/11 (27%) 4 years: 2/11 (18%) 5 years: 3/11 (27%)
STILL USING NAPPIES Total n=23 Yes: 8 No: 15	Yes: 5/12 (42%) No: 7/12 (58%)	Yes: 3/11 (27%) No: 8/11 (73%)
SHOWED SYMPTOMS OF DIARRHOEA Total n=23 Yes: 8 No:15	Yes: 3/12 (25%) No: 9/12 (75%)	Yes: 5/11 (45%) No: 6/11 (55%)

PROFILE D

Demographics of participants in this study (Table 4.4) had an age distribution of one (57%) and two (45%) years in DCC A and two (87%) and three (13%) in DCC B of the sampled stool samples. The gender showed a higher number of males (57%) to females (43%) in DCC A whereas in DCC B had a higher number of females (67%) in comparison to males (33%). The sampled participants showed symptoms of diarrhoea (47%) in DCC B while DCC A had only asymptomatic cases. The type of stools differed in both DCC with mushy (57%), watery (29%) and sausage (14%) in DCC A. DCC B showed types of stools with mushy (47%), watery (33%) and sausage (20%).

Table 4.4: Demographics of study participants for stool samples in the Vhembe region, Limpopo Province, South Africa.

VARIABLE	DCC A (rural) (n=7)	DCC B (peri-urban) (n=15)
AGE Total n=22: 1 year: 4 2 years: 16 3 years: 2	1 year: 4/7 (57%) 2 years: 3/7 (43%) 3 years: 0	1 year: 0 2 years: 13/15 (87%) 3 years: 2/15 (13%)
GENDER Total n=22: Males: 9 Females: 13	Male: 4/7 (57%) Female: 3/7 (43%)	Male: 5/15 (33%) Female: 10/15 (67%)
TYPE OF SYMPTOM SHOWN Total n=22 Diarrhoea: 7 None: 15	Diarrhoea: 0 None: 7/7 (100%)	Diarrhoea: 7/15 (47%) None: 8/15 (53%)
TYPE OF STOOL Total n=22 Mushy: 11 Watery: 7 Sausage: 4	Mushy: 4/7 (57%) Watery: 2/7 (29%) Sausage: 1/7 (14%)	Mushy: 7/15 (47%) Watery: 5/15 (33%) Sausage: 3/15 (20%)

4.2 BACTERIAL ANALYSIS

4.2.1 DETECTION OF *E. COLI* USING COLILERT®QUANTI TRAY®/2000 IN DCC A AND DCC B

Total coliforms (TC) and *E. coli* counts (Most Probable Number) were compared to the standards set by WHO (2012) as low-risk contamination (when equal to 1-10 MPN/100 ml), intermediate to high-risk (11-100 MPN/100 ml) and when MPN/100 ml is >100 MPN very high-risk contamination (Table 4.5). Of the ten *E. coli,* positive samples in both DCC A (7) and DCC B (3) five samples were found to be of low-risk contamination, one was intermediate-risk and four were of very high contamination-risk.

Table 4.5: Total coliforms and *E. coli* counts (Most Probable Number) in DCC A and DCC B in the Vhembe region, Limpopo Province; South Africa.

	DCC A	**DCC B**	**DCC A**	**DCC B**
Variable name	**Total coliforms (MPN)**	**Total coliforms (MPN)**	***E. coli* (MPN)**	***E. coli* (MPN)**
HANDWASH SAMPLES				
Hand wash of daycare worker	>2419.6 MPN	104.7 MPN	>2419.6 MPN	1.0 MPN
Hand wash of daycare worker	727.0 MPN	1732.9 MPN	**-**	**-**
Hand wash of daycare worker	>2419.6 MPN	866.4 MPN	866.4 MPN	**-**
Hand wash of daycare worker	>2419.6 MPN	>2419.6 MPN	**-**	**-**
Hand wash of daycare worker	307.6 MPN	>2419.6 MPN	**-**	>2419.6 MPN

Hand wash of child	>2419.6 MPN	517.2 MPN	-	-
Hand wash of child	214.2 MPN	261.3 MPN	-	-
Hand wash of child	727.0 MPN	235.9 MPN	-	-
Hand wash of child	547.5 MPN	150.0 MPN	1.0 MPN	-
Hand wash of child	224.7 MPN	179.3 MPN	-	-
Hand wash of child	*	193.5 MPN	*	-
Hand wash of child	248.1 MPN	365.4 MPN	-	-
Hand wash of child	178.9 MPN	>2419.6 MPN	1.0 MPN	-
Hand wash of child	613.1 MPN	1203.3 MPN	-	-
Hand wash of child	>2419.6 MPN	>2419.6 MPN	6.3 MPN	-
Hand wash of child	435.2 MPN	172.2 MPN	-	-
Hand wash of child	>2419.6 MPN	104.7 MPN	-	-
ENVIRONMENTAL SAMPLES				
Kitchen table	2419.6 MPN	32.1 MPN	-	-
Kitchen sink	*	1119.9 MPN	*	-
Fridge door handle	15.3 MPN	-	-	-
Kitchen floor	>2419.6 MPN	9.6 MPN	-	-
Playroom table	*	1.0 MPN	*	-
Playroom pillows/sponge	686.7 MPN	344.8 MPN	-	3.1 MPN
Toilet seat for boys	-	1 MPN	-	-
Toilet seat for girls	36.4 MPN	3.0 MPN	-	-

Toilet floor for boys	55.4 MPN	325.5 MPN	**-**	**-**
Toilet floor for girls	36.4 MPN	16.1 MPN	23.1 MPN	**-**
Nappy changing area	686.7 MPN	4.1 MPN	**-**	**-**
Diaper pail lid	*	3.1 MPN	*	**-**
Toy 1	1.0 MPN	410.6 MPN	**-**	**-**
Toy 2	21.8 MPN	>2419.6 MPN	**-**	**-**
Tap water	**-**	**-**	**-**	**-**
Storage water	>2419.6 MPN	2.0 MPN	158.5 MPN	**-**

Negative result (**-**); Not available on sampling site (*****); Positive result (e.g. 1.0 MPN)

4.2.2 BIOCHEMICAL TEST RESULTS OF SAMPLES IN BOTH DCC A AND DCC B

The results in Table 4.6 show that all stool samples were presumptively positive for *E. coli* on EMB agar, showing metallic green shining colonies. On the KIA test, one sample was presumptively negative from DCC B and all others were positive. The Indole test had six presumptively negative for *E. coli* in DCC B with the rest being positive. All samples in both DCC A and DCC B were presumptively negative for urease.

Table 4.6: Results of presumptive *E. coli* culture on EMB, KIA test, Indole test and Urease test.

SAMPLE NUMBER	EMB	KIA TEST	INDOLE TEST	UREASE TEST
DCC B				
P01	**+**	**+**	**+**	**-**
P02	**+**	**+**	**-**	**-**

P03	+	+	+	-
P04	+	+	+	-
P05	+	+	-	-
P06	+	+	+	-
P07	+	-	+	-
P08	+	+	+	-
P09	+	+	-	-
P10	+	+	+	-
P11	+	+	-	-
P12	+	+	+	-
P13	+	+	-	-
P14	+	+	-	-
P15	+	+	+	-
DCC A				
P16	+	+	+	-
P17	+	+	+	-
P18	+	+	+	-
P19	+	+	+	-
P20	+	+	+	-
P21	+	+	+	-
P22	+	+	+	-

Positive (**+**); Negative (**-**)

4.2.3 PREVALENCE AND GENETIC DIVERSITY OF DIARRHOEGENIC *E. COLI* STRAINS CIRCULATING IN ENVIRONMENTAL SAMPLES, STOOL SAMPLES, HANDWASH OF DAYCARE WORKERS AND CHILDREN IN DCC A AND DCC B.

The prevalence of *E. coli* was 13.6% for handwash samples of the children, 40% for handwash samples of the daycare workers, 10% for environmental samples and 100% for stool samples in both DCCs. Different pathotypes were detected in the various sample types shown in the two DCCs (Table 4.7). Single diarrheagenic *E. coli* pathotypes were not detected. All detections where mixed infections with two or more pathotypes. Both DCC A and DCC B showed co-infection of strains EHEC+ETEC+EAEC (20%) on the hand wash samples of the DCC workers. Co-infections of pathotypes were prevalent in DCC A with EPEC+EIEC+ETEC+EAEC (4.6%) EPEC+ETEC+EAEC (4.6%), and EPEC+EHEC+ETEC+EAEC (4.6%) recorded. The environmental samples had various co-infections with pathotypes in both DCCs.

Table 4.7: Characterization of *E. coli* from hand wash samples, environmental samples and stool samples at DCC A and B in the Vhembe region, Limpopo Province, South Africa.

SAMPLE TYPE	**DCC A (rural)**	**DCC B (peri-urban)**
Hand wash of children Total n=22 Positive: 3/22 (13.6%)	**N=11**	**N=11**
	E. coli Positive: 3/11 (14%) *E. coli* strains: • EPEC+EIEC+ETEC+EAEC (4.6%) • EPEC+ETEC+EAEC (4.6%) • EPEC+EHEC+ETEC+EAEC (4.6%)	***E. coli*** Positive: 0 *E. coli* strains: 0

Handwash of workers Total n=10 Positive: 4/10 (40%)	**N=5**	**N=5**
	E. coli Positive: 2/5 (40%) *E. coli* strains: • EHEC+ETEC+EAEC (20%) • EPEC+EIEC+ETEC+EAEC (20%)	**E. coli** Positive: 2/5 (40%) *E. coli* strains: • EHEC+ETEC+EAEC (20%) • EPEC+ETEC+EAEC (20%)
Environmental samples Total: n=29 Positive: 3/29 (10%)	**N=13**	**N=16**
	E. coli Positive: 2/13 (15%) *E. coli* strains: • EPEC+EHEC+EIEC+ETEC (7.5%) • EIEC+ETEC+EAEC (7.5%)	**E. coli** Positive: 1/16 (6%) *E. coli* strains: • EPEC+EHEC+ETEC+EAEC (6%)
	N=7	**N=15**

Stool samples from diapers Total n=22 Positive: 22/22 (100%)	**E. coli:** Positive: ALL *E. coli* strains: • EHEC+ETEC+EAEC (14%) • EPEC+EHEC+EIEC+EAEC (43%) • EPEC+EHEC+EAEC (14%) • EPEC+EHEC (14%) • EPEC+EHEC+EIEC+ETEC+EAEC (14%)	**E. coli:** Positive: ALL *E. coli* strains: • EPEC+EHEC+EIEC+ETEC+EAEC (20%) • EPEC+EHEC+EIEC+EAEC (20%) • EHEC+EIEC+ETEC+EAEC (7%) • EPEC+EIEC+ETEC+EAEC (7%) • EHEC+ETEC+EAEC (27%) • EPEC+EHEC+EIEC+ETEC (7%) • EPEC+ETEC (7%) • EPEC+EHEC (7%)

In this study, all *E. coli* positive samples were analysed using the 11-gene m-PCR (Omar and Barnard, 2014). Comparisons of PCR product sizes were done using the 11-gene positive control standard shown in figure 4.1 lane 3.

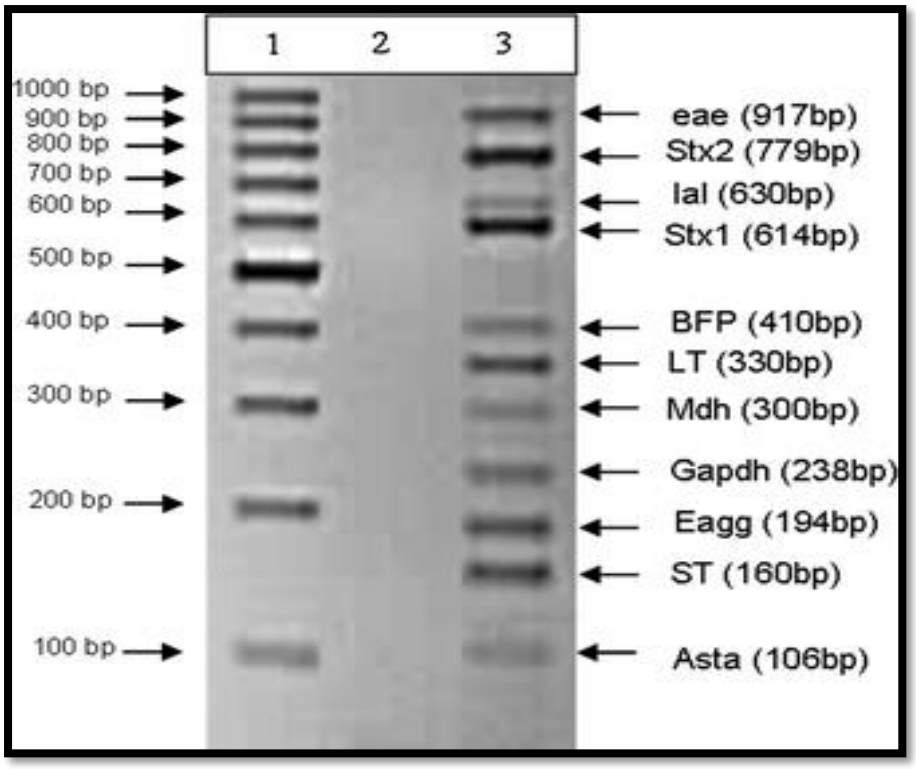

Figure 4.1: *Agarose gel for m-PCR positive control with 100 bp marker (lane 1), PCR negative control (lane 2) and 11-gene positive control (lane 3),* (Omar and Barnard 2014).

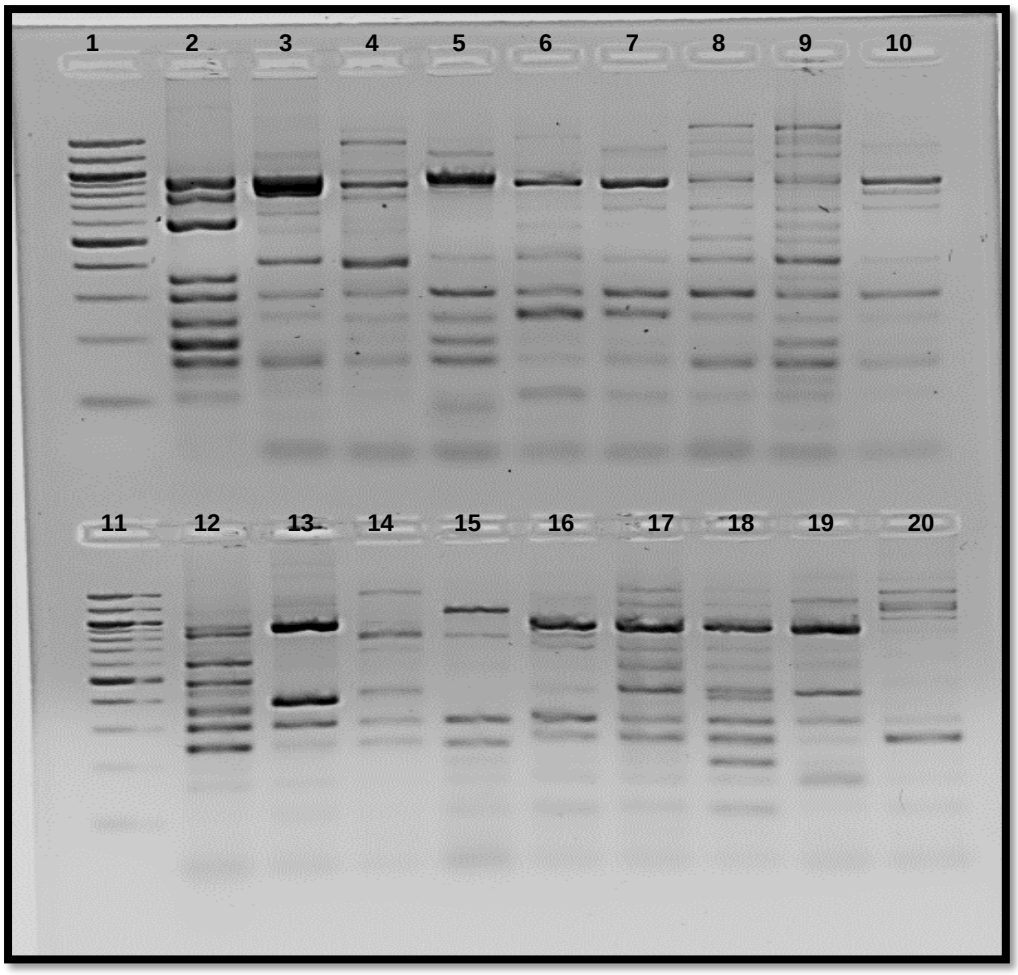

Figure 4.2: *Agarose gel electrophoresis results for multiplex PCR amplification of diarrhoeagenic E. coli. Lane 1 and 11 molecular weight markers; lane 2 positive control eae (917 bp), stx2 (779), ial (630 bp), bfp (410 bp), LT (330 bp), Gapdh (238 bp), Eagg (194 bp), ST (160 bp), Asta (106 bp); Lane 3-10 and 12-20 samples that tested positive for multiple pathogens.*

For *E. coli mdh* is the house-keeping gene and was seen in all samples in both DCC A and B (Table 4.8). The gene *bfp* (has been associated with infantile diarrhoea) was seen in DCC A (71%) and DCC B (61%). All DCC A and B had the *Asta* gene which has been reported to be responsible for persistent diarrhoea.

Table 4.8: Genetic diversity of the 32 *E. coli* positive samples from DCC A and DCC B in the Vhembe region, Limpopo Province; South Africa.

GENES	**DCC A (rural) (n= 14)**	**DCC B (peri-urban) (n=18)**

Mdh	14/14 (100%)	18/18 (100%)
Bfp	10/14 (71%)	11/18 (61%)
Eal	9/14 (64%)	13/18 (72%)
Stx1	8/14 (57%)	7/18 (39%)
Stx2	6/14 (43%)	14/18 (78%)
Ial	5/14 (36%)	6/18 (33%)
Lt	3/14 (21%)	8/18 (44%)
St	12/14 (86%)	17/18 (94%)
Gapdh	14/14 (100%)	18/18 (100%)
Eagg	12/14 (86%)	15/18 (83%)
Asta	14/14 (100%)	18/18 (100%)

4.3 VIRUS ANALYSIS

4.3.1 PREVALENCE OF NOROVIRUS CIRCULATING IN ENVIRONMENTAL SAMPLES, STOOL SAMPLES, HANDWASH OF DAYCARE WORKERS AND CHILDREN IN DCC A AND DCC B.

The prevalence of NoV was 5% on handwash samples of the children, 10% on handwash samples of the daycare workers, 27% on stool samples in both DCC A and DCC B (Table 4.9). Most of the detected samples, however had high Ct values. Of the eight detected, one sample was detected from the handwash of a child and one from the daycare worker of DCC A. A higher number of NoV GII (6 samples) was detected compared to NoV GI (2 samples).

Table 4.9: Prevalence of NoV in DCC A and B in the Vhembe region, Limpopo Province, South Africa.

SAMPLE TYPE	DCC A (rural)	DCC B (peri-urban)
Hand wash of children Total n=22 Positive: 1/22 (5%)	**N=11** **NOROVIRUS:** Positive: 1/11 (9%) NoV strain: NoV GII	**N=11** **NOROVIRUS:** Positive: 0 Strains: 0
Hand wash of workers Total n=10 Positive: 1/10 (10%)	**N=5** **NOROVIRUS:** Positive: 1/5 (20%) NoV strain: NoV GII	**N=5** **NOROVIRUS:** Positive: 0 NoV strain: 0
Environmental samples Total n=29 Positive: 0/29 (0%)	**N=13** **NOROVIRUS:** Positive: 0 NoV strain: 0	**N=16** **NOROVIRUS:** Positive: 0 NoV strain: 0
Stool samples from diapers Total n=22 Positive: 6/22 (27%)	**N=7** **NOROVIRUS:** Positive: 1/7 (14%) NoV strain: NoV GII	**N=15** **NOROVIRUS:** Positive: 5/15 (33%) NoV strains: NoV GII, NoV GII, NoV GII, NoV GI, NoV GI

4.3.2 AMPLIFICATION OF NOROVIRUS USING ONE-STEP AHEAD RT-PCR

The eight (6 NoV GII and 2 NoV GI) detected samples were amplified using the one-step ahead RT-PCR. Only one sample was successfully amplified for the NoV GII capsid gene as seen in Figure 4.3 from DCC A.

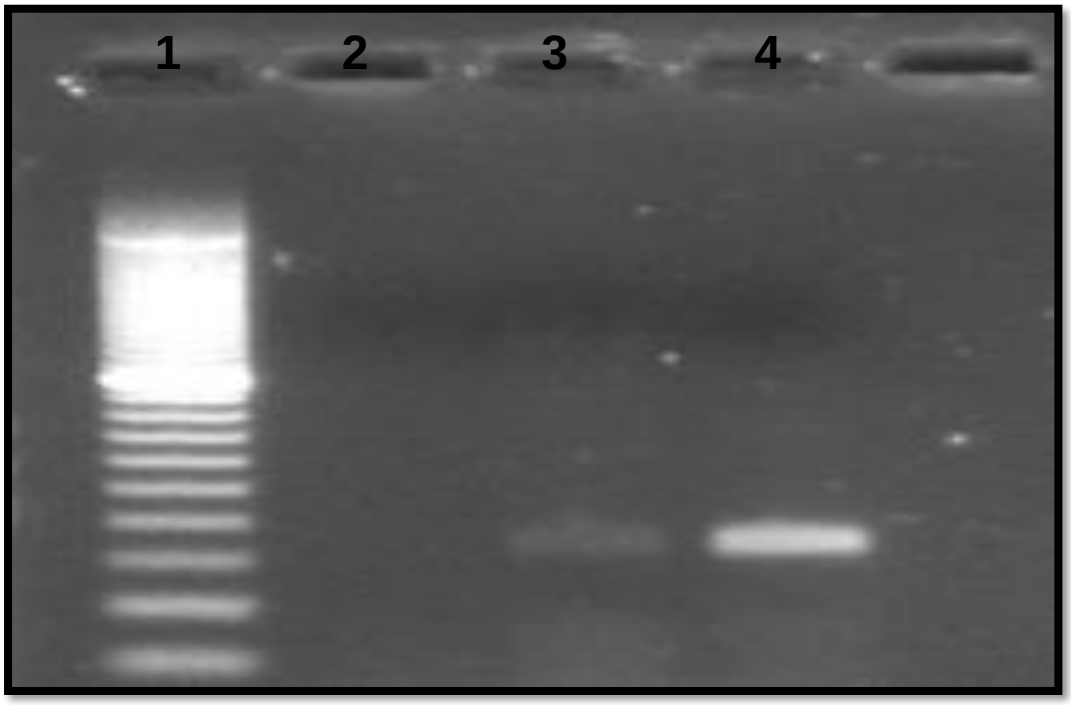

Figure 4.3: *Agarose gel electrophoresis results for the amplification of NoV GII capsid gene. Lane 1 molecular weight marker (100 bp); lane 2 Negative control, lane 3 positive control; Lane 4. Sample 7 showed NoV GII capsid (344 bp).*

4.3.3 GENOTYPING

Only one sample was sequenced successfully, and the genotype was identified as GII.1 based on the capsid sequence of NoV GII. The sequence was submitted to GeneBank and assigned accession number MT123332. There was no genetic diversity observed in this study.

4.3.4 PHYLOGENETIC ANALYSIS OF NOROVIRUS

In the evaluation of genetic relationships among positive NoV samples, a phylogenetic tree was generated based on 344-nucleotide sequence of GII capsid gene using MEGA 10 software (Kumar et al., 2018) (Figure 4.4). The blast search similarity with the reference strains used in the study for GII.1 was between 89-99%.

The study results (Figure 4.4), showed a close association with previously found strains in water and stool samples in the study area as well as other regions of South Africa and Spain.

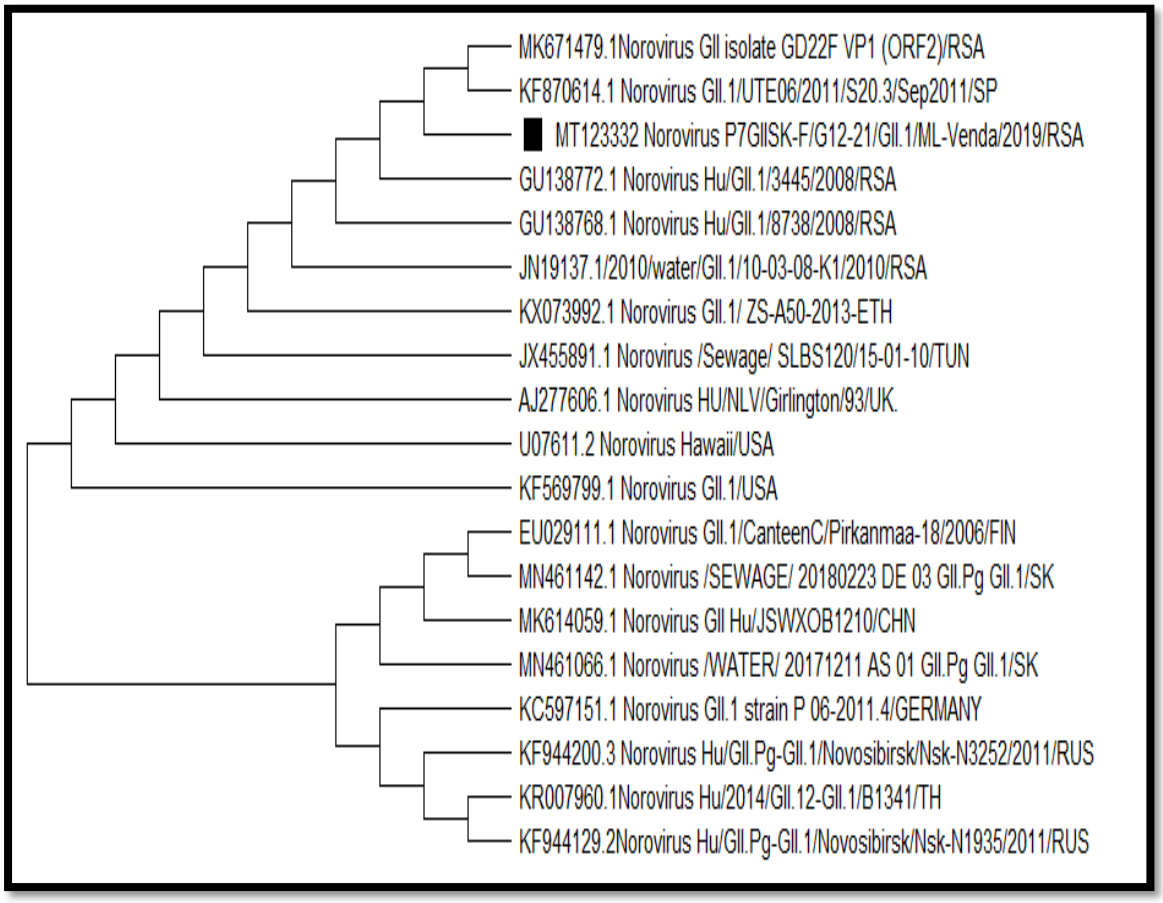

Figure 4.4: Phylogenetic tree based on 344-nucleotide sequence of NoV GII capsid gene fragment. The Neighbor-Joining tree of the GII capsid strains circulating between September 2019 and November 2019 in Daycare centers in Vhembe district, Limpopo province/South Africa. Squared black dot indicated the GII.1 with accession number MT123332 capsid genotype. Eighteen reference strains of NoV were selected from Genbank with their respectively accession numbers. Evolutionary analyses were conducted in MEGA 10.

4.4 DISCUSSION

The study aimed to characterize diarrhoeagenic *E. coli* and human NoV from stool samples, environmental samples, handwash of children and daycare workers in two (one peri-urban and one rural) DCCs in the Vhembe region of the Limpopo Province,

South Africa. For *E. coli*,results showed five samples to be of low-risk contamination, one sample was intermediate-risk and four were high contamination risk. Co-infections of pathotypes where prevalent in all samples in both DCC's. Norovirus GII was predominant in the DCC settings in the Vhembe District. The genotype GII.1 was found in this study.

In this study a prevalence of DEC in environmental samples (10%), stool samples (100%), handwash sample of daycare workers (40%) and handwash sample of children (13.6%) was observed, with all being bacterial co-infections among pathotypes. Ledwaba et al. (2018) reported under-five children infected with ETEC, EPEC, EAEC and other diarrhoeal causing pathogens in the same region. Similarly, virulent strains of *E. coli* were in combinations which is associated with acute gastroenteritis (Ledwaba et al., 2018; Omar and Barnard 2014). Daycare center A accounted for most of the *E. coli*-positive samples from environmental and handwash samples. This could have been due to the low number of staff workers to a high number of enrolled children. Taylor et al. (2008), observed that a high child/staff ratio reduces the ability of individual daycare workers to supervise hygiene, thus could lead to the exposure to enteric pathogens. This suggests increasing the risk of transmission of *E. coli* among children (Li et al., 2014b; Berg et al., 1991; Wald et al.,1991; Alexander et al., 1990).

Poor hygiene by workers (not washing their hands regularly when encountering the children), as well as allowing the children to wash their hands in the same water container (not constantly replacing water in the water containers for drinking throughout the day), leads to the children re-exposing themselves and others to their own and others bacteria (Ledwaba et al., 2019; Zipursky et al., 2013).

Handwash of the children was highly contaminated in DCC A, probably due to poor hygiene. Improper handling practices and poor hygiene are associated with high levels of bacterial contamination (Oliveira and Pinto., 2018; Ejemot-Nwadiaro et al., 2015). Vishwanath et al. (2019), reported a low prevalence of *E. coli* on handwash samples; however, the study was from children attending primary school.

A Norovirus prevalence of 5% was detected in the handwash of the children, 10% from handwash of the workers, 27% stool specimen in this study and no NoV was detected

in environmental samples in both DCCs. A higher number of NoV GII was detected compared to NoV GI in this study. Low detections of NoV have been reported in environmental samples (Lee et al., 2018). This study, however, did not observe genetic diversity of NoV in the daycare settings. Although there was a low level of contamination on the surfaces, the low infectious dose of NoV still presents a potential health risk (Park et al., 2015; Atmar et al., 2014; Calderon., 2008).

In stool samples, NoV is known to be a cause of sporadic infections in children under-five years (Xue et al., 2019; Patel et al., 2008). Previously in the same study area of Vhembe, studies on enteric pathogens showed a high prevalence of asymptomatic carriage in under-five years children in rural communities (Ledwaba et al., 2018; Kabue et al., 2016; Potgieter et al., 2010). Asymptomatic and symptomatic carriage of enteric pathogens has been reported in daycare center attendees in the Netherlands and Sweden with a high prevalence of NoV GII in under 5 children (Kaarme et al., 2016; Enserink et al., 2014; Enserink et al., 2013). Previous studies observed a relatively lower prevalence of infection with NoV in the UK (Borrows and Turner, 2014), Tanzania (Moyo et al, 2007), and Uruguay (Varela et al., 2015).

The genotype GII comprises of strains GII.1-GII.29; however, the predominant genotypes have surged to be GII.4 and GII.17, which are associated with sporadic infections (Shen et al., 2020; Xue et al., 2019; Tran et al., 2013; Patel et al., 2008). The found genotype GII.1 capsid in this study, was also previously reported mainly in environmental samples and clinical cases (Fumian et al., 2019). The genotype showed close relatedness to other strains detected in South Africa in clinical and water samples (Mans et al., 2010). Genotypes reported in other African countries, Ethiopia (Sisay et al., 2016) and Tunisia (Hassine-Zaafrane et al., 2014) showed close relatedness to the genotype found in this study. Another developed country that reported a genotype showing close relatedness is Spain (Sabria et al., 2014). Similarly, in Brazil, Marques et al. (2013), reported findings of GII.1 in asymptomatic and symptomatic cases of children in DCCs. Several studies in Argentina (Degiuseppe et al., 2020), Thailand (Phumpholsup et al., 2015), Russia (Zhirakovskaia et al., 2015), Germany (Hoffmann et al., 2013), Finland (Makary et al., 2009), and London (Green et al., 2000), however, reported the genotype GII.1 in diarrhoeal outbreaks. The presence of NoV GII.1 in under-five years children is of concern as the

genotype has been reported in diarrhoeal outbreaks. Contrary to the findings in this study, Mans. (2019) reported GII.3 to be predominant in low-income countries.

All *E. coli*-positive samples detected in this study revealed bacterial strain co-infections of (90.4%) and viral-bacterial co-infections (9.6%). In both DCCs EHEC+ETEC+EAEC co-infections were seen from handwash samples of the workers. In the clinical samples, co-infections among all five pathotypes (EPEC+EHEC+EIEC+ETEC+EAEC) were seen in both DCCs. Youseff et al (2000), reported detecting ETEC + EAEC co-infections in children aged less than five years. In China, Zhu reported a high prevalence of viral-bacterial co-infections in same aged children (Zhu et al., 2016).

The high prevalence of *E. coli* and NoV detected in this study will provide knowledge on setting guidelines relating to the DCC workers as well as the children's hygiene in the DCCs. Furthermore, the data obtained provide epidemiological data that could aid in vaccine production.

CHAPTER 5: CONCLUSION, RECOMMENDATIONS & LIMITATIONS

5.1 CONCLUSION

Daycare center environments host a variety of enteric microorganisms which are known to cause diarrhoea worldwide in children under the age of 5 years. These pathogens are a burden in developing countries with high numbers of diarrhoeal diseases leading to a high mortality rate. The study results showed that *E. coli* and

NoV are circulating in DCCs environments, handwash as well as the clinical samples in DCCs around Vhembe District. The study findings showed genetic diversity of *E. coli* in these settings. The phylogenetic analysis revealed that the NoV genotype GII.I capsid sequenced in this study to share a common ancestor with previously reported strains associated with outbreaks with NoV. This study contributes to other studies done in the region by providing an insight on the circulating *E. coli* pathotypes and NoV genotype in the daycare settings. This will aid in setting guidelines for hygiene in the DCC environment. In addition, the obtained data will provide a rationale for further studies so that measures can be put in place to avoid outbreaks caused by these pathogens. Also, the data will aid in identifying the type of strains circulating in the region which is of importance for vaccine production.

5.2 RECOMMENDATIONS

- ✓ Sample collection can be done in different seasons which will include the seasonality factor as it is known to play a role in these enteric pathogens.
- ✓ Further analysis of the participating children can be done also by sampling their home environment as to understand the pathway of contamination from all different points.
- ✓ Educating the daycare workers on the importance of practicing good hygiene.
- ✓ Further studies with a broader number of enteric pathogens, larger sample size and also include the urban setting.

5.3 LIMITATIONS

- ✓ The collected data from each DCC principal (Profile A) was mainly received through self-report subjecting to recall bias.
- ✓ Several unannounced sampling visits in different seasons may have reported a broader spectrum of the epidemiology of the enteric pathogens in the DCC with respect to seasonality.
- ✓ The small number of samples collected limited getting the broad spectrum of the presence of the selected enteric pathogens.

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APPENDIX 1: ETHICAL CLEARANCE

RESEARCH AND INNOVATION
OFFICE OF THE DIRECTOR

NAME OF RESEARCHER/INVESTIGATOR:
Mr L Munzhedzi

Student No:
11636213

PROJECT TITLE: **Characterisation of diarrhoegenic Escherichia coli and human norovirus from day care centers in the Vhembe region of Limpopo Province, South Africa.**

PROJECT NO: **SMNS/19/MBY/04/2204**

SUPERVISORS/ CO-RESEARCHERS/ CO-INVESTIGATORS

NAME	INSTITUTION & DEPARTMENT	ROLE
Prof N Potgieter	University of Venda	Supervisor
Prof AN Traore	University of Venda	Co - Supervisor
Dr JP Kabue	University of Venda	Co - Supervisor
Mr L Munzhedzi	University of Venda	Investigator – Student

ISSUED BY:
UNIVERSITY OF VENDA, RESEARCH ETHICS COMMITTEE

Date Considered: April 2019
Decision by Ethical Clearance Committee Granted
Signature of Chairperson of the Committee: .....
Name of the Chairperson of the Committee: Senior Prof. G.E. Ekosse

UNIVERSITY OF VENDA
DIRECTOR
RESEARCH AND INNOVATION
2019 -05- 10
Private Bag X5050
Thohoyandou 0950

University of Venda
PRIVATE BAG X5050, THOHOYANDOU, 0950, LIMPOPO PROVINCE, SOUTH AFRICA
TELEPHONE (015) 962 8504/8313 FAX (015) 962 9060
"A quality driven financially sustainable, rural-based Comprehensive University"

APPENDIX 2: PROFILES A, B, C and D

RESEARCH ETHICS COMMITTEE

UNIVEN Informed Consent for Daycare Centre
PROFILE A

LETTER OF INFORMATION

Title of the Research Study : Characterisation of diarrhoegenic *Escherichia coli* and human norovirus from daycare centers in the Vhembe region of the Limpopo province, South Africa.

Principal Investigator/s/ researcher : Mr Lutendo Munzhedzi (BSCHMB)

Co-Investigator/s/supervisor/s : Prof Natasha POTGIETER (PhdMed Virol)
Prof Afsatou TRAORE (PhdBCM)
Dr Jean Pierre KABUE (PhdMby)

Brief Introduction and Purpose of the Study: This study will include swab samples from the Day care center environment and handwash samples from day care workers as well as children in the day care center. About 120 samples from the day care environment, 16 handwash samples from children attending the day care centers and 24 handwash samples from the day care workers will be included in this study.
The project aims to characterize diarrhoegenic Escherichia coli and human norovirus from day care centers in the Vhembe region of the Limpopo province, South Africa. This information will help decision making in prevention strategies against enteric pathogen disease transmission particularly human norovirus and Escherichia coli, improvement of sanitary environments and hygienic practices. The findings of this study will provide also provide information on human norovirus and Escherichia coli prevalence and diversity in the day care environment.
General information will be taken from the day care center such as hygienic practices used, total number of staff, total number of children attending the day care and one handwash sample will be collected from two children and 3 day care workers and will be transported to the laboratory for analysis. The information used will not expose the Day care centers identity.

Outline of the Procedures : See attached proposal

Risks or Discomforts to the Participant: There are no risks involved in participating. Collection of handwash sample will be done once during the sampling period.

Benefits : No monetary compensation is offered for participation, but the day care centre will be receiving the results of bacteriological and virological analysis if positive.

Reason/s why the Participant May Be Withdrawn from the Study: Participation in this study completely voluntary. The day care centre may refuse to provide information or handwash sample.

Remuneration : None

Costs of the Study : None

Confidentiality : Medical information will not be accessed. Identity of the day care centre will not be made public and if the results of this study are published or presented, the day care centre will only be referred to by a code number. Identity will be kept confidential.

Research-related Injury : None

Persons to Contact in the Event of Any Problems or Queries:
(Prof Natasha Potgieter (Department of Microbiology/ University of Venda) Please contact the researcher (071 2112 472), my supervisor (015-962-8474 or 015-962-8107) or the University Research Ethics Committee Secretariat on 015 962 9058. Complaints can be reported to the Director: Research and Innovation, Prof GE Ekosse on 015 962 8313 or Georges Ivo.Ekosse@univen.ac.za

General:
Potential participants must be assured that participation is voluntary and the approximate number of participants to be included should be disclosed. A copy of the information letter should be issued to participants. The information letter and consent form must be translated and provided in the primary spoken language of the research population

CONSENT

Statement of Agreement to Participate in the Research Study:

- I hereby confirm that I have been informed by the researcher, (Lutendo Munzhedzi), about the nature, conduct, benefits and risks of this study - Research Ethics Clearance Number: _,
- I have also received, read and understood the above written information (*Participant Letter of Information*) regarding the study.
- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerized system by the researcher.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.
- I understand that significant new findings developed during the course of this research which may relate to my participation will be made available to me.

Full Name of Participant Date Time Signature

I, …… …… ……
……..

(*Name of researcher*) herewith confirm that the above participant has been fully

Informed about the nature, conduct and risks of the above study.

Full Name of Researcher

…… Date…… Signature……

Full Name of Witness (If applicable)

…… Date …… Signature……..

Full Name of Legal Guardian (If applicable)

…… Date…… Signature……

Please note the following:

Research details must be provided in a clear, simple and culturally appropriate manner and prospective participants should be helped to arrive at an informed decision by use of appropriate language (grade 10 level- use Flesch Reading Ease Scores on Microsoft Word), selecting of a non-threatening environment for interaction and the availability of peer counseling (Department of Health, 2004)

If the potential participant is unable to read/illiterate, then a right thumb print is required and an impartial witness, who is literate and knows the participant e.g. parent, sibling, friend, pastor, etc. should verify in writing, duly signed that informed verbal consent was obtained (Department of Health, 2004).

If anyone makes a mistake completing this document e.g. a wrong date or spelling mistake, a new document has to be completed. The incomplete original document has to be kept in the participant's file and not thrown away, and copies thereof must be issued to the participant.

References:

Department of Health: 2004. *Ethics in Health Research: Principles, Structures and Processes*

http://www.doh.gov.za/docs/factsheets/guidelines/ethnics/

Department of Health. 2006. *South African Good Clinical Practice Guidelines*. 2nd Ed. Available at:

http://www.nhrec.org.za/?page_id=14

RESEARCH QUESTIONNAIRE:

TITLE: Characterisation of diarrhoeagenic Escherichia coli and human norovirus from daycare centers in the Vhembe region of the Limpopo province, South Africa.

PROFILE A: OWNER

1. How many staff workers are employed at this day care center?	
2. What are the hygienic practices applied by the staff (Washing hands, cleaning of floor with detergents, washing of kitchen utensils)?	
3. What are the daily activities of the facility?	
4. How many children are there in this day care center?	
5. What is the overall age distribution of this day care center?	
6. How is the waste handled (where is it kept, for how long, where is it disposed)?	
7. How often is the day care center cleaned?	

RESEARCH ETHICS COMMITTEE

UNIVEN Informed Consent for Day Care Worker

PROFILE B

LETTER OF INFORMATION

Title of the Research Study : Characterisation of diarrhoegenic *Escherichia coli* and human norovirus from daycare centers in the Vhembe region of the Limpopo province, South Africa.

Principal Investigator/s/ researcher : Mr Lutendo Munzhedzi (BSCHMB)

Co-Investigator/s/supervisor/s : Prof Natasha POTGIETER (PhdMed Virol)
Prof Afsatou TRAORE (PhdBCM)
Dr Jean Pierre KABUE (PhdMby)

Brief Introduction and Purpose of the Study: This study will include swab samples from the Day care center environment and handwash samples from day care workers as well as children in the day care center. About 120 samples from the day care environment, 16 handwash samples from children attending the day care centers and 24 handwash samples from the day care workers will be included in this study.
The project aims to characterize diarrhoegenic Escherichia coli and human norovirus from day care centers in the Vhembe region of the Limpopo province, South Africa. This information will help decision making in prevention strategies against enteric pathogen disease transmission particularly human norovirus and Escherichia coli, improvement of sanitary environments and hygienic practices. The findings of this study will provide also provide information on human norovirus and Escherichia coli prevalence and diversity in the day care environment.
General information will be taken from you such as age, gender, use of toilet and one handwash sample will be collected from you and will be transported to the laboratory for analysis. The information used will not expose your identity.

Outline of the Procedures : See attached proposal

Risks or Discomforts to the Participant: There are no risks involved in participating. Collection of handwash sample will be done once during the sampling period.

Benefits : No monetary compensation is offered for your
participation, but you will be receiving the results of bacteriological and virological analysis if positive.

Reason/s why the Participant May Be Withdrawn from the Study: Participation is entirely voluntary and is free to decline to participate.

Remuneration : None

Costs of the Study : None

Confidentiality : Medical information will not be accessed. Your identity will not be made public and if the results of this study are published or presented, you will only be referred to by a code number. Your identity will be kept confidential.

Research-related Injury : None

Persons to Contact in the Event of Any Problems or Queries:
(Prof Natasha Potgieter (Department of Microbiology/ University of Venda) Please contact the researcher (071 2112 472), my supervisor (015-962-8474 or 015-962-8107) or the University Research Ethics Committee Secretariat on 015 962 9058. Complaints can be reported to the Director: Research and Innovation, Prof GE Ekosse on 015 962 8313 or Georges Ivo.Ekosse@univen.ac.za

General:
Potential participants must be assured that participation is voluntary and the approximate number of participants to be included should be disclosed. A copy of the information letter should be issued to participants. The information letter and consent form must be translated and provided in the primary spoken language of the research population

CONSENT

Statement of Agreement to Participate in the Research Study:

- I hereby confirm that I have been informed by the researcher, (Lutendo Munzhedzi), about the nature, conduct, benefits and risks of this study - Research Ethics Clearance Number: _,
- I have also received, read and understood the above written information (*Participant Letter of Information*) regarding the study.
- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerized system by the researcher.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.
- I understand that significant new findings developed during the course of this research which may relate to my participation will be made available to me.

Full Name of Participant Date Time Signature

I, …….. …….. ……. ……..

(*Name of researcher*) herewith confirm that the above participant has been fully

Informed about the nature, conduct and risks of the above study.

Full Name of Researcher

…… Date**……** Signature**……**

Full Name of Witness (If applicable)

…… Date …… Signature……..

Full Name of Legal Guardian (If applicable)

…… Date…… Signature……

Please note the following:

Research details must be provided in a clear, simple and culturally appropriate manner and prospective participants should be helped to arrive at an informed decision by use of appropriate language (grade 10 level- use Flesch Reading Ease Scores on Microsoft Word), selecting of a non-threatening environment for interaction and the availability of peer counseling (Department of Health, 2004)

If the potential participant is unable to read/illiterate, then a right thumb print is required and an impartial witness, who is literate and knows the participant e.g. parent, sibling, friend, pastor, etc. should verify in writing, duly signed that informed verbal consent was obtained (Department of Health, 2004).

If anyone makes a mistake completing this document e.g. a wrong date or spelling mistake, a new document has to be completed. The incomplete original document has to be kept in the participant's file and not thrown away, and copies thereof must be issued to the participant.

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http://www.doh.gov.za/docs/factsheets/guidelines/ethnics/

Department of Health. 2006. *South African Good Clinical Practice Guidelines*. 2nd Ed. Available at:

http://www.nhrec.org.za/?page_id=14

RESEARCH QUESTIONNAIRE:

PROFILE B: CRECHE STAFF

1. Do you wash your hands before receiving the kids in the morning (If **yes** with what do you wash)?	

2. After receiving the kids where are they taken to (floor, chairs or play area)?	
3. At what time are the children fed? Which foods are they eating and how are they fed?	
4. How are the foods handled and utensils cleaned?	
5. Which type of water source, water storage and type of toilet are used in this day care center?	
6. Do all the kids use one utensil to drink water?	
7. How many times do the kids wash their hands and what are they using to wash their hands?	
8. How are the children's nappies changed?	

RESEARCH ETHICS COMMITTEE

UNIVEN Informed Consent for Child
PROFILE C

LETTER OF INFORMATION

Title of the Research Study : Characterisation of diarrhoegenic *Escherichia coli* and human norovirus from daycare centers in the Vhembe region of the Limpopo province, South Africa.

Principal Investigator/s/ researcher : Mr Lutendo Munzhedzi (BSCHMB)

Co-Investigator/s/supervisor/s : Prof Natasha POTGIETER (PhdMed Virol)
Prof Afsatou TRAORE (PhdBCM)
Dr Jean Pierre KABUE (PhdMby)

Brief Introduction and Purpose of the Study: This study will include swab samples from the Day care center environment and handwash samples from day care workers as well as children in the day care center. About 120 samples from the day care environment, 16 handwash samples from children attending the day care centers and 24 handwash samples from the day care workers will be included in this study.
The project aims to characterize diarrhoegenic Escherichia coli and human norovirus from daycare centers in the Vhembe region of the Limpopo province, South Africa. This information will help decision making in prevention strategies against enteric pathogen disease transmission particularly human norovirus and Escherichia coli, improvement of sanitary environments and hygienic practices. The findings of this study will provide also provide information on human norovirus and Escherichia coli prevalence and diversity in the day care environment.
General information will be taken from your child such as age, gender, use of toilet and one handwash sample will be collected from your child and will be transported to the laboratory for analysis. The information used will not expose you or your child's identity.

Outline of the Procedures : See attached proposal

Risks or Discomforts to the Participant: There are no risks involved in participating. Collection of handwash sample will be done once during the sampling period.

Benefits : No monetary compensation is offered for your child's participation, but you will be receiving the results of bacteriological and virological analysis if positive.

Reason/s why the Participant May Be Withdrawn from the Study: Your child's participation is entirely voluntary and is free to decline to participate.

Remuneration : None

Costs of the Study : None

Confidentiality : Medical information will not be accessed. Your childs identity will not be made public and if the results of this study are published or presented, your child will only be referred to by a code number. Identity of your child will be kept confidential.

Research-related Injury : None

Persons to Contact in the Event of Any Problems or Queries:
(Prof Natasha Potgieter (Department of Microbiology/ University of Venda) Please contact the researcher (071 2112 472), my supervisor (015-962-8474 or 015-962-8107) or the University Research Ethics Committee Secretariat on 015 962 9058. Complaints can be reported to the

Director: Research and Innovation, Prof GE Ekosse on 015 962 8313 or Georges Ivo.Ekosse@univen.ac.za

General:
Potential participants must be assured that participation is voluntary and the approximate number of participants to be included should be disclosed. A copy of the information letter should be issued to participants. The information letter and consent form must be translated and provided in the primary spoken language of the research population

CONSENT

Statement of Agreement to Participate in the Research Study:

- I hereby confirm that I have been informed by the researcher, (Lutendo Munzhedzi), about the nature, conduct, benefits and risks of this study - Research Ethics Clearance Number: _,
- I have also received, read and understood the above written information (*Participant Letter of Information*) regarding the study.
- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerized system by the researcher.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.
- I understand that significant new findings developed during the course of this research which may relate to my participation will be made available to me.

Full Name of Participant Date Time Signature

I, ...

(*Name of researcher*) herewith confirm that the above participant has been fully

Informed about the nature, conduct and risks of the above study.

Full Name of Researcher

…… Date…… Signature……

Full Name of Witness (If applicable)

…… Date …… Signature……..

Full Name of Legal Guardian (If applicable)

…… Date…… Signature……

Please note the following:

Research details must be provided in a clear, simple and culturally appropriate manner and prospective participants should be helped to arrive at an informed decision by use of appropriate language (grade 10 level- use Flesch Reading Ease Scores on Microsoft Word), selecting of a non-threatening environment for interaction and the availability of peer counseling (Department of Health, 2004)

If the potential participant is unable to read/illiterate, then a right thumb print is required and an impartial witness, who is literate and knows the participant e.g. parent, sibling, friend, pastor, etc. should verify in writing, duly signed that informed verbal consent was obtained (Department of Health, 2004).

If anyone makes a mistake completing this document e.g. a wrong date or spelling mistake, a new document has to be completed. The incomplete original document has to be kept in the participant's file and not thrown away, and copies thereof must be issued to the participant.

References:

Department of Health: 2004. *Ethics in Health Research: Principles, Structures and Processes*

http://www.doh.gov.za/docs/factsheets/guidelines/ethnics/

Department of Health. 2006. *South African Good Clinical Practice Guidelines*. 2nd Ed. Available at: http://www.nhrec.org.za/?page_id=14

RESEARCH QUESTIONNAIRE:

PROFILE C: CHILD

1. How old is the child?	
2. What is the gender of the child?	

3. Did the child show any clinical symptoms of coughing, running nose, dehydration or diarrhoea in the past 1 month or 24 hours?	
4. Is the child still using nappies?	

RESEARCH ETHICS COMMITTEE

UNIVEN Informed Consent form in TSHIVENDA

PROFILES IN VENDA

Thalutshedzo ya thandela ya thoduluso na fomo ya thendelo vharangaphanda vha thoduluso edzi.

Thoho ya thandela iyi : Characterisation of diarrhoegenic *Escherichia coli* and human norovirus from daycare centers in the Vhembe region of the Limpopo province, South Africa.

Principal Investigator/s/ mutodulusi : Mr Lutendo Munzhedzi (BSCHMB)

Co-Investigator/s/vha hulwane : Prof Natasha POTGIETER (PhdMed Virol)
Prof Afsatou TRAORE (PhdBCM)
Dr Jean Pierre KABUE (PhdMby)

Kha ngudo dza mushumo uyu wa thoduluso hu do vha hu kho itwa ni?
Kha ngudo idzi hu do todea mashika abvaho kha mupo wa crèche na madi ane vhashumi na vhana vha crèche iyi vha do vha vho tambela khao zwanda. Nga u anganyela hu do todea mashika ane a nga lingana 30 samples dzibvaho kha mupo, madi o tambelwaho zwanda nga vhana a linganaho 30 samples na madi o tambelwaho zwanda nga vhashumi vha crèche a linganaho 10 samples.
Ngudo idzi dzi lusa u todulusa tshitshili tshine tshavha *Escherichia coli* na Human Norovirus kha zwiimiswa zwa u thogomela vhana zwine zwa wanala kha tshitiriki tsha Vhembe kha Vundu la Limpopo, Afrika Tshipembe. Tsedzuluso idzi dzi do thusa kha u dzhia tsheo ya u thivhela u phadalala ha malwadzwe a diswaho nga zwi Human norovirus na *Escherichia coli*, u khwinifhadza ndila ya u thogomela mutakalo, tsedzuluso idzi dzi do dovha hafhu dza thusa kha u humbudza vhathu uri vha dzulele u ita ndowe ndowe dza vhudele kha masia othe hu u itela mutakalo wavhudi. Mawanwa a tsedzuluso idzi a do andadza na ndila dzine ha nga thivhelwa ngadzo u dalesa ha zwitshili kha mupo wa zwiimiswa izwi zwa u thogomela vhana.

Hu na khombo dzine dza nga bvelela kha vho nga u vha tshipida tsha tsedzuluso idzi:
A huna na khombo na nthihi ine ya nga bvelela kha vho nga u vha tshipida kha mushumo uyu. U kolekiwa ha madi o tambelwaho khao zwanda zwi do itwa luthihi nga tshifhinga tsha tsedzuluso iyi.

Malamba ane vha nga a wana kha uvha tshipida tsha thodisiso kana tsedzuluso iyi ndi afhio?
A hu nga do vha na tshelede ine vha nga lambedzwa ngayo, fhedzi vhado newa vhutanzi ha mawanwa nga murahu ha musi ndingo dzono itwa.

Ndi nnyi ane anga kona u swikelela rekhodo ya dzilafho lavho?
A vha nga do vhudziswa nga mafhungo a dzilafho lavho. Vhune havho a vhu nga do adadzwa kha tshitshavha. Arali mawanwa a tsedzuluso idzi a nga andadzwa, vha do sumbedzwa nga nambara ya khoudu. Vhune havho vhu do vhewa nga ndila yo tsireledzwaho.

Vhudifhinduleli havho vhu dovha vhufhio?
U dzhenelela havho kha itshi tshipida tsha ngudo idzi hu do vha u takalela havho. Vho tendelwa u landula u disa Ndivho ya zwine vha divha kana u disa sample ya madi e vha tamba ngao zwanda.

Malamba ane vha nga a wana
Ahuna

Khombo ine ya nga itea kha mu jeneleli?
Ahuna khombo.

Ara vha na mbudziso dzinwe dzine vha nga takalela u vhudzisa kana vha tangana na thaidzo, vha nga kwama vho:
(Prof Natasha Potgieter (Department of Microbiology/ University of Venda) Please contact the researcher (071 2112 472), my supervisor (015-962-8474 or 015-962-8107) or the University Research Ethics Committee Secretariat on 015 962 9058. Complaints can be reported to the

Director: Research and Innovation, Prof GE Ekosse on 015 962 8313 or Georges Ivo.Ekosse@univen.ac.za

THENDELO

U tenda nga mudzheneleli:

- Ndi kho u tenda uri ndo vhala nda pfesesa zwothe zwo nwalaho kha fomo iyi, nahone zwo nwalwa nga luambo lwune nda amba lwone nda dovha nda lwu pfesesa.

- Ndi kho u tenda uri ndo newa tshifhinga tsha u vhudzisa mbudziso dzothe dze nda vha nadzo, mbudziso idzo dzo dovha hafhu dza fhindulwa nga ye nda I pfesesa.

- Ndi kho u tenda uri ndo pfesesa uri u vha tshipida tsha tsedzuluso idzi ho to u vha u di dzhenisa na u takalela hanga, nga u ralo a thingo newa mutsiko kana u kombetshedzwa uri ndi vhe tshipida kha mushumo uyu.

Dzina la mujeneleli datumu tshifhinga Sain

Nne , ……. …… ……. ……..

(*Lutendo munzhedzi*) kho tenda uri mujeneleli o taluchedziwa na hone a pfesesa ngaha heyi tandela.

Dzina la motodulusi

……. Datumu…….. Sain……

Dzina la mutalutshedzi

……. Datumu…….. Sain……..

Dzina la mubebi

…… Datumu…… Sain……

DEPARTMENT OF MICROBIOLOGY, SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES, UNIVERSITY OF VENDA

Research project data capture form DATE : **DD/MM/2019**

Subject Number……

Details			
Date:	Visit Number:	DCC name:	
CHILD INFORMATION			
Name _____	Date of birth _____	Gender: M ☐ F ☐	Contact details _____
DCC CONDITION			
Water source:	Tap ☐	Spring/wells ☐	Boreholes ☐ River ☐
Sanitation:	VIP/Pit latrine ☐	Flush toilet ☐	
No Clinical symptoms of :	Diarrhea ☐	Vomiting ☐	Fever ☐
Sample collection ***Date of collection:***	_____		
Type of sample:	Type of stool: Watery ☐	Sausage ☐	Mushy ☐
Treatment			
Current :			
Previous :			
Laboratory Results			
PCR:			
Sequencing:			

APPENDIX 3: BIOCHEMICAL TEST PREPARATION

KLIGLER'S IRON AGAR

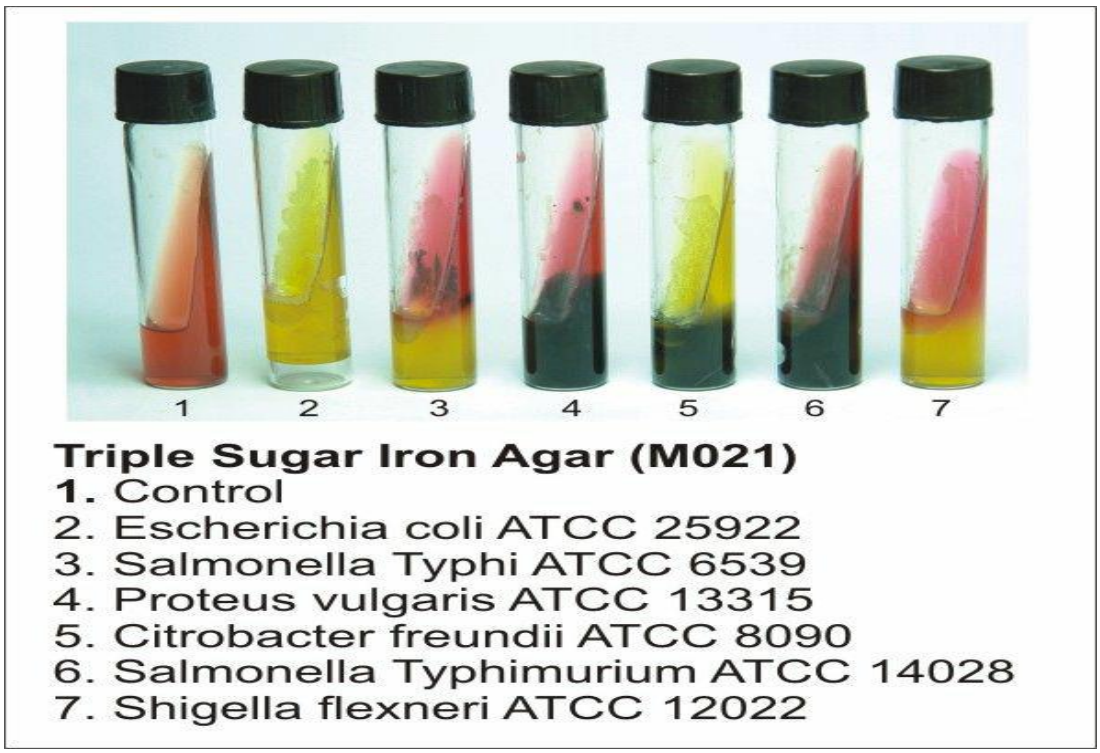
Objective of kligler's

- Differentiating certain members of Enterobacteriaceae by demonstrating hydrogen sulphide production and the fermentation of dextrose and lactose**.**

Procedure of Kligler's

1. With an inoculating needle, pick the centre of well-isolated colonies obtained from solid culture media. The medium is recommended for the identification of colonies picked off from plating media from EMB.
2. Stab the centre of the medium into the deep of the tube to within 3-5mm from the bottom.
3. Withdraw the inoculating needle and streak the surface of the slant.
4. Loosen closure on the tube before incubating.
5. Incubate aerobically at 35ºC for 18-48 hours.
6. Read tubes for acid production of the slant/butt, gas, and hydrogen sulphide reactions.

Interpretation: A yellow slant and a yellow butt indicate that both glucose and lactose are fermented *E. coli.* Bubbles, mostly in the butt of the tube, specify gas production.



Kliglers Iron agar expected results of *E. coli* at 2

UREASE TEST

Objective

- To check whether the *E. coli* can produce urease enzyme for the degradation of urea or not.

Principle of Urease Test

- Urea is common metabolic waste product of protein digestion in most vertebrates that is toxic to most living organism. Urease catalyses the breakdown of urea into ammonia and carbon dioxide. The test organism is cultured in a medium containing urea and the indicator phenol red. If the bacterial strain is urease-producing, the enzyme will hydrolyse the urea to give ammonia and carbon dioxide. With the release of ammonia, the medium become alkaline shown by change in color of indicator to reddish pink.

Media preparation

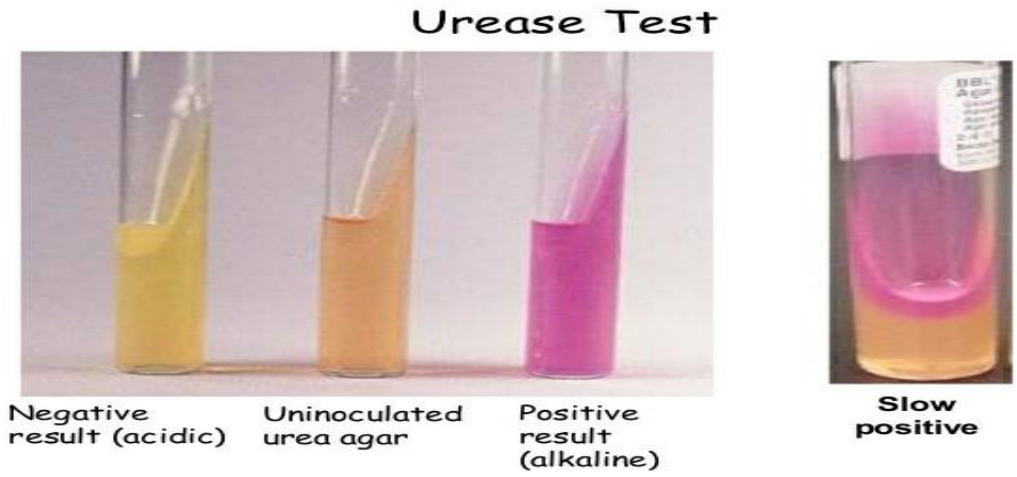
1. Urea test broth is prepared by adding 20 g of urea, 9.5 g of Na_2HPO_4, 9.1 g of KH_2PO_4, 0.1 yeast extract, 0.01 g of phenol red and agar. The pH is made to 6.8 at 25^{o}C.
2. Mix thoroughly and dispense aseptically in sterile tubes.
3. Cool the tubed medium in a slanted position so that deep butts are formed.

Procedure for urease test

1. Sterilize the loop in the blue flame of the Bunsen burner till red hot and then allowed to cool.
2. Take out a loopful organism from the tryptic broth culture tube with the cooled loop aseptically.
3. Again, flame the neck of the tube and replace the tube in the test tube rack.
4. Take a sterile urea slant tube remove the cap and flame the neck of the tube.
5. Inoculate the entire surface of the urea slant (slope) with the provided growth from the tryptic broth culture using the inoculating loop (do not stab the butt). The slant of the medium is inoculated by streaking the surface of the agar in a zigzag manner.
6. Again, flame the neck of the urea tube and place it in the test tube rack.
7. Tighten the cap and incubate at 37°C for 24-48 hours.

8. Obtain the tubes from the incubator and observe the colour change

Interpretation:



INDOLE TEST

Objective of indole test

- To detect the ability of *E. coli* to produce enzyme tryptophanase.

Principle of indole test

- Indole test is a biochemical test which differentiates the coliform from other members of Enterobacteriaceae by detecting their ability to produce the enzyme tryptophanase. This enzyme hydrolyses the amino acid tryptophan into indole, pyruvic acid and ammonia.

Procedure of indole test

1. Prepare peptone broth in test tube
2. Autoclave the tubes at 121^{O}C for 15 min.
3. Using sterile wire, inoculate the broth with the *E. coli* and label the tubes
4. Incubate the tubes at 37°C for 24 hours.
5. After proper incubation, add 4-8 drops of kovac's reagent to the tube touching the wall of glass tube.
6. Roll each tube between your palms to mix the reagent through the culture.
7. Wait and observe for the development of cherry red color at the surface of media.

Interpretation: Expected results of indole test for *E. coli* is Positive which is indicated by formation of a red ring at the surface of the medium and no color change indicates a negative test.