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Molecular Epidemiology and Characterization of Rotavirus strains isolated from dogs' stool

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Dissertation submitted in fulfilment of the requirements for the degree *Masters of Science in Biology* at the North West University

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DECLARATION

I, **Lerato Peggy Kgosana** wish to declare that this mini dissertation is my own work unless where acknowledged. It has not been submitted to any institution for the purpose of obtaining a qualification.

.....

Signature of candidate

Signed at ..... on this ..... day of..... 2017

.....

Signature of supervisor

Signed at ..... on this ..... day of..... 2017

DEDICATION

I would like to dedicate this thesis to my loving parents, Simon and Jeanett Kgosana.

ACKNOWLEDGEMENTS

First of all I would like to thank the Almighty God for being my rock, strength and fortress. I wouldn't have come this far if it weren't of Jesus.

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LIST OF ABREVIATIONS

AGE - Agarose Gel Electrophoresis

BLAST: Basic Local Alignment Search Tool

cDNA - complementary Deoxyribonucleic acid

dNTP- deoxynucleotide triphosphate

DNA - Deoxyribonucleic acid

dsRNA - double stranded ribonucleic acid

EIA – Enzyme immunoassay

EDTA - ethylene di-amine tetra acetic acid

GE- Gastroenteritis

ICT- Immunochromatography Technique

NCBI- National Centre for Biotechnology Information

NSP5- Non-Structural Protein 5

PAGE - polyacrylamide gel electrophoresis

PCR - polymerase chain reaction

RNA - ribonucleic acid

RT-PCR - reverse transcription polymerase chain reaction

SPCA- Society for the Prevention of Cruelty against Animals

TAE - tris acetic ethylenediamine tetra acetic acid

TEM – Transmission electron microscopy

TEMED - N,N.N,N tetra methlylene diamene

VP- Viral Protein

WHO- World Health Organisation

DEFINITION OF TERMS

Ubiquitous- Present, appearing or found everywhere.

Antibody- A glycoprotein made by plasma cells in respond to the introduction of an antigen.

Antigen- A foreign substance which can be a protein, nucleoprotein etc, to which lymphocytes respond; also known as an immunogen because it induces the immune respond.

Enterocytes- Also known as intestinal absorptive cells, are simple columnar epithelial cells found in the small intestines and colon.

Enterotoxigenecity- The ability of a pathogen to cause toxins within the gastrointestinal tract.

Gastroenteritis- Inflammation of the gastrointestinal tract.

Immunocompromised- Having an impaired immune system and therefore incapable of an effective immune response, usually as a result of disease.

Monoclanal antibody– Antibody of a single type that is produced by a population of genetically identical plasma cells.

Reassortment- The formation of a hybrid virus containing parts from the genomes of two distinct viruses in a mixed infection.

Self-limiting- A disease process that resolves spontaneously with or without specific treatment

Sero-group- A group of viral species that are closely related antigenically.

Virulence- The degree or intensity of pathogenesity of an organism as indicated by case fatality rates or ability to invade host tissue and cause disease.

Viroplasm- part in a cell where viral replication and assembly occurs

PUBLICATIONS AND PRESENTATIONS

1. **Manuscripts submitted for review during this study**

   i. **Canine rotavirus infection: Interrelationship with human**
   LP Kgosana, I Du Preez. K Malatji and NP Sithebe

   Indian Journal of Virology

   ii. **Detection of Rotavirus antigen from bovine calves in Mafikeng using RNA-PAGE and PCR methods**
   K K Malatji, **LP Kgosana** I Du Preeze and NP Sithebe

   J of Virology

2. **Conference Presentations**

   i. **L.P Kgosana** and N.P Sithebe: Molecular characterization of rotavirus strains isolated from dogs Stools. 11th African rotavirus symposium (May 2017), Malawi, (Poster)

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ABSTRACT

Background: Rotavirus is one significant viral cause of acute gastroenteritis in humans and animals. It is estimated to cause approximately 218 000 deaths annually in children of less than 5 years of age globally, predominantly in developing countries. Dogs are considered as reservoirs of viral, bacterial, or parasitic zoonosis for persons of all ages due to the close relationship of dogs and humans. Contamination of the stools with rotavirus of infected dogs embodies a solemn hygienic problem in many capacities and this may be of epidemiological importance and therefore the need to conduct rotavirus prevalence studies amongst dogs especially in rural areas where animal health facilities are a scarcity.

Methods: Two hundred and forty five (245) stool samples (diarrhoeal and none diarrhoeal) were randomly collected from dogs in Mafikeng and its immediate proximity, in the North West Province, between 2014 and 2016. The samples were screened for the presence of Rotavirus using a VIKIA Rota/Adenovirus Kit to determine antigen presence. Samples positive for rotavirus antigens were further analysed by Polyacrylamide Gel Electrophoresis (PAGE) to determine electrophoretic patterns and the morphology of the virus was studied using Transmission Electron Microscopy (TEM). Only five (5) PAGE Positive samples were confirmed using Real Time RT-PCR and from the five real time positive samples only two were positive by conventional RT-PCR. The resulting PCR products were sent to Inqaba for sequencing and BLASTING was completed through the National Center of

Biotechnology Information (NCBI). The relatedness of the strains was analysed through a phylogenetic tree.

Results: The overall prevalence based on antigen detection of rotavirus from this study was found to be 9.76% (24/245). Comparing the 3 years of data collection, the prevalence of rotavirus was shown to be higher in 2015 (16.84%) than 2014 (10.84%) and 2016 (2.94%) and when the winter and the summer season collection were compared, rotavirus prevalence was found to be higher during the winter season at 15.46%, than during the summer season which was 6.08% in line with most human rotavirus reports. The age distribution of the dogs studied was estimated to be less than 3 months and older and rotavirus prevalence considering age, was found to be 15.9% in dogs less than 3 months and 8.46% in older dogs. The prevalence between diarrhoeal and non-diarrhoeal animals was found to be 7.84% and 8.46% respectively. Two samples visualised by TEM for structural morphology revealed viral like particles of about 50nm. From PAGE analysis, only 5 samples showed rotavirus migration patterns. All (5), PAGE positive samples were amplified successfully with Real Time RT-PCR and only two samples were successfully amplified by conventional RT-PCR.

Discussion: Currently there are no rotavirus prevalent studies published concerning dogs in the country. This study was considered, therefore, as a baseline study. The prevalence results obtained need to be interpreted with discretion as we do not have any other data in the country to compare with except studies from abroad. Therefore the antigen/antibody of 9.46% indicates an exposure of some dogs to the virus. The

confirmation tests also revealed a low molecular prevalence of 2.04%. The two phylogenetic trees constructed indicated that the isolated rotavirus strains are not of canine origin but of human origin. The strains were also placed in Groups A and C which are common in humans.

We may therefore **conclude**, with caution, that rotavirus infection among dogs in the area of study is currently not a problem corresponding with some reports from abroad. Though the study will contribute to the missing body of scientific knowledge further cross-sectional studies with higher numbers are recommended in order to be able to make informed conclusions about the molecular epidemiology of rotavirus infection among dogs, not only from the rural areas of Mafikeng but also from other rural areas in the country.

CHAPTER 1

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1.0. Introduction and Literature Review

1.1. Introduction

Rotavirus was first discovered by Dr Ruth Bishop and her team at The Royal Children Hospital in the year 1973 in Australia (Bishop *et al.*, 1973) and has become one of the mostly distributed gastrointestinal virus that infects infants of mammalian species worldwide (Gentsch *et al.*, 2005). Rotavirus infection previously caused approximately 600 000 deaths per year worldwide and the largest proportion of mortality occurs in the lowest-income countries (Glass *et al.*, 2006), however the mortality rate has dropped by 57%, now amounting to 213 000 deaths per year post the vaccine era (Lui *et al.*, 2016). Patients who die from rotavirus infections die from the complications that come with rotavirus such as severe dehydration and secondary bacterial infection and not from the virus itself (Takeshi and Hoshino., 2008).

Rotavirus is highly contagious among the same group of species and also affects adults though the infection is known to be self-limiting, meaning that the virus will clear off after a certain period of time without treatment (Kang *et al.*, 2007). However there are vaccines available which are licensed and prequalified by the World Health Organization (WHO) and are already in use in national immunization programs in over 100 countries worldwide (Tate *et al.*, 2016).

Canine rotavirus is often asymptomatic with mild diarrhoea and may thus be taken lightly as the infection present itself not to be so much of threat and this therefore makes the canine rotavirus investigations of importance to the society. Rotavirus infection may

be overlooked and yet may play a great role in the transmission of the virus. While asymptomatic cases of rotavirus infections make the virus to be shed faster and without control, thus increasing the burden of disease among human.

Environmental contamination by dogs' stools also represents a serious sanitary problem in many areas and thus rotavirus infections become an important epidemiological factor especially in areas with inadequate water purification systems (Gabby *et al.,* 2003). However, the virus is not preventable by improved sanitation or water supply, Reports also indicate that dogs may be infected with rotavirus from different species and that transmission may occur through direct contact, indicating that dogs may have a role in the transmission and dissemination of rotavirus between humans and other species resulting in interspecies rotavirus transmission which is thought to be one of the important contributors involved in the evolution and diversity of rotavirus in nature (Takesh and Hoshino., 2008). Since there is a possibility of getting infected with more than one strain at a time, the chances of genetic re-assortment also increase thus making it difficult to study the different types and components of their structure (Gentsch *et al.,* 2005; Hoshino *et al.,* 1985).

Currently, there are only a few reports on the isolation of canine rotavirus and thus fewer knowledge of the circulating strain (Naumann, S. & Vahlenkamp, T. W., unpublished observations and Sieg *et al.,* 2015) and the status of rotavirus distribution in South Africa is unknown. This research study, therefore, focussed on screening for the presence of rotavirus from dogs' stool collected from the rural areas of Mafikeng and its immediate surroundings. The study also sought to characterize the isolated dog-rotavirus strains to determine the predominant circulating groups in these areas.

1.2. Problem Statement

Several local and international studies have reported rotavirus as a major health problem associated with high morbidity and mortality, and causing major economic losses for both the communities and families (Seheri, *et al.*, 2010) amongst humans. However, there is little to no reporting on the mortality and morbidity of rotavirus infections among canines. Due to the rotavirus' ability to go through genetic variation, and evolving into new unrecognized strains the virus has become more difficult to eradicate. The virus can also be transmitted or can circulate among animals of different species, thus making its spread more rapid and difficult to control. Therefore molecular epidemiology and characterization on this virus among dogs are a necessity. The study, considered a baseline study, was conducted in order to contribute to the body of scientific knowledge for future studies.

1.3. Aim

The aim of the study was to determine the molecular distribution of rotavirus from dogs' stools collected in Mafikeng, North West Province.

1.4. Objectives

- To screen for the presence of rotavirus from stool collected from dogs using Immunochromatography Technique (ICT) and Polyacrylamide Gel Electrophoresis (PAGE)
- To detect the morphology of rotavirus by Transmission Electron Microscopy (TEM)
- To confirm the amplification of rotavirus using Real Time RT-PCR and Conventional RT-PCR

- To phylogenetically analyse the resulting strains

1.5. Literature Review

1.5.1. Virology

1.5.1.1. Genomic structure

Rotavirus is a double stranded RNA virus which is in the family of Reoviridae (Attoui, *et al.,* 2000, Kanai *et al.,* 2017). The name originates from the Latin word *rota* which means a wheel. This is because rotavirus has a wheel-like structure (Takeshi and Hoshino., 2008) (Figure 1.1). It is made up of 11 segments of RNA which encodes 6 structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and 6 non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6) (Matthijnssens *et al.,* 2008; Takeshi and Hoshino., 2008) (Table 1.1). Structurally the virus is made up of a triple shelled capsule which is the main factor to its virulence. It is made up of the outer, inner and middle layer (Takeshi and Hoshino., 2008). The outer part of the capsule encodes the proteins VP4 and VP7 which determines the protein P-type and G-type respectively. These proteins are responsible for evoking the neutralizing and protective antibody of the host (Kang *et al.,* 2007).

The 11 segments range in size from 667 to 3302 base pair (Estes and Cohen., 1989). The proteins encoded from each segments are listed in the tables below:

Table 1.1. Segments number with protein they code for (Estes and Cohen., 1989, Patton., 1995).

Segments	Proteins	Size (bp)	Size (kDa)
1	VP1	3 302	125
2	VP2	2 687	95
3	VP3	2 591	88
4	VP4	2 364	86
5	NSP1	1 581	58
6	VP6	1 356	44
7	NSP2	1 059	36
8	NSP3	1 104	34
9	VP7	1 062	37
10	NSP4	751	20
11	NSP5/6	667	21/12

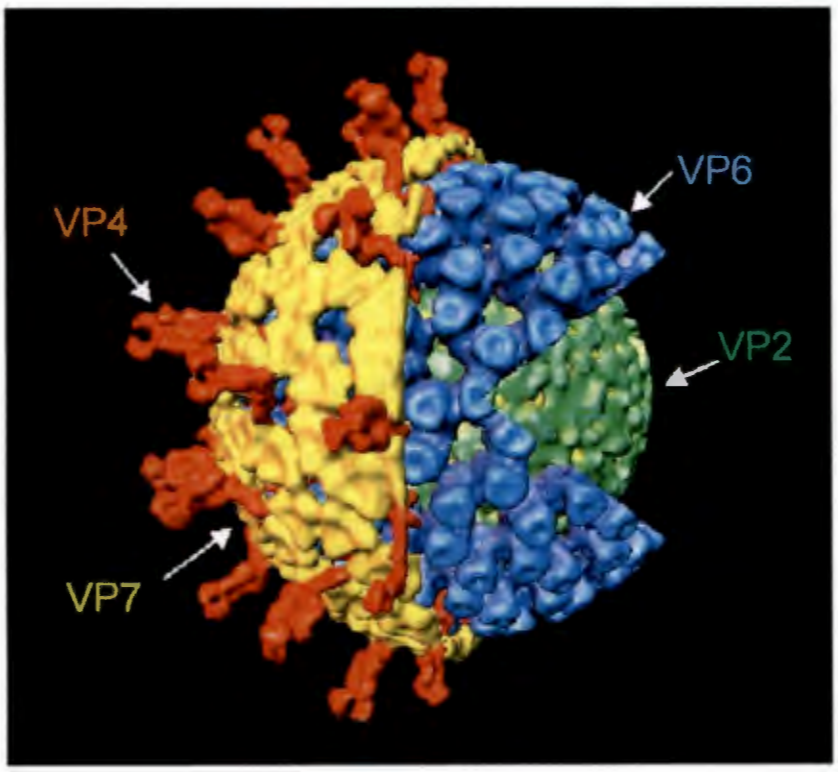

Figure1.1.The structure of the enveloped tripled shell of a rotavirus. Picture by Sarah M McDonalds (http://galleryhip.com/sarah-mcdonald-rotavirus.html)

1.5.1.2. Proteins

Proteins are encoded from a single viral protein with the exception of gene 11 which encodes for NSP5 and NSP6 (Figure1.3). These proteins are responsible for different processes which are vital for replication, growth and survival of the virus. The inner layer of the rotavirus virion is mainly composed of VP2, which encases VP1, the viral RNA-dependent RNA polymerase, and VP3, the viral capping enzyme. The middle layer of the virion is composed entirely of VP6 trimers, which determine rotavirus groups. VP7 and VP4 comprise the outer layer of the virion and are the basis of a binary classification system defining G types (glycoprotein) and P types (protease sensitive), respectively. VP7 and VP4 are capable of independently eliciting neutralizing

antibodies, initially used to define rotavirus G and P serotypes (Matthijnssens *et al.*, 2011).

The non-structural proteins are variously involved in replication (NSP1 to NSP3, NSP5, and NSP6) and morphogenesis (NSP4), and with the exception of NSP1 and NSP4, all interact with nucleic acids. De Grazia *et al.*, (2007) suggested that non-structural protein NSP4 is beneficial for assembly of epidemiologic information and locating the basis of uncommon rotavirus strains. NSP4 is responsible for causing the disease in that it is the enterotoxin (Figure 1.3) which is capable of initializing mobilization of calcium, now an increase in calcium is involved in causing diarrhoea (Tian *et al.*, 1996).

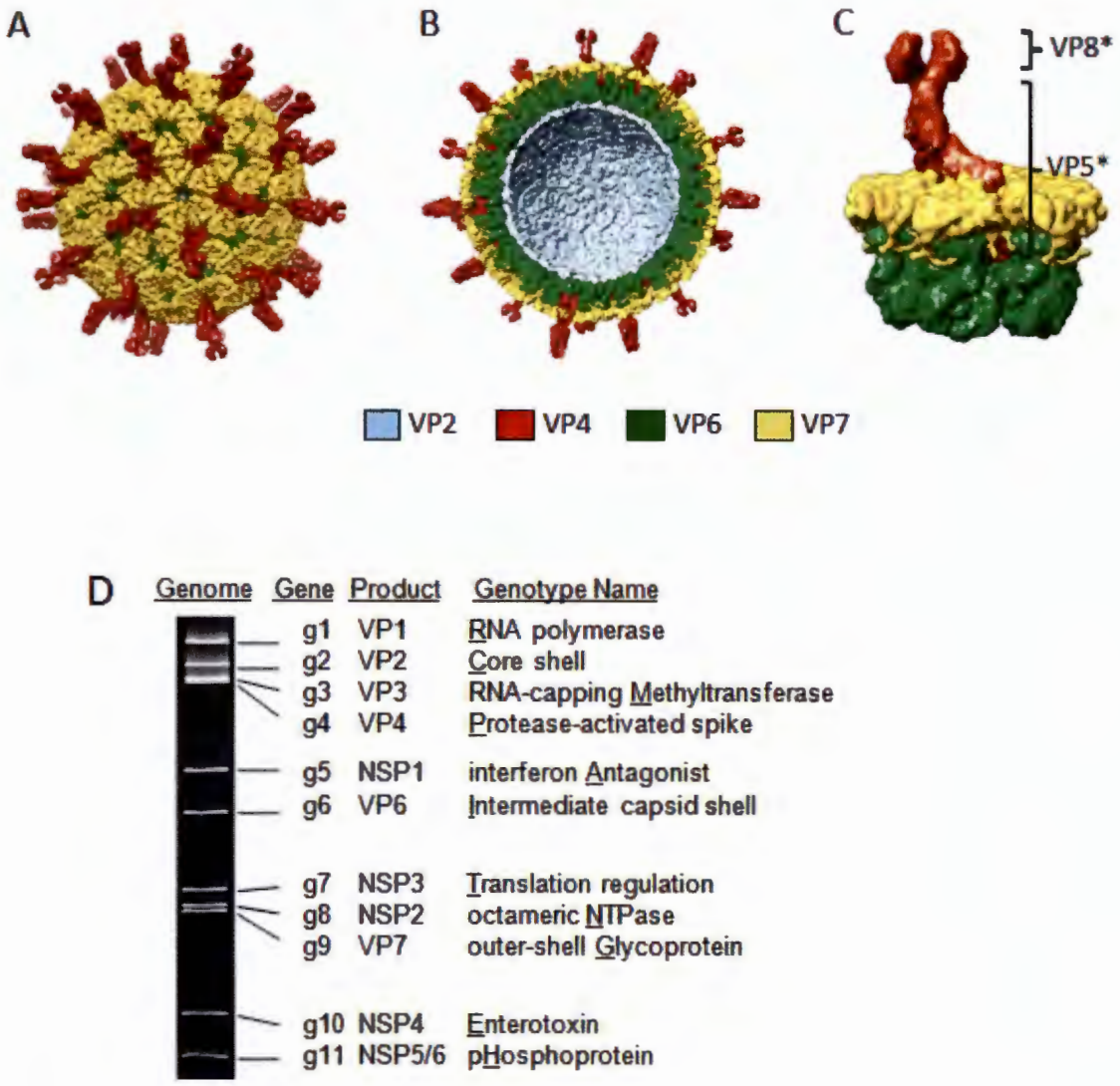

Figure 1.3. The illustration above shows on (A) the structure of rotavirus, (B) shows the three layers of the virus, (C) shows a close-up part of the outer shell where VP4 (spike) in which VP8 is also encoded and VP7 shaded yellow, where VP5 is encoded and lastly (D) shows a picture of electrophoretic pattern of genes, protein encoded together with the genotype name (Patton., 2012).

NSP1: A protein coded from segment number 5 and weight of 58 kDa (figure 1.3). The protein acts an interferon antagonist and function in supressing the induction of apoptosis (Anold *et al.,* 2011; Bagchi *et al.,* 2010)

NSP2: Coded from gene 7 and weighs 36 kDa (figure 1.3) which is RNA binding protein accumulating in the viroplasms. This protein is mostly important for the formation of viroplasms and genome replication (Silvestri *et al.,* 2004). It is unique in that it does not depend on any viral protein for RNA-binding activity.

NSP3: This protein is coded from gene 8 and weighs 34 kDa (figure 1.3). The protein is slightly acidic and moderate to abundant in infected cells. Together with NSP1 localized in the cytoskeleton, However it is unknown if they interact. Functions by suppressing host protein synthesis via antagonism of the poly A binding protein and enables the translation of the rotaviral mRNA transcripts (Vende *et al.,* 2000; Piron *et al.,* 1998).

NSP4: As illustrated on figure 1.3, VP4 is coded from gene 10, weighs 20 kDa and is called an enterotoxin. This protein is mostly known for the fact that it is an enterotoxin which causes symptoms in infected individuals. This is achieved by stimulating chloride secretion through the calcium-dependant signalling pathway after activation of the putative receptor on the intestinal epithelium (Horie *et al.,* 1999). However further studies show that it cannot be concluded that NSP4 does not play a major role in causing symptoms, thus the enterotoxigenicity of this protein is yet to be confirmed. "The non-structural protein NSP4, encoded by gene 10 in Group A rotaviruses, has been implicated in a pathway shown to play a role in viral assembly (Kirkwood and Palombo., 1997). Several studies and analyses of the NSP4 genes have demonstrated that NSP4 is an intracellular receptor for a number of human and animal Group A

rotaviruses has for single-shelled particles (those lacking the outer cap revealed a reasonably well-conserved proteins, VP4 and VP7) and assists in the translocation of these particles across the endoplasmic reticulum sequence conservation and divergence in the gene cod-(ER) membrane by budding (Kirkwood and Palombo., 1997).

NSP5/6: They both accumulate in viroplasms with different molecular mass of 21kDa for NSP5 while NSP6 has a molecular mass of 12kDa. NSP5 is an RNA-binding protein involved in the formation of viroplasms (Silvestri *et al.,* 2004). It is coded from gene 11 together with NSP6. NSP6 is not coded by all rotaviruses and degrades within two hours of synthesis (Rainsford and McCrae., 2007; Mattion *et al.,* 1991). The function of NSP6 in replication still needs to be define (Hu *et al.,* 2012)

VP1: Coded from gene 1 and has a molecular mass of 125 kDa being the largest of all proteins (figure 1.3). VP1 is a nucleotide-binding protein and RNA polymerase (Patton., 1995). The co-expression of VP1 and VP2 have been reported to synthesis dsRNA from viral mRNA in vitro (Wentz *et al.,* 1996).

VP2: Coded from gene 2 and weighs 95 kDa (figure 1.3). It is the core shell where the virus is a single layered particle. This layer house the dsRNA genome (Lawton *et al.,* 1997). The attraction of VP2 for ssRNA may be linked with the packaging of viral mRNA into the core during the replication (Patton., 1995)..

VP3: coded from gene 3 and weighs 88kDa (figure 1.3). It is an RNA capping emzyme and important component of the transcriptase compleses (Patton ., 1995)

VP4: Made up of 60 spikes that are formed by dimers of VP4. It is coded from gene 4 and weighs 86 kDa (figure 1.3). The spikes of VP4 directly associated with specific monoclonal antibodies (Tihova *et al.,* 2001). Together with VP7, these proteins are important during cell entry through attachment and penetration.

VP6: This proteins has a molecular mass of 44 kDa and is coded from gene 6(figure 1.3). Forms part of the DLP which is is the transcriptionally competent form of the virus during the replication cycle (Pesavento *et al.,* 2006). By interacting with VP4 and VP7, it plays an important role in the organization of rotavirus generally.

VP7: Formed part of the outter shell making the TLP of the virus and responsible for inducing production of specific antibodies. It is coded from gene 9 and weighs 37 kDa (figure 1.3). VP7 makes the largest part of the outer shell and glycoprotein (Estes., 2001).

1.5.1.3. Classification

Rotavirus is classified using the nomenclature kind of method which classifies viruses based on their nucleic acid (Matthijnssens *et al.,* 2008b). The virus has seven sero-groups (A-H) and may be distinguished on the basis of their antigenic relationship and the pattern of the double stranded RNA segments in the polyacrylamide gel electrophoresis (Matthijnssens *et al.,* 2012). The most common sero-group is group A which causes diarrhoea in mammalian species (Matthijnssens *et al.,* 2012).

A new genome based classification system was introduced to differentiate rotavirus strains (Matthijnssens *et al.,* 2008a). This system is based on nucleotide-sequences for classification where all the 11 segments of rotavirus genome are allocated in harmony

to their nucleotide percent cut off value (Matthijnssens *et al.,* 2008b). With these nucleotide cut off percentages, a complete nomenclature and classification system was proposed to nominate the complete genetic constellation of rotavirus (Matthijnssens *et al.,* 2008b). This is the suggested schematic nomenclature: Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx which delineates the genotype of the genes VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 respectively, the x represents the number of corresponding genotypes (Matthijnssens *et al.,* 2008b).

1.5.1.4. Epidemiology

Rotavirus affects young animals such as cats (felines), dogs (canines), cattle (bovine), pigs (porcine), etc. The virus does affect adults too but it is self-limiting thus it is not a threat in adult groups (Riepenhoff-Talty *et al.,* 1987; Morris and Estes., 2001). However, there are cases in which adult dogs present symptoms like infants (Martella *et al.,* 2000). The virus can be isolated in contaminated water and also sewage where the virus can be found in abundance due to the fact that the virus is shed through the faeces (Metcalf *et al.,* 1995).

The environmental contamination by dogs' stools represents a serious sanitary problem in many urban and suburban areas since the detection of rotavirus in stools from these animals has been previously reported, rotavirus has become an important epidemiological factor (Gabby *et al.,* 2002). Moreover Gabby *et al.,* (2002) also indicated that dogs may be infected with rotavirus from different species and that transmission may occur through direct contact, indicating that dogs may have a role in the transmission and dissemination of rotavirus between humans and other species. According to Mochizuki *et al.,* (1985) in spite of the prevalence of anti-rotavirus

antibodies in dog populations, detection of rates using molecular techniques has been low, however the human and bovine rotavirus could propagate in dogs without any clinical signs and thus it was concluded that canine rotavirus infection may be unapparent and dogs may have a role in the transmission and dissemination between human and other animal species.

1.5.1.5. Transmission

Rotavirus is mostly isolated from the stool of an infected host and that is how the virus is shed. Transmission is thorough the faecal oral route (Parashar *et al.,* 1998). Puppies are normally kept together in a box after birth, thus transmission occurs if one puppy gets infected and spread the virus by its diarrhoeal stool in the box (Dennehy and Penelope., 2000). The healthy one will definitely ingest some the stool and that is how they get infected. Only a relatively small dose (i.e. amount found in nails) of stool is enough to induce the signs of gastroenteritis (Dennehy and Penelope., 2000).

With every case of watery diarrhoea in animals, one must make sure that the place where the animal was is properly disinfected. Failure to disinfect the area where the infected animal was living will play a huge role in the spread of the disease, because the virus can persist for a long time in the environment (Dennehy and Penelope., 2000). Drinking contaminated water does contribute towards spreading the virus (Dennehy and Penelope., 2000).

There have been cases where the animals were found with strain of humans. This means that puppies can get infected by ingesting stool of infected human. When a person prepares food for the dogs without washing their hands after using the bathroom

or after changing children's nappies, one can spread the virus to dogs (Dennehy and Penelope., 2000).

Depending on the severity of the infection and the virus itself, the virus can be spread through air droplets (Chen *et al.,* 2007). This can be common in animals which live in close contacts such as those kept in cages and those kept in boxes. Hospitalized animals also live in close contacts and these are the animals which are immunocompromised, this will play a role in the transmission of virus (Parashar *et al.,* 1998; Dennehy and Penelope., 2000).

1.5.1.6. Replication

The main location of rotavirus infection is the small intestinal villi, where the virus replicates in the cytoplasm of mature enterocytes (McDonald and Patton., 2011). The complex architecture of the mature rotavirus particle, with three concentric capsid layers, elegantly integrates the necessary elements required for cell entry and endogenous transcription of viral mRNAs (*Hu et al.,* 2012). "The triple layered particle is the infectious form of the virus that attaches to and enters into host cells. However, during the cell entry process, the outer VP4–VP7 layer of the TLP is shed, depositing a double-layered particle (DLP) into the cell cytoplasm" (Lawton *et al.,* 2000; Long and McDonalds., 2017). Removal of the outer layer during the process of cell entry triggers the endogenous transcriptase activity of the resulting double-layered particles (*Hu et al.,* 2012). DLP works as a Transcriptional machine in the cytoplasm, generating eleven topped (+) RNAs (Charpilienne *et al.,* 2002). Rotavirus DLPs in cytoplasm generate capped, non-polyadenylated, (+) ssRNA transcripts which are needed to aid discharged from the particles, now these transcripts serve possibly for translation of virus-encoded

proteins as templates for replication (Silvestri *et al.,* 2004). The capped transcripts are released through aqueous channels at the 5-fold axes of these intact particles. Once these initial transcripts are translated, the rotavirus NSPs then coordinate various stages of genome replication and viral assembly by adapting and modifying the cellular machinery, which leads to productive release of mature particles through cell lysis (*Hu et al.,* 2012). The process is illustrated in Figure 1.2.

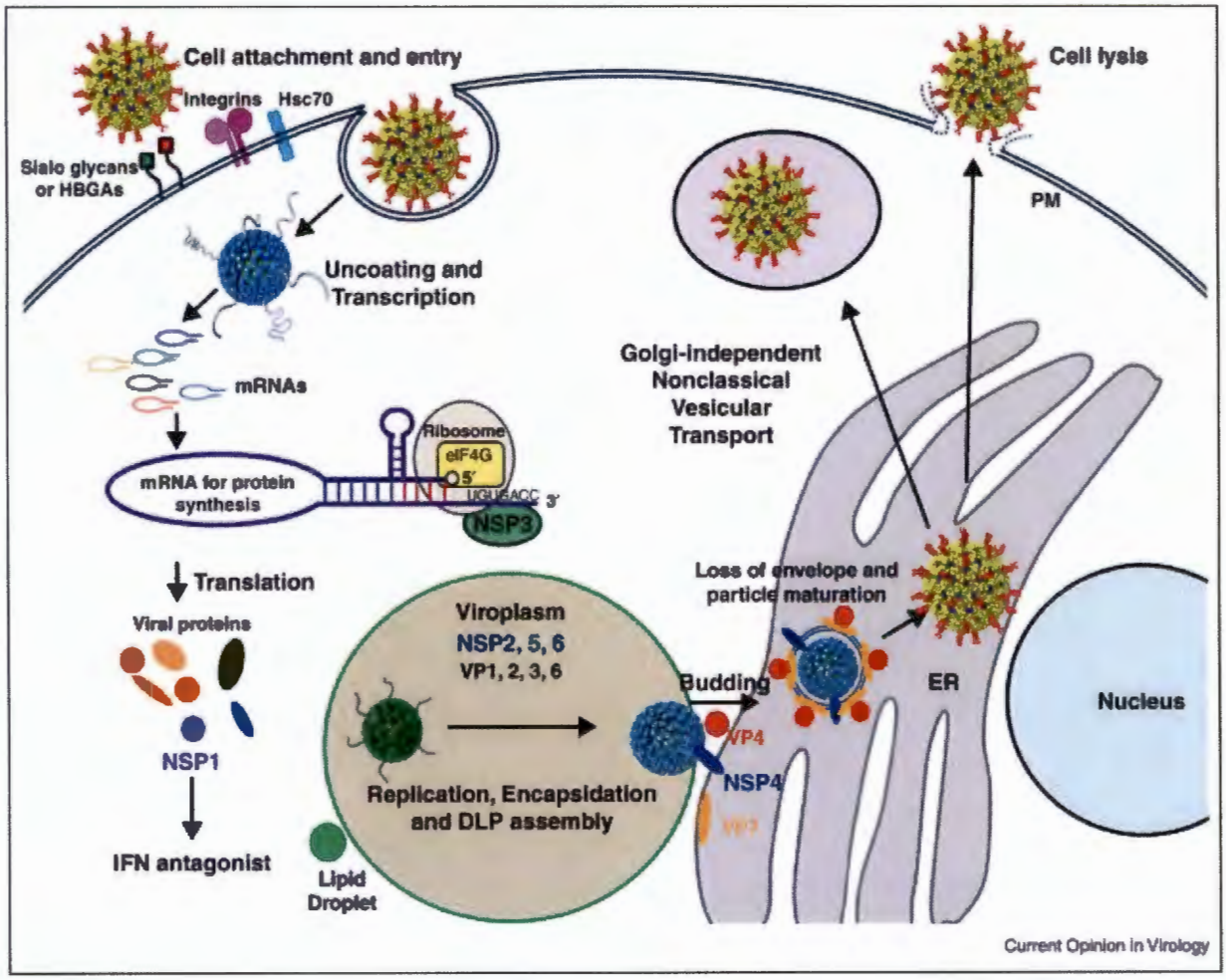

Figure 1.2. The diagram shows all the processes that happen during replication of the rotavirus. (*Hu et al.,* 2012).

NSP2 and NSP5 are the two proteins which are directly involved in the formation of viroplasms and act as recruiters of the protein components of the core. According to

Silvestri *et al.,* (2004) the involvement of these proteins had been established by siRNA-mediated knockdown experiments where the loss of NSP2 or NSP5 expression has been interconnected to the lack of viroplasm formation in the infected cell (Patton *et al.,* 2006). Functional proteasomes and components of the autophagicpathway are essential for viroplasm formation and RV replication (Contin *et al.,* 2011; López *et al.,* 2011; Arnoldi *et al.,* 2014; Desselberger., 2014). NSP2 forms a doughnut-shaped octamer and four grooves related by a four-fold axis on the sides of the octamer, these groves are the RNA binding sides. The formation of octamers by NSP1 and NSP5 is required for the NTPase activity to localise in the monomeric subunit and their ability to bind RNA (Pesavento *et al.,* 2006). Viroplasms are caused by the assembly of insoluble cytoplasmic complexes (Sen *et al.,* 2007). NSP4 protein forms vesicular structures in cells after a calcium switch, and reported to be associated with viroplasmic NSP5 (Berkova *et al.,* 2006). As NSP4 expression assembles intracellular calcium, NSP4 functions as a viroplasm assembly trigger that leads NSP5 to form viroplasms (Berkova *et al.,* 2006; Sen *et al.,* 2007).

DLP leaves the viroplasms by budding through the endoplasmic reticulum (ER) for the purpose of maturation, this is done through NSP4 which will serve as intercellular receptor by interacting with VP6 (Taylor *et al.,* 1996). Budding rotaviral particles are briefly enveloped, but the envelope is lost when RV particles procure the outer layer consisting of VP4 and VP7 within the ER, nevertheless, there's a possibility that VP4 spikes react with VP6 first and are then embedded in a continuous layer of VP7 trimers (Estes and Greenberg., 2013; Trask and Dormitzer., 2006). Rotaviral particle maturation results from blockage NSP4 expression by siRNA thus replication will also be inhibited

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through the interaction with viroplasm. Rotaviral TLPs will leave the epithelial cells by a kind of budding process which does not kill the cell immediately (Gardet *et al.,* 2006); Silvestri *et al.,* 2005; Berkova *et al.,* 2006)

1.5.1.7. Pathogenesis

Rotavirus will attach to the target cells which are the enterocytes of the small intestines. The virus will use the spike-like projection it possesses to attach to the cell (McDonald and Patton., 2011). This is the outer part of the capsule which is VP4. The second layer of the capsule VP7 will be released; this triggers the immune system of the host to produce antibodies against the antigen.

Following this will be the release of the second layer of the capsule which is VP6. The protein VP6 is considered to be highly antigenic, so this protein will trigger antibody production more than other proteins and will result in disease or symptom (Estes and Cohen., 1989). The inner part of the capsule will then be released. All these proteins play a role in the virulence characteristics. The protein NSP4 will disturb homeostasis of calcium and this will results in problems with absorption of the nutrients such as carbohydrates, other valuable electrolytes and body fluids (Gajardo *et al.,* 1997). Then all this will be lost in the watery stool. Symptoms like dehydration result from this excessive fluid loss. The virus also irritates the lining the intestines resulting in inflammation of this lining (Ramig., 2004).

1.5.1.8. Clinical signs

Canine rotavirus is usually responsible for subclinical or mild forms of enteritis, associated with anorexia and vomiting, especially in puppies younger than two weeks of age. This is best described above in the pathogenesis which says the virus is found in

the small intestines and disturbs absorption of fluids together with electrolytes. Other signs which may follow would be fever which is common with any viral infection (Figure 1.4). Gastroenteritis result in abdominal pains, however this may be difficult to observe in animals (Bridger., 1987).



Figure 1.4. Pie chart of symptoms that diarrhoea and vomiting are most likely to co-occur in patients with rotavirus and fever is shown to almost occur in every patient (Staat and Merchant, 2005).

1.5.1.9. Diagnosis

Rotavirus in dogs can be detected using a wide range of methods. The following list describes a few methods that can be used for the detection of rotavirus.

VIKIA® Rota/Adenovirus Kit

The Rota-Adeno test is a rapid test which is based on the immunochromatography technique (ICT) and brings about dual detection of rotavirus and adenovirus in a single

stool extract. This is a qualitative test based on the association of monoclonal antibodies specific to rotavirus and adenovirus respectively (Kaplon *et al.,* 2008).

Premier Rotaclone

Premier Rotaclone is an enzyme immunoassay (EIA) used for the detection of rotavirus antigen in faecal samples. It is a monoclonal antibody test which is simple and highly sensitive protocol that provides rapid detection. Rotaclone uses the monoclonal antibodies in a solid phase sandwich type EIA where the plastic microtiter wells are coated with a monoclonal antibody that is directed against the product of the VP6, the group specific antigen known for rotavirus (Dennehy *et al.,* 1999).

Prospect rotavirus test

The ProSpecT™ Rotavirus test is a subjective catalyst immunoassay for the detection of rotavirus (Group A) in human faecal specimens as a guide in the determination of intense gastroenteritis. The test uses a polyclonal immune response to distinguish particular proteins, including the major inward capsid protein (VP6), present in Group A rotaviruses.

TEM

The presents of rotavirus in the stool can be detected by passing through a beam of electrons in order to form an imagine. Rotavirus was discovered using an electron microscope (Bishop *et al.,* 1973)

RT-PCR

The main purpose of PCR is to generate multiple numbers of copies of a certain gene. The targeted DNA must be recognised by primers to begin replication. PCR take place

in 3 steps namely Denaturation of double stranded DNA at 95°C, Annealing which takes place at 42°C and lastly Extension at 72°C. These conditions were reported by Gouvea and colleagues (Gouvea *et al.*, 1990). The RNA from rotavirus must first be converted to cDNA before replication can start. Primers for VP4 (con 2 and con3) and VP7 (sBeg 9 and EndA) were constructed by Gentsch *et al.*, (1992) and Gouvea *et al.*, (1990) respectively.

Genotyping

Rotavirus is suitable for the surveillance determination principally where rotavirus vaccination has been implemented (Liu *et al.,* 2015). Genotyping allows specific strains to be typed and identification of the new putative reassortant strain (Iturriza-Gomara *et al.,* 2004). Primer used for genotyping of VP4 and VP7 were described by Gentsch *et al.,* (1992) and Gouvea *et al.,* (1990) respectively.

Real Time PCR (qPCR)

Detection and quantification of rotavirus by qPCR has been defined by Schwarz *et al.,* (2002) targeting VP6 region. The Syber green dye used in qPCR binds to double stranded DNA in the reaction. The Rotorgene 2000® Software will record the fluorescence produced as the numbers of DNA copies increases resulting in peak on the curve. Studying of the melting temperature curves is vital to determine if the peak on the curve represents true amplicon.

Sequencing

The purpose of sequencing is to determine the order of the nucleotides of a gene from a PCR fragments. The reaction happens like PCR with Denaturation (94°C), Anneal (50°C) and Extension (60°C). After this reaction comes the separation of DNA molecules based on their size on an agarose gel by electrophoresis. An automated sequencer will detect the fragment migrating on gel through laser that excites fluoresces thus producing a light of a distinct colour. Finally the assembling of the parts of sequenced gene by the computer program using both forward and reverse primers until all the required bases are produced.

Phylogenetic analysis

Phylogenetic analysis of rotavirus allows the relationship among strains isolated across the world to be studied. The ancestral evolution of these strains allows monitoring of similarities based on genetic characteristics. Phylogenetic analysis of rotavirus strains is important because natural changes of the prevalence of rotavirus is common and thus continuous monitoring of the circulating genotypes in the community is crucial to guarantee the successful delivery of available vaccines (Intamaso *et al.*, 2017).

1.5.1.10. Prevention

Control of either the initiation or the spread of the disease can be maintained by proper hygiene and sanitation (Dennehy and Penelope., 2000). The following will be useful in the prevention of infection of rotavirus:

- Any area where there was a diarrhoeal stool must be disinfected
- Puppies which have any form of diarrhoea must be isolated from the healthy ones
- Proper hand washing before preparing food for dogs.

1.5.1.11. Rotavirus vaccines

Rotavirus vaccine development began in the mid-70s through animal rotavirus strain (Zissis *et al.*, 1983). The vaccines demonstrated variable efficacy in field trials but gave unsatisfactory outcomes in developing countries (Lanata *et al.*, 1989; Hanlon *et al.*, 1987). First generation rotavirus vaccines used naturally attenuated animal strains; however effectiveness of these monovalent vaccines against rotavirus was variable. Besides lamb-derived vaccine that is presently used in China, all these vaccines were

not licensed (De Mol *et al.,* 1986; Vesikari *et al.,* 1984; Yen *et al.,* 2011). While second generation vaccines as well as currently licensed vaccines uses either naturally attenuated human-animal reassortant strains or an attenuated human strain (Tate etal., 2010).

RotaShield was multivalent live oral reassortant vaccine which was lisenced in the US in 1998, the vaccine contained a combination of four virus strains representing the most commonly seen G types which were G1, G2, G3 and G4 (Bernstein *et al.,* 1995; Kapikian *et al.,* 1996). The use of the vaccine was discontinued followed a large number (1 in 10 000) (Peter and Meyer., 2002) of vaccine association intussusception were reported by Centers for Disease Control and Prevention.

The live vaccines are RV1 and RV5, where RV1 is a monovalent vaccines which contains an attenuated human G1P8 rotavirus strain and RV5 is a pentavalent human-bovine reassortant vaccine which comprises 5 reassortant viruses, 4 expressing human G-type protein (G1–G4) and one expressing a human P-type protein (P[8]) (Vesikari *et al.,* 2006; Liakopoulou *et al.,* 2005; Vesikari *et al.,* 2007).

Two effective rotavirus vaccines, namely RotaTeq (Merck and Co, PA, USA) and Rotarix (GSK Biologicals, Rixensart, Belgium) have been approved since 2006, and they are recommended for use in all countries by WHO, especially in countries with high related mortality in children than 5 years (Linhares *et al.,* 2008). There is currently no vaccines approved for use in dogs.

Rotateq is human-animal reassortant rotaviruse vaccine that includes either human VP7 or VP4 genes, developed by Merck research. It is a Pentavalent live attenuated vaccine

given orally. It contains VP7 protein G1-G4 from human rotavirus parent strains and attachment protein P7[5] from bovine rotavirus parent strain WC3 (Dehehney., 2008). RotaTeq reduced the occurrence of office visits by 86%, emergency department visits by 94%, and hospitalizations for rotavirus gastroenteritis by 96% (Dehehney., 2008).

Rotarix is a live attenuated human rotavirus vaccine which represents the most common stain (P1A[8]G1. Rotarix established 85% defence rate against severe rotaviral gastroenteritis and 100% protection against the most severe dehydrating rotaviral gastroenteritis however the vaccine was not efficacious against G2 serotype (Ruiz-Palacios *et al.,* 2006).

Vaccines denote the best anticipation for preventing the severe complications of rotavirus infection, especially in disadvantaged counties where resources and admission to care may be limited. South Africa was the first African country to introduce rotavirus vaccine and the related mortality has declined (Madhi *et al.,* 2010)

CHAPTER 2

2.0 Methods and Materials

2.1 Design of the Study

The study was exploratory and laboratory based.

2. 2 Ethical Clearance

The study was cleared by the Research Ethics Committee of the North West University Mafikeng Campus, (S9/NWU-00217-15-S9). The stool samples were collected from the Society for the Prevention of Cruelty to Animals (SPCA) facilities and nearby areas in Mafikeng, North West Province following good laboratory practices (GLP) and good animal handling practices with the help of a Veterinarian. A letter outlining the study aims and its significance was presented to the SPCA facility officials and dog owners requesting their permission as well as their assistance.

2.3. Sample Collection

2.3.1. Sample Size

According to Charan and Biswas (2013) determination of sample size is an important part of research, saying that the evidence is as good as the research is based on. Thus the sample size for this study was determined using the Charan and Biswas method according to the following formula.

Sample (n) = $\frac{Z_{(1-\alpha/2)}{}^2 P(1-P)}{d^2}$

Z1-α/2 = is standard normal variable (at 5% type I error (P< 0.05) it is 0.05)

P = Expected prevalence in population based on previous studies

d = Absolute error or precision (which is 5%)

$$\text{Sample (n)} = \frac{(1.96)^2(0.8\ )(1-0.8)}{(0.05)^2}$$

$$= 245.8624$$

The prevalence of Canine rotavirus as described by Mosallanejad *et al.,* (2015) was 80%. Therefore this prevalence will be our expected prevalence with a 95% confidence level and desired precision of 5%. The expected sample size according to our calculations was 245 samples and we collected that amount.

The samples were collected between 2014 and 2016 during the winter and summer months. Diarrhoeal and non-diarrhoeal stools were collected from puppies less and more than 3 months of age.

2.3.2. Sample Collection Sites

Samples were randomly collected from the SPCA, Mohlabeng, Dibate, Signal Hill and Seweding (Figure 2.1) based on stool availability and accessibility to the facility or site.

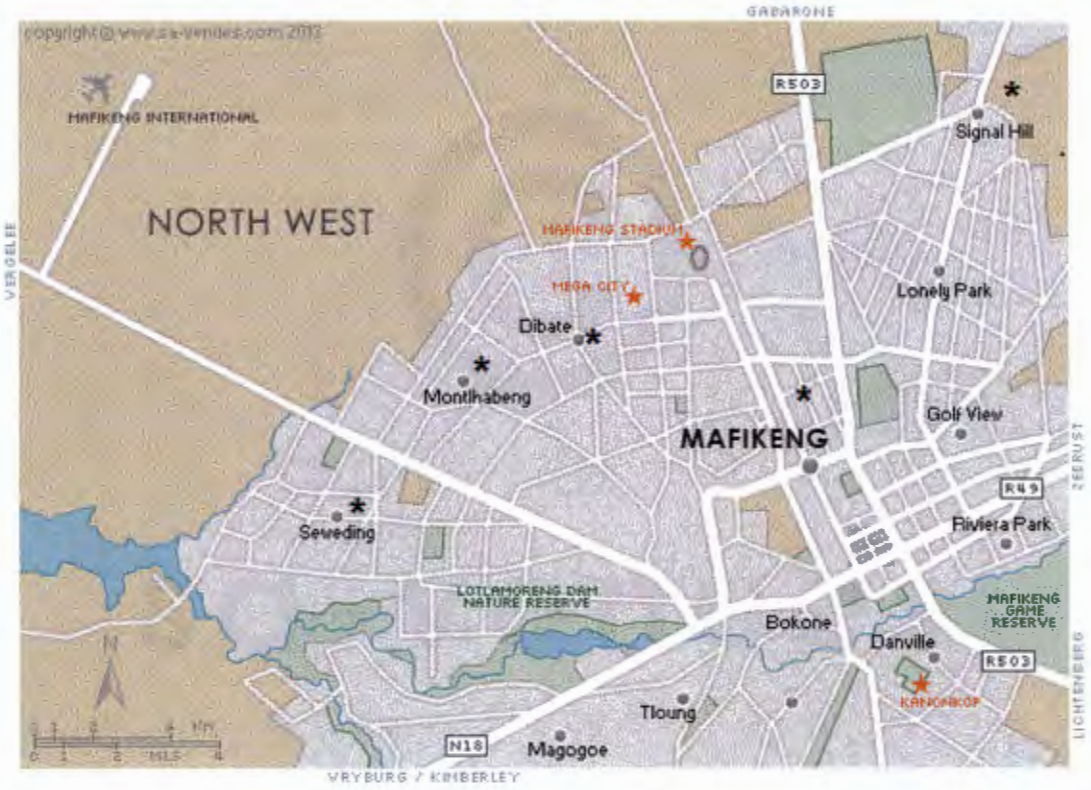

Figure 2.1. Map showing collection sites indicated with a star. https://www.sa-venues.com/maps/northwestprovince/mafikeng.php Access date: 23.06.2017

2.3.3. Methods of Collection

The stools were collected using two methods. The first method was by directly collecting from the rectum. This is the most preferred method because there is minimal contamination. The second method was the free flow in which the stool was only collected when the dog is defecating or collected after it had defecated. Collection was done by trained personnel under the supervision of a Veterinarian. Samples were stored in sterile tubes and transported in cooler bags on ice. The stools were stored at -80°C upon arrival to laboratory until further use.

2.4. Laboratory Techniques

Different screening and confirmation methods were used to determine the presence of the rotavirus from the stool as summarized in the following flow diagram (Figure 2.2).

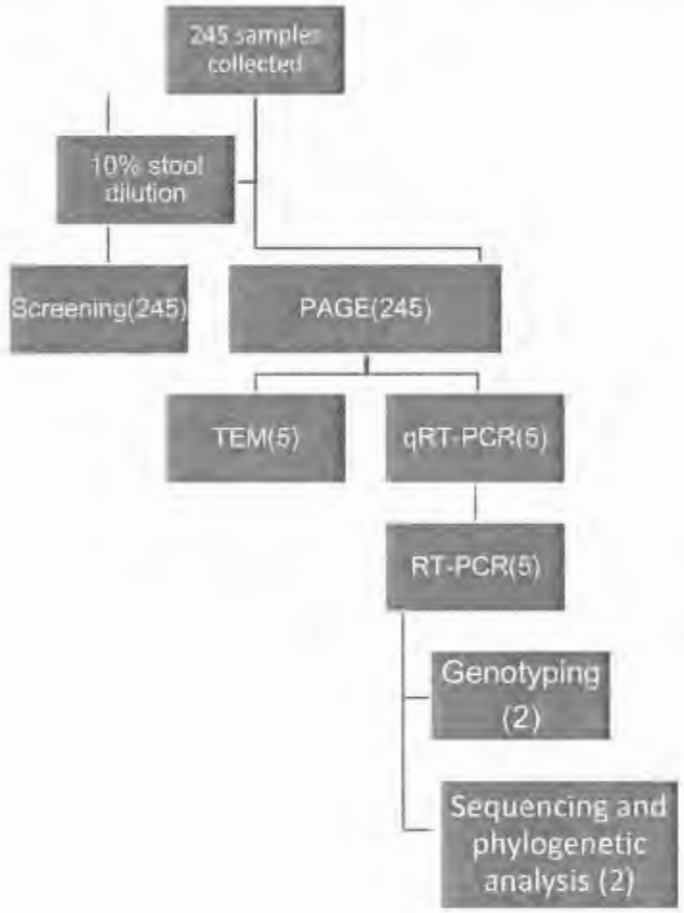

Figure 2.2. Flow diagram showing the order of the processes carried out

2.4.1. Screening Methods

2.4.1.1. VIKIA Rota/Adenovirus Kit

For the detection of rotavirus in the stool samples we used VIKIA® Rota-Adeno. The "Rota-Adeno" test device is a rapid test which is based on the immunochromatography technique (ICT) that brings about dual detection of rotavirus and adenovirus in a single

stool extract. This is a qualitative test based on the association of monoclonal antibodies specific to rotavirus and adenovirus respectively.

The procedure involves a three (3) step process which was exactly followed as described by the manufacturer (VIKIA ® Rota-Adeno, Biomerieux ® SA). Briefly, the first step was to place 50 µl of 10% stool suspension into the collection tube with diluent and was mixed thoroughly. This was followed by dispensing 2 drops of the homogenized solution into the cassette sample well. Finally the last step was the reading of results. Blue line indicated positive for rotavirus while red line indicated positive for adenovirus and if ever there was no control line the test was invalid.

2.4.1.2 . Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was used to separate the RNA present in our sample. For this study PAGE was used to separate the 11 segments of Rotavirus. The procedure involves two steps, namely RNA extraction and gel electrophoresis.

RNA Extraction

500µl of 10% stool dilution was transferred to a clean and sterile 1.5 Eppendorf, followed by addition of 2µl DNAse and 5µl RNAse A. This solution was incubated at 37°C for 30 minutes. Following this was addition of 0.1m DTT and RNAsin, incubated at 37°C for 10 minutes. Then 50µl of 0.5M EDTA and 50µl 10% SDS was added, followed by 620µl of phenol-chloroform and briefly vortex and centrifuged at maximum speed for 10 minutes. After the upper part layer was removed and chloroform was added, briefly vortex and centrifuged for 10 minutes. Again we removed the upper layer and added 620µl isopropanol and the solution was left -20°C overnight.

The solution was then spun at maximum speed and the liquid part was removed without disturbing the pellet followed by an addition of 1ml of 70% alcohol which was spun at maximum speed for 15 minutes. The alcohol was removed and the pellet air dried. Last step was to dissolve the pallet with 20µl RNase free water.

Polyacrylamide Gel Electrophoresis

Reagents measurements for the resolving and stacking gel layer were prepared as indicated on the appendices 1.

A seal to avoid leakage was prepared by adding 10µl TEMED to 1ml the resolving gel solution, poured in the gel tray. Once the seal had polymerized we added TEMED to the resolving gel solution, mixed gently and immediately cast over the seal layer. A tiny amount of saturated butanol was added over the resolving gel layer and left to polymerise.

Once the resolving gel (Appendices) had polymerized, the butanol was washed out with water. On the stacking gel solution (Appendices), we only added APS and TEMED once the resolving gel had polymerized. The solution was mixed gently followed by the addition of APS and TEMED and cast over the resolving gel and then insert combs.

After approximately an hour when the gel has polymerized, the combs were carefully removed the gel was inserted into gel rack filled with 1x Laemmli buffer. The samples were pop-spun thawed and added 20µl of 4x Laemmli loading buffer and then loaded into the wells. The gel was left to run at 30 mAmp overnight.

The gel was removed from glass plates into 100ml of 1x TAE and 10µl of cybergold 10000x and then covered with a foil and put on shaker for 30 minutes. Finally results were viewed under UV light.

2.4.1.3. Transmission Electron Microscopy

Samples were sent to the North-West University, Potchefstroom Campus to study the morphology of rotavirus by Transmission Electron Microscopy (TEM). The 10% stool suspensions were first cleaned using Vertrel before being shipped off. The method for is briefly defined below and preparation of solutions is found the appendix.

Fixation

Fix 10% stool suspension in 4% formaldehyde and 1% glutaraldehyde in 0.1 M PB (pH 7.4) by mixing equal volume of fixative and stool suspension. The suspension was transferred to a centrifuge tube and span for 10 minutes then carefully pipetted off fixative. A fresh fixative was added and left overnight. Fixative was pipetted off and replaced with 8% (0.2M) sucrose in 0.1 M PB overnight at 4°C. The reaction was finally fixed with 1% OsO4 in 0.1 M PB for 1 hour then pipetted off OsO4, rinsed in 0.1 M PB 3x10 minutes.

Dehydration

This step was fulfilled by adding the following solutions: 50% ethanol for 15min, 70% Ethanol for 15min, 95% Ethanol for 15min, 100% Ethanol for 15min (twice), 100% Propylene oxide for 15min (twice), 1:1 EMBed 812 and Propylene Oxide for 2 hours. Finally 2:1 EMBed 812:Propylene Oxide overnight in desiccator with top off.

Embedding

Embed in Beam capsules, and baked in 60 °C oven for 48 hours.

Sectioning

Semi-thin (thick section, 0.5-1 um) and toluidine blue staining were observed the thick section, and trimmed in EM block. Then ultrathin sectioning, and collected on grids.

Staining

Staining grids with uranyl acetate for 15 minutes, rinsed with distilled water and then stained with lead citrate for 3-5 minutes, rinse with distilled water.

2.4.2. Confirmation Methods

2.4.2.1. Qiagen Viral Extraction

Qiagen Viral extraction Kit was used following the manufacturers' instructions with minor modifications. Briefly, 560 µl of prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 ml micro-centrifuge tube then added 180 µl 10% sample dilution to the Buffer AVL-carrier RNA in the micro-centrifuge tube. The solution was mixed by vortexing for 15 seconds and then incubated at room temperature for 10 minutes. And the mixture was briefly centrifuged to remove moisture from the inside of the lid.

Some 560 µl of ethanol (96-100%) was added to the sample, and mixed by vortexing for 15 seconds then briefly centrifuged to remove moisture from inside of the lid. An amount of 630 µl of the solution from the previous step was carefully transferred to the QIAamp Mini spin columns (in a 2-ml collection tube) without wetting the rim and centrifuged at 6 000 x g (8 000 rpm) for 1 minute. Then we replaced the QIAamp spin columns into a clean 2-ml collection tube, and discarded the tube containing the filtrate. This step was done twice until the initial solution was finished.

Some 500 µl of Buffer AW1 was carefully added to the QIAamp Mini spin columns and centrifuged at 6 000 x g (8 000 rpm) for one minute. The QIAamp Mini spin column was

placed in a clean 2-ml collection tube, and the tube containing the filtrate was discarded. A volume of 500 µl of Buffer AW2 was carefully added to the QIAamp Mini spin columns and centrifuged at full speed (20 000 x g; 14 000rpm) for 3 minute. The QIAamp Mini spin column was placed in a new 2-ml collection tube and centrifuged at full speed for 1 minute.

The QIAamp Mini spin column was placed in a fresh sterile clean labelled 1.5-ml microcentrifuge tube, and then discarded the old collection tube containing the filtrate. Lastly 60 µl of Buffer AVE equilibrated to room temperature was added to the QIAamp spin column, incubated at room temperature for 1 minute and centrifuged at 6 000 x g (8000 rpm) for 1 minute. The 1.5 ml Eppendorf with the eluent containing the viral RNA was stored at -80°C until needed for all PCR reactions.

2.4.2.2. Real time RT-PCR

This method was used as a confirmatory test because it is more sensitive and able to amplify as little as 10 copies of RNA (Schwarz *et al.,* 2002). The primers used for this reaction were for gene VP6, designed by McKell *et al.,* (2013).

Quantification of the virus was carried out using the non-probe methods of Sybergreen which works by detecting all double stranded DNA within the reaction. Reverse Transcription was carried out with the SuperScript III First-Strand Synthesis System for RT-PCR. The following procedure was based on Invitrogen's protocol with fewer modifications. RNA/primer mixture was prepared as described below in PCR tubes: Total RNA 5 µl, random hexamers (50 ng/µl) 3 µl, 10 mM dNTP mix 1 µl, DEPC H_2O 1µl.

The mixture was incubated at 65°C for 5 min and then on ice for at least 1 min. Following this a reaction master mix was prepared as described below: 25mM MgCl2 4μl, 0.1 M DTT 2μl and RNAaseOUT 1μl.

The reaction mixture was added to the RNA/primer mixture, mixed briefly, and then incubated at room temperature for 2 min. following this was the addition of 1 μl of SuperScript II RT to each tube, mixed and incubated at 25°C for 10 min. The tubes were incubated at 42°C for 50 min, heat inactivate at 70°C for 15 min, and then chilled on ice. Lastly an addition of 1 μl RNase H and then incubated at 37°C for 20 min. At this stage the cDNA was stored at -20°C until use for real-time PCR.

A real-time PCR reaction mixture of 25 μl was prepared following the description as follows: 12.5 μl SYBR Green Mix, 0.2 μl liver cDNA, 1 μl primer pair mix (5 pmol/μl each primer), 11.3 μl H_2O

After loading the mixture in the plates and loaded in thermocycler. Real Time PCR was run under the following conditions: 50°C 2 min, 95°C 10 min, (95 °C 15 s -> 60 °C 30 s -> 72 °C 30 s) 40 cycles; 72°C 10 min, 1 cycle

2.4.2.3. First round RT-PCR

PCR was used to study the genetic variety of VP4, VP7 and NSP5/6. This was our choice of our method because it offers 11% more sensitivity than EIA (Gouvea *et al.*, 1990). For PCR reactions we used Con2 and Con 3 for VP4, sBeg and EndA for VP7 and NPS5/6 forward and reverse universal primers (Gentsch *et al.*, 1994 Gouvea *et al.*, 1990).

Some 0.2 ml thin walled (PCR) tubes were used for each amplification reaction and a mixture of 8µl RNA and 1µl each primer were used. The mixture was heated for 5 minutes at 95°C then immediately put on ice. This mixture stayed on ice for not more than 5 minutes until the next step.

A 1.5 ml eppendorf tube for the reverse transcription (RT) master mix was prepared prior to denaturing of RNA. The master mix was prepared as follows: 10mM dATP 0.25µl, 10mM dCTP 0.25µl, 10mM dGTP 0.25µl, 10mM dTTP 0.25µl, (AMV) RTase 0.2µl, AMV Buffer 2.0µl.

Addition of AMV enzyme was prior to use. The solution was vortexed and spun for 10 seconds then added to primers and RNA. Mixing of the solution was done by pipetting followed by spinning for 10 seconds and incubated the tubes at 42°C for 30 minutes.

The amplification master mix was prepared as follows: dATP 1 µl, dCTP 1 µl, dGTP 1 µl, dTTP 1 µl, 10 x *Taq* Buffer 5 µl, 50 mM $MgCl_2$ 1.2µl, ddH_2O 29.5µl and *Taq* Polymerase, 0.3µl.

Sample tubes were centrifuged for 10 seconds followed by addition of 40 µl of amplification master mix. Samples were mixed by pipetting then put in PCR-thermocycler with a lid heated at 105°C, 95°C for 1min, 1 cycle (95 °C for 1min-> 42°C for 1min-> 72 °C for 1min) this was repeated for 30 cycles then the tube was heated to 72°C for 7min, after which the equipment was placed at 4C indefinitely. PCR fragments (cDNA) and a 100bp molecular weight marker were run on a 2% TBE- agarose gel.

2.4.2.4. Genotyping

The purpose of this reaction was to determine the genotype of the strains circulating. Primer 1 was Con2 while Primer 2 was a mixture of P4, P6, P7, P9 and P11 for VP4 and Primer 1 was EndA while Primer 2 was a mixture of G3, G5, G6, G8 and G11 for VP7 (Table 3.1). Taq polymerase was pour 5 minutes prior to reaction. The solution was mixed by pipetting. The master mix for genotyping was prepared as indicated as follows: Sterile dH_2O 23.5 µl, 10mM dTTP 1 µl, 10mM dGTP 1 µl, 10mM dATP 1 µl, 10mM dCTP 1 µl, 50 $MgCl_2$ 1.2 µl, 10 x *Taq* Buffer 5 µl, 10pmol Primer 1 1 µl, 10pmol Primer2 5 µl, *Taq* Pol. (5U/µl) 0.3 µl, cDNA 4µl. PCR was run using the same conditions as the ones used for the first round PCR. PCR products were run on a 2% agarose gel at 90 volts with 100bp molecular weight marker.

Table 2.1. The list of primers and the sequences used for Real time RT-PCR, first round RT-PCR and genotyping.

Strain primer	**Animal – Sequence (5'-3')**	**References**
P[6]	GCTTCAACGTCCTTTAACATCAG	(Gentch *et al.*, 1992)
P[7]	CTTTATCGGTGGAGAATACGTCAC	Itturriza-Gomara *et al.*, 2004)
P[4]	GCCAGGTGTCGCATCAGAG	(Gentch *et al.*, 1992)
P[1]	CGAACGCGGGGGTGGTAGTTG	Itturriza-Gomara *et al.*, 2004)
P[11]	GGAACGTATTCTAATCCGGTG	(Gentch *et al.*, 1992)2004)
G3	GACGTAACAACGAGTACATG	(Gouvea *et al.*, 1990)
G5	GATTCTACACAGGAACTAG	(Gouvea *et al.*, 1990)
G6	GTGTCTAATCCGGAACCG	(Gouvea *et al.*, 1990)
G8	GAAGTCGCAACGGCTGAA	(Gouvea *et al.*, 1990)
G11	GCAACTCAGATTGCTGATGAC	(Gouvea *et al.*, 1990)
Sbeg	GGCTTTAAAAGAGAGAATTTC	(Gouvea *et al.*, 1990)
EndA	ATAGTATAAAATACTTGCCACCA	(Gouvea *et al.*, 1990)
Con2	ATTTCGGACCATTTATAACC	(Gentsch *et al.*, 1994)
Con3	TGGCTTCGCTCATTTATAGACA	(Gentsch *et al.*, 1994)
NSP6/5	GCTGGCGTGTCTATGGATTCATCA	(McKell *et al.*, 2013)
NSP6/5	GGTCACAAAACGGGAGTGGGGAGC	(McKell *et al.*, 2013)

2.4.2.5 Agarose gel electrophoresis (AGE)

This is a detailed method for the electrophoresis for all the PCR products for this study. Agarose gel used was 2% prepared by with 1X TBE buffer. 4g of powdered agarose was mix with 200ml 1X TBE buffer in a 1liter glass bottle. The mixture was put in microwave for 3 minutes and left to cool down before pouring it in an electrophoresis tray. Some 5ul of ethidium bromide was added after the mixture had cooled down. The mixture was poured in tray with combs inserted and left to solidify for approximately one hour. The combs were removed when the gel had solidified. The gel was used for electrophoresis for RNA and PCR products. The samples were loaded using the ratio 1:1 to 6X loading dye. Electrophoresis was run at 100V for 2 hours and results were visualised under UV light

2.4.2.6. Sequencing

The VP4, VP7 and NSP5/6 positive samples were sent to Inqaba for sequencing. The main aim of sequencing was to determine of the order of the nucleotides. The first step is a PCR like reaction consisting of denaturation, annealing and extension followed by separation of molecules, then detection on automated sequencer and lastly assembling of the sequenced parts the gene.

2.4.2.7. Phylogenetic Tree

A phylogenetic tree was prepared to determine/study the relatedness of the strains obtained by Basic Local Alignment Search Tool (BLAST). Through this we were able to study the distribution of the strains found in the North West province and also their relatedness to human strains. The sequences were first cleaned using Chromas version 2.6.4 and a blast search was done by submitting the consensus sequences on NCBI

(http://blast.ncbi.nlm.nih.gov). The sequences were aligned by ClustalW protein alignment and neighbour-joint trees were constructed using Genenious software. Analysis of the trees was done by bootstrapping with 1000 replicates data sets.

CHAPTER 3

3.0. Results.

3.1. Overall Results

From the 245 samples analysed, the following summary describes the overall research findings

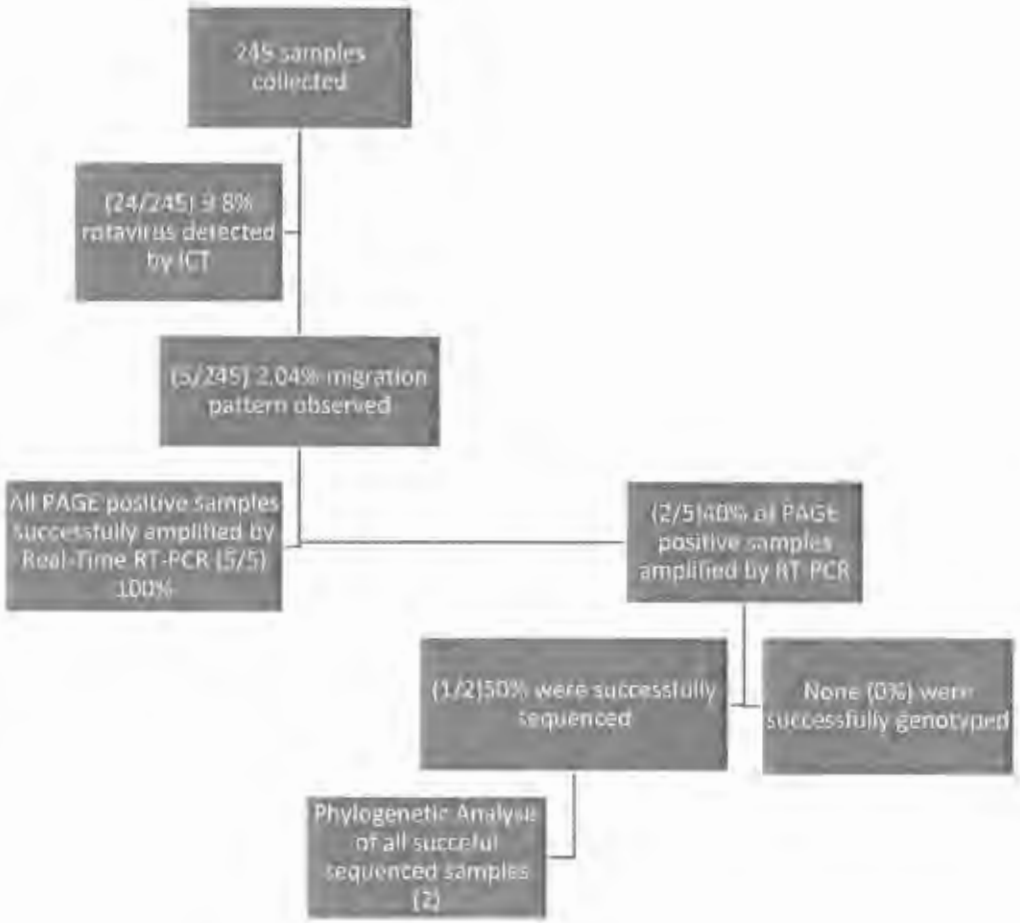

Figure 3.1. Summary of the results obtained in this study

Screening of the virus was done using VIKIA Rota/Adeno kit. A total of 245 sample where tested and prevalence of rotavirus was found to be 9.76%. The prevalence of

rotavirus was to be higher on 2015 than 2014 and 2016 with 16.84%, 10.42% and 2.94% respectfully.

The prevalence of rotavirus was also studied based the following demographics (Results also demonstrated on table 3.1):

- Season (Winter and Summer)
- Health status (Diarrhoea and Non-diarrhoea)
- Age (<3 Months and >3 Months)

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Rotavirus was found to have the prevalence of 15.46% during winter and 6.08% in Summer. This finding may indicate that rotavirus is more prevalent during winter than it is in summer. The age distribution of rotavirus amount dogs aged less than 3 month and older was found to be 15.9% and 8.46% respectful. Lastly the prevalence between diarrhoeic and on-diarrheic animals was found to be 7.84% and 8.46% respectfully.

The morphology of the virus was studied by Transmission Electron Microscopy (TEM). Two samples from the 24 positive samples showed the expected structure of the virus. The same positive samples were run for Polyacrylamide gel electrophoresis (PAGE) to determine the migration pattern of the virus. We were only able to obverse 5 rotavirus migration patterns for all those samples. Confirmation of the presence of rotavirus done by Real time RT-PCR from PAGE positive samples and amplification were observed.

Through conventional RT-PCR (First Round), we successfully amplified two samples from the Real Time RT-PCR positive samples for VP4, VP7 and NSP5. None of these samples were successfully genotyped. All RT-PCR positive samples were sent out for

sequencing; however only VP7 and NSP5 were successfully sequenced and Phylogeneticallyanalyse

Table 3.1 The summary of results obtained in this study

YEAR	TOTAL	POSTIVE	NEGATIVE	PERCENTAGE OF POSITIVE SAMPLES
2014	48	5	43	10.42%
2015	95	16	79	16.84%
2016	102	3	99	2.94%
TOTAL	245	24	221	9.8%
SEASON				
Winter	97	15	82	15.46
Summer	148	9	139	6.08%
STOOL STATUS				
Diarrhoeal	51	4	47	7.84%
Non-Diarrhoeal	194	20	174	10.31%
AGE OF DOGS				
<3 months	44	7	37	15.1%
>3 moths	201	17	184	8.46%

3.3. Screening Results

3.3.1. VIKIA Rota-Adeno Screening

VIKIA Rota-Adeno allows detection of rotavirus by the association of monoclonal antibodies which are specific for it. Rotavirus antigens were detected from 24 out of the 245 (9.8%) samples analysed. All positive samples showed low colour intensity, an indication of a possible low concentration.



Figure 3.2. The image above shows the results which were positive for Rotavirus and Adenovirus. C=control while R=rotavirus and A=adenovirus.

3.3.2. Polyacrylamide Gel electrophoresis (PAGE)

A total of 245 samples were analysed using PAGE. Different types of segment migrations were observed. From the 245 samples analysed, only 5 (2.04%) samples positive for rotavirus.

3.3.3. Transmission Electron Microscopy (TEM)

The general morphology of rotavirus was studied using Transmission Electron Microscopy (TEM). Prior to analysis the samples where cleaned using vertrel solution. Five (5) PAGE positive samples were tested and rotavirus-like morphological features were observed (Figure 3.5) on samples D290 and D270.

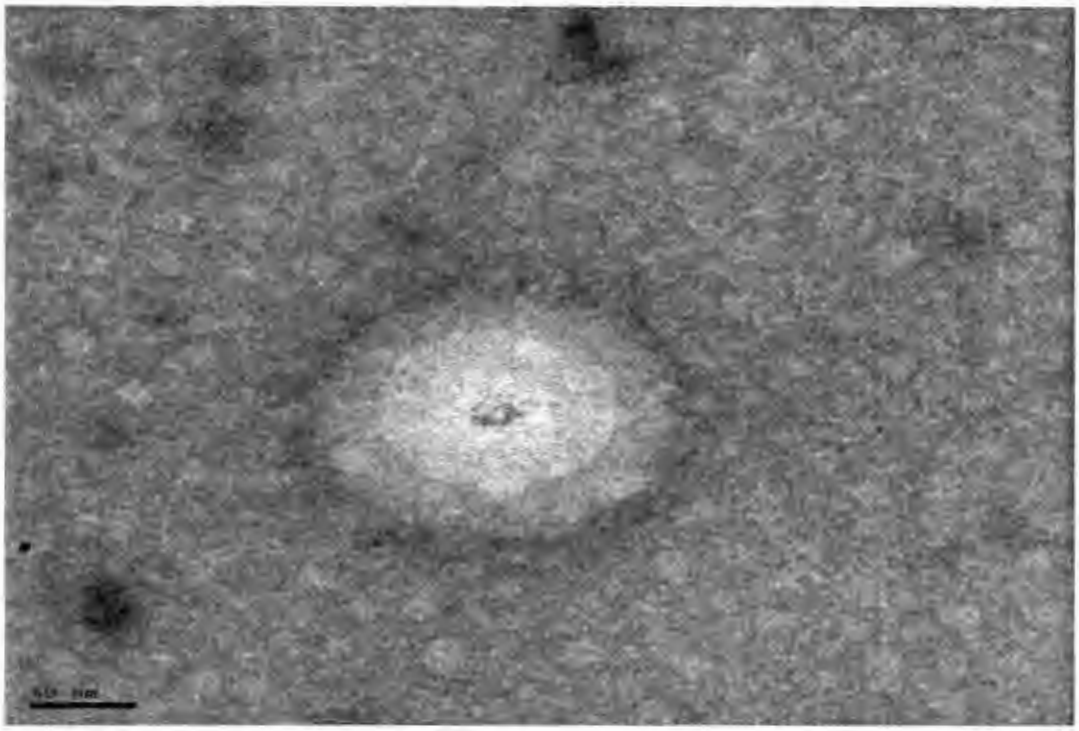

Figure 3.3. Viral-like particle showing triple shelled layers which is common for rotavirus, with size of 50nm.

3.4. Confirmatory Results

3.4.1. Real Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

All the PAGE positive samples (5) were subjected to Real Time RT-PCR. Figure 3.6 shows all the sample amplified. VP6 primers were used for this reaction.

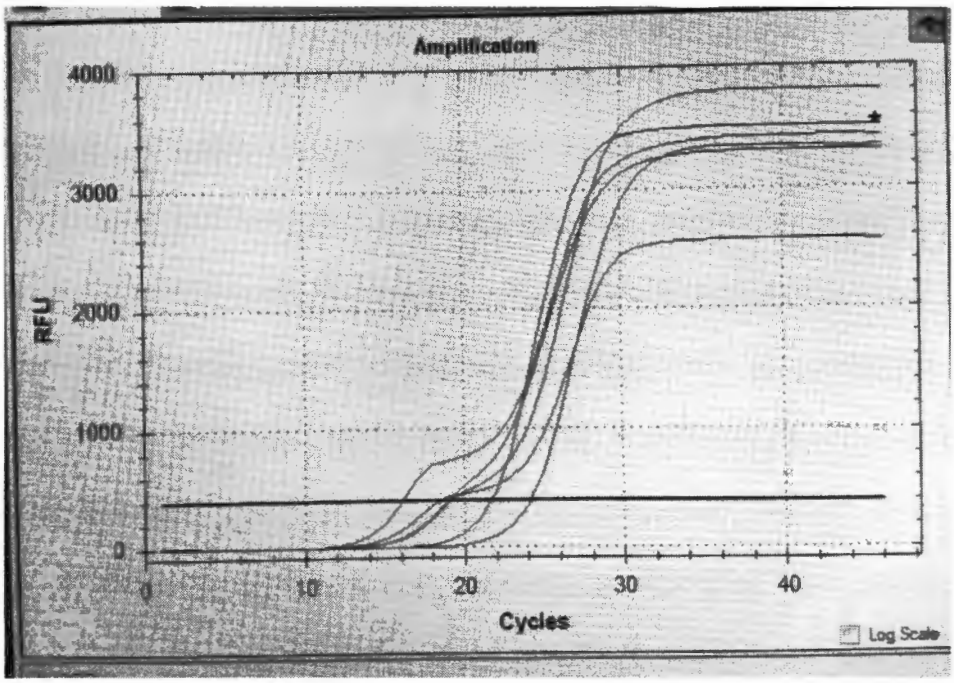

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Figure 3.4. Amplification curve of cDNA of positive samples. Positive control is indicated by * sign.

Real time RT-PCR efficiency

The efficiency of the Real Time RT-PCR was determined by the amplification curve. A single peak (Figure 3.7) on the graph shows that the amplifications observed were true amplicons. The PCR products were run on 1% agarose gel stained with Ethidium Bromide and the image are presented (Figure 3.8). The true amplicons were also seen on the agarose gel (Figure 3.8) with the expected size.

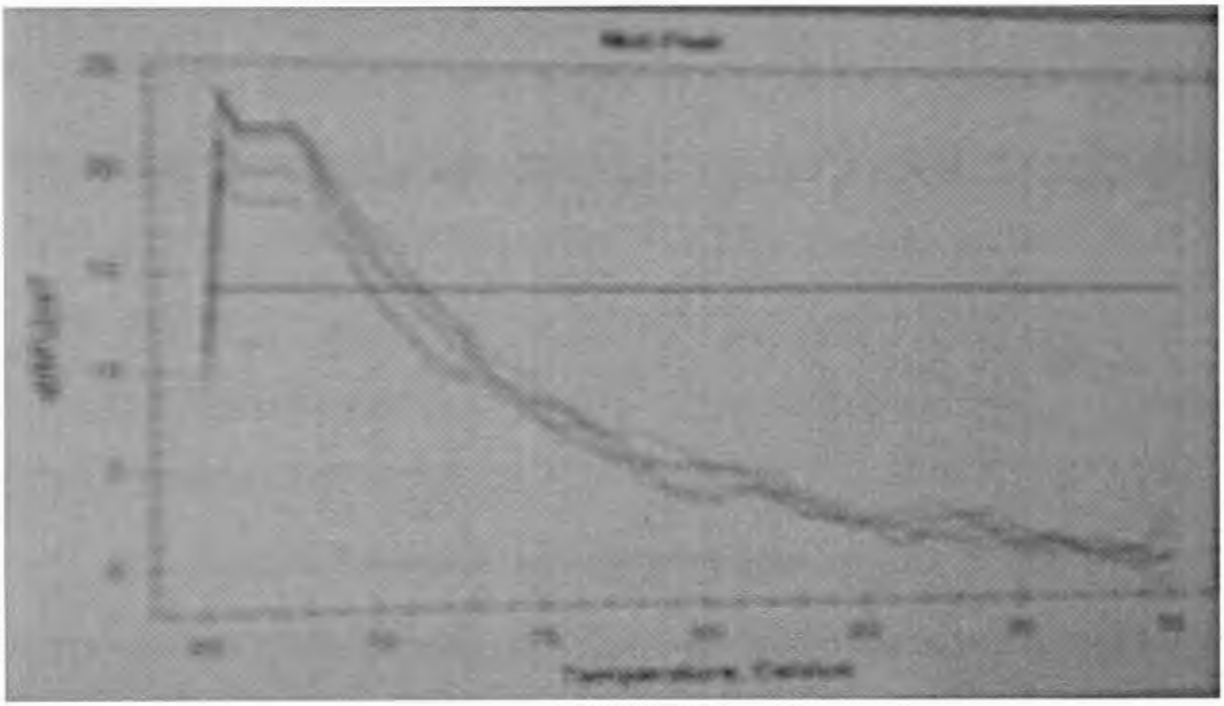

Figure 3.5. The melt curve observed from the reaction showed a single peak with a melting temperature of 67 Celsius.

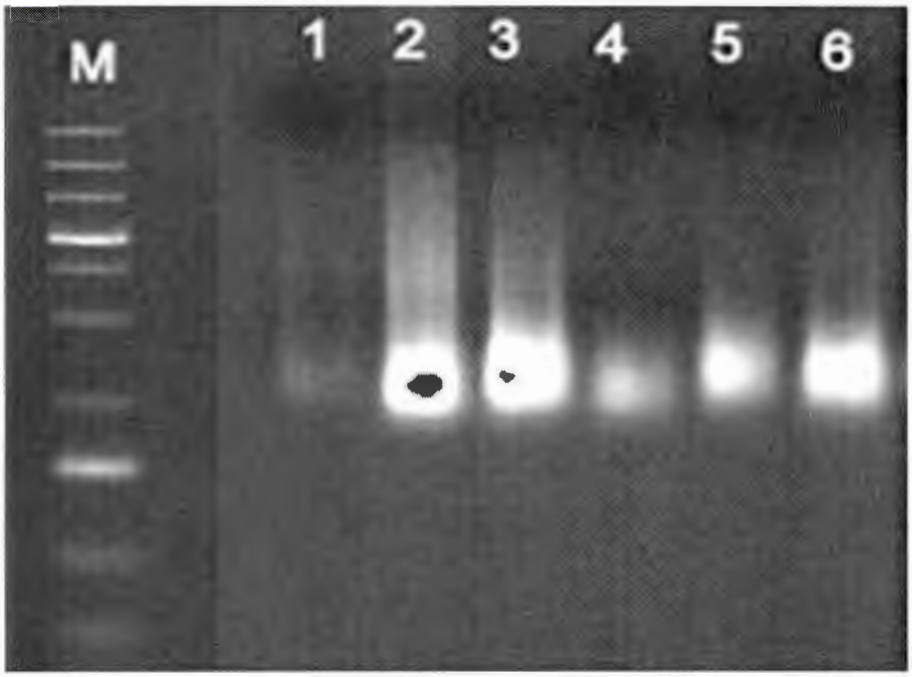

Figure 3.6. Agarose Gel of qPCR products with M representing DNA marker. Lane 1 to 5 represent samples isolated from dogs and Lane 6 is positive control. Lane1= D320, 2=D290, 3=D270, 4=D18 5=D32.

3.4.2. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

All Real Time PCR positive samples were further subjected to conventional RT-PCR for VP4 (Figure 3.9), VP7 (Figure 3.10) and NSP5/6 (Figure 3.11). Two samples were succefully amplified for the selected regions. Lane 1 represents sample D290 while 2 represent sample D270 and 3 sample D170.

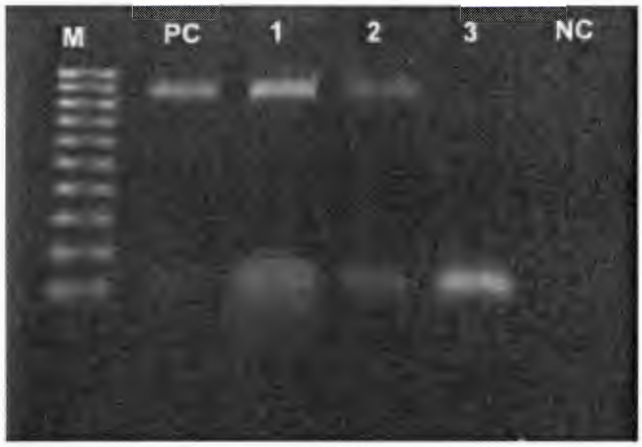

Figure 3.7. Electrophoresis of VP4 (876bp) rotavirus isolated from dogs stool and M represents DNA marker (100bp). PC represents Positive and NC represents Negative control.

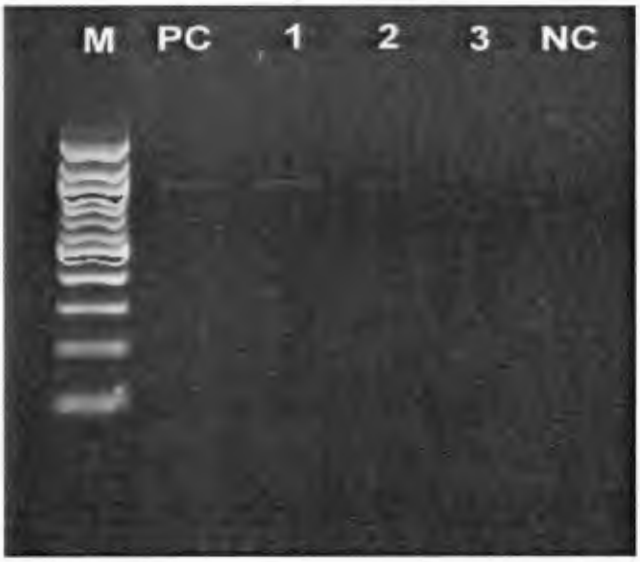

Figure 3.8. Electrophoresis of VP7 (944bp) of the rotavirus isolated from dogs. DNA marker used is 100bp. NC= Negative control and PC= Positive

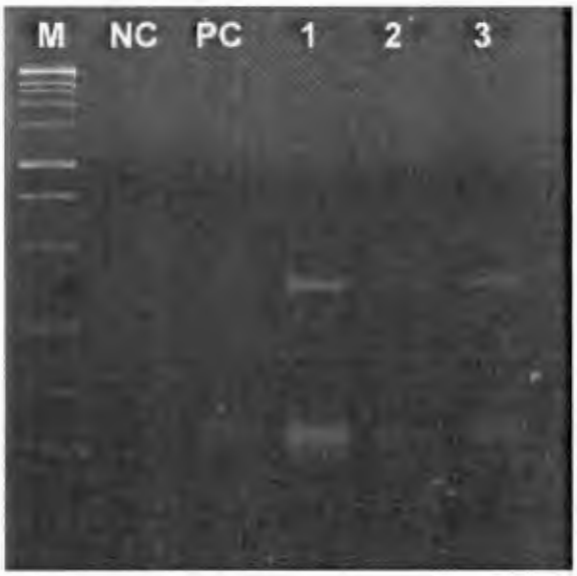

Figure 3.9. The NSP5/6 (385bp) PC=Positive represented with faint band, NC=Negative control.

3.4.3. Sequencing

The resulting sequence nucleotides (VP4, VP7 and NSP5/6) for samples D290 and D270 were submitted to GenBank for a BLAST search through NCBI. The sequences were aligned and manually edited using Chromas 2.6.4. Sample D290 was the only sample that was sequenced successfully for VP4 and NSP5, both forward and reverse sequences.

3.4.4. Phylogenetic Tree

The phylogenetic tree was constructed with VP4 and NSP5 strains isolated from dogs. Alignments of the sequences were compared with a selection of reference strains from the GenBank database.

Phylogenetic Tree of VP4

The phylogenetic tree of VP4 (Figure 3.12) was constructed using a strain isolated from dog and 52 reference from Gene Bank. Most of the reference strains were isolated from humans. All the strains had 98% and 99% identity to the strain isolated from dogs. On the tree the D290 strain was clustered together with Rotavirus strain isolated from stools isolated from bat in Zambia. The strain also showed to have strong relatedness with Cow strain from Thailand. The strain was a distant relative to the strain isolated from humans. The description the strains used from Gene bank are as follows: KJ094915, KX171547, KX171549, KX171553, KX171556, JN706682. KT695030, KT695008, KT695140 ,KT694975, JN849149, KU356588, KU356577, KU248419, KU248397, KU248375, KU199273, KU356665, KU356643, KU356632, KU356610, KU361044, KF371925, KT694942, KU738598, LC105012, LC105067, LC105122, LC105131, LC105193, LC105204, LC105226, LC105246, LC105512. LC105523, KU048747, KU048750, KU048762, LC215252, LC215263, KP902560, KP902556, KP902555, KP902551, KP902544, KP902541, KP902538, KP902537, KP902534, KP902533, KX362896, KX363104.

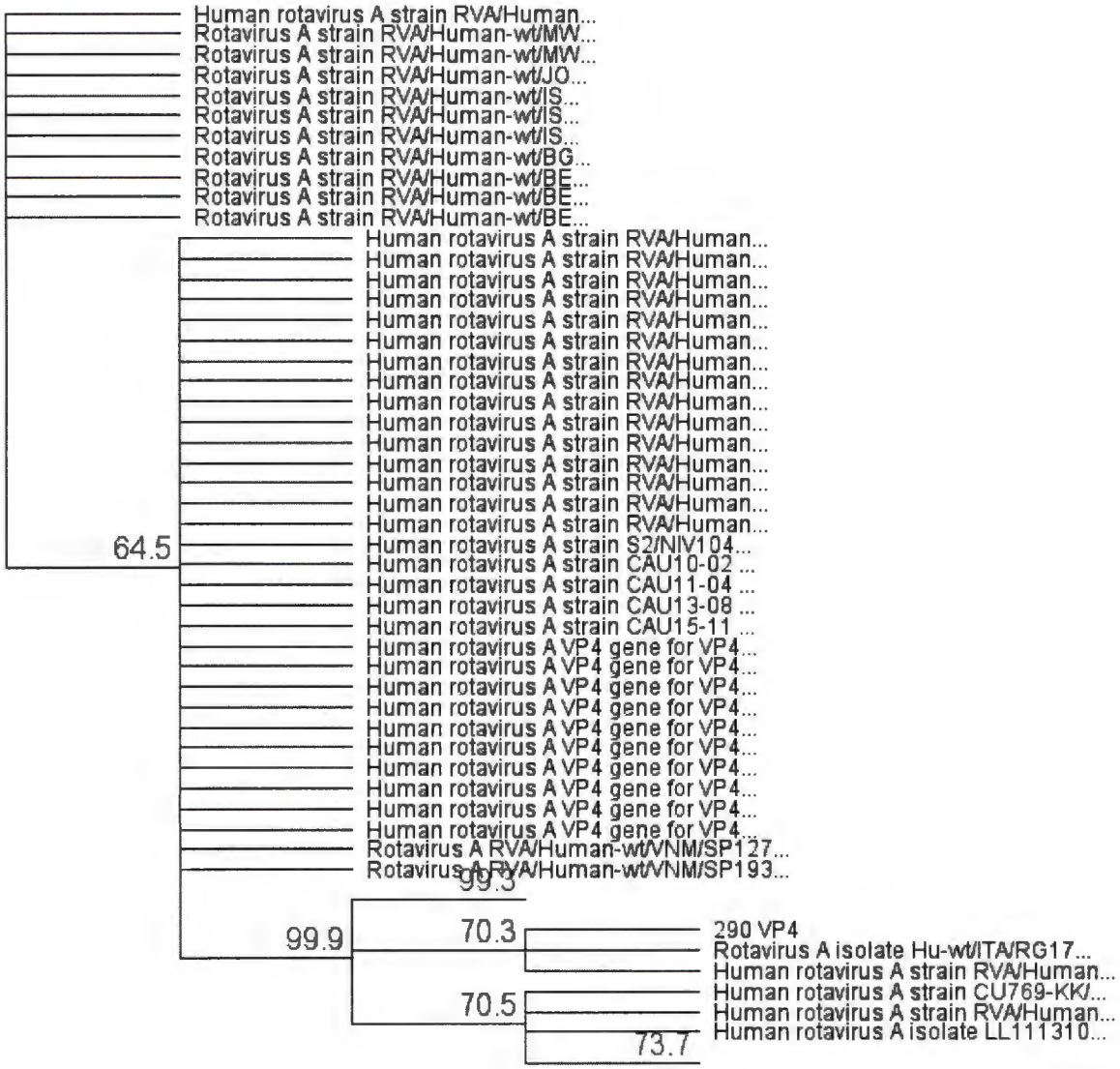

Figure 3.10. Phylogenetic tree displaying the relatedness of rotavirus strains. The strain isolated from a South African dog is represented as VP4 290 strain and rest of them are gene bank deposit.

Phylogenetic Tree of NSP5

The phylogenetic tree of NSP5 (Figure 3.13) was constructed using a strain isolated from dog and 54 references from Gene number of the strains taken from gene bank: HQ609576, KJ094915, AB008663, AB008664, AB861955, AB861966, LC019073, LC019095, JQ043313, GU288652, JQ043319, JN706673, JN706664, JN706675, JN706677, JN706682, JN706683, JN706680, HQ670645, AY803730, JX416226, JQ087433, KT695147, KF907271, KF371707, KF371740, KF371784, KF371795, KF371806, KF371861, KF726054, KF371872, KF371947, KF372002, KC020046, KU048767, KU048749, KU048766, KU048754, JX195073, FJ794026, FJ794039, FJ794034, DQ146670, DQ146659, KJ870932, KJ870921, KT921090, KT919336, KX362994, KX363104, KX363157, KX363303, HQ657148. The strain showed to possess strong similarities with a human strain isolated from China and clustered together also with a strain from the USA. The dog strain had an identity ranging from 96% to 98% assertion.

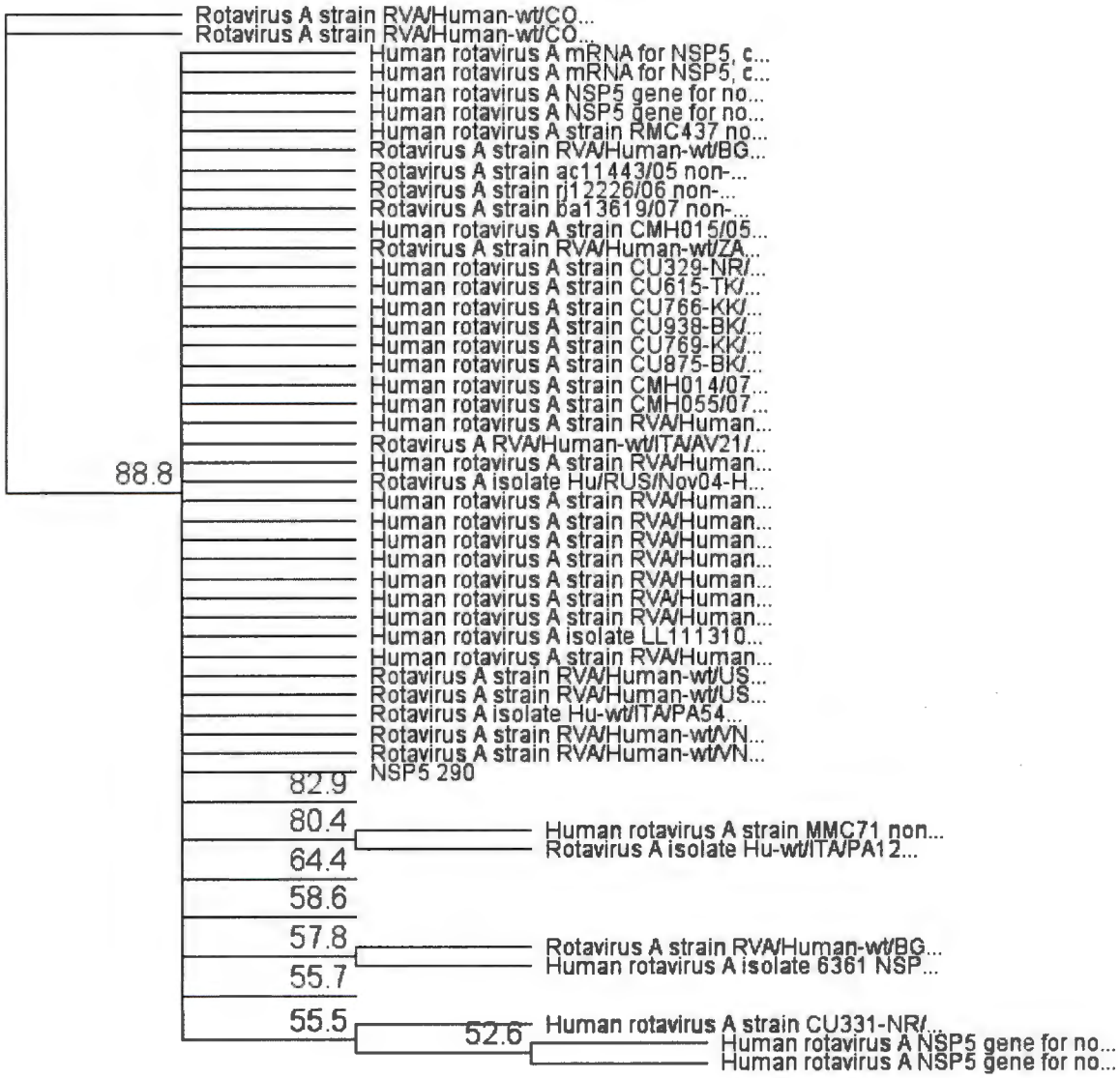

Figure 3.11. The demostration of the ancestral relationship between rotavirus isolated from a dog (NSP5 290) and human strain from Gene Bank.

CHAPTER 4

4.0. Discussion, Conclussion and Recommendations

4.1 Discussion

4.1.1. Overview

Rotavirus is one of the etiological agents of gastroenteritis, with approximately 213 000 annual mortality rate amongst children of less than 5 years predominantly in developing countries. In dogs rotavirus is most prevelent in puppies less than two weeks of age. However, the virus presents asypmtomatic infection in older dogs. Due to the proximity relationship between children and dogs, this interaction may likely render children susceptible to interspecies transmission. The purpose of this study, therefore, was to identify the presence of rotavirus in dogs' stools collected from rural areas of Mafikeng, in the North West Province and to charaterise the prevailing strains.

Two hundred and forty five (245) samples were collected between 2014 and 2016 and were demographically compared. The presence of rotavirus in this study, was shown to be higher in 2015 (16.84%) when compared to 2014 (10.42%) and 2016 (2.94%). We believe that the drop in the presence of rotavirus between 2015 and 2016 is dirrectly linked to effectiveness of the vaccines.

Rotavirus seasonal results showed the frequency of 15.46% in winter and 6.08% in summer, confirming that rotavirus is more predominant in winter than in summer. The study also showed that rotavirus was higher (15.9%) in dogs less than three months of age than those older than 3 months (8.46%) and thus indicaditing that young dogs are more prone to rotavirus infection. The different stool types analysed showed that

diarrhoeal stools had a slightly higher rotavirus presence (8.46%) as compared to the non-diarrhoeal stool type (7.84%) –(Figure 3.1 and Table 3.1)

Laboratory results from this study revealed some interesting information with regard to the prevalence of rotavirus among dogs. Screening detected a 9.76% rotavirus prevalence while PAGE and Real Time RT-PCR results confirmed only 2.83%. The phylogenetic analysis of the dogs' strains isolated revealed high relatedness (93% to 100%) to strains isolated from humans.

4.1.2. Major Dermographic Factors Measured

4.1.2.1 Age Distribution of Rotavirus

In this study, it was established that Rotavirus had a prevalence of 15.9% in dogs aged 3 months and less and 8.46% in dogs older than 3 month. Similary to our study, the prevalence of rotavirus in dogs was found to be significantly higher in dogs aged less than three months than those aged three months and above. An article by Vetary (2017) decribes rotavirus as not serious with symptoms resolving in about 8-10 days. In the very same article it was further stated that dogs will get in contact with rotavirus at one stage or another of their existence, that rotavirus will present asymptomatic infection in older dogs but presents diarrhoea in dogs of less than two weeks of age. In a study conducted in Hungary, 2015, a new-born puppy was detected with rotavirus as well as other enteric infection. We believe dogs of all age are likely to get in contact with rotavirus however symptoms are most likely to be present in puppies aged less than 3 months.

4.1.2.2. Stool status

In a study conducted by Gabby *et al.,* (2003) they found that 80% of dogs had antibodies for rotavirus. These scholars suggested that most dogs presented with the asymptomatic rotavirus, but an article by Vetary (2017) states that the rotavirus is rarely symptomatic in dogs and will carry the antiboties of the virus onwards.

This study was able to detect 7.84% and 8.46% prevalence in diarrheic and non-diarrheic dogs respectfully, supporting claims that rotavirus is more likely to present as asymptomatic infection than infection which is characterization of diarrhoea. However, our claim might not be significant, as the prevalence difference between diarrhetic and non-diarrhetic is marginal.

4.1.2.3 Seasonal distribution of Rotavirus

Rotavirus was found to have the prevalence of 15.46% during winter (May to August) and 6.08% in summer (November to March). Rotavirus has been reported by several authors to occur in both extreme and moderate temperatures across the world, including a study by Levy *et al.,* (2009). Rotavirus exhibits distinct seasonality, and has been known as a 'winter' infection in some parts of the world. Winter gastroenteritis' and 'winter vomiting disease' were recognized illnesses of early childhood before rotavirus was identified and found to be their cause according to Levy *et al.,* (2009).

In a study conducted by Mwenda *et al.,* (2010), it was reported that the prevalence of rotavirus infection carried out in Ghana peaked throughout the cool, dry months of January and February while conversely, a different seasonal peak was perceived in Uganda, where the climate is temperate year-round. The same scholars further added that in Zimbabwe a seasonal peak in the prevalence of rotavirus diarrhoea happened

throughout the winter months (May–August) and lastly in Ethiopia, the occurrence of rotavirus infection peaked from October through December. Levy *et al.,* (2009) further added that rotavirus infections tend to be highest under cool, dry conditions in the tropics. Our finding suggests that Rotavirus occurence is most likely to occur during winter and dry seasons as opposed to other seasons. This finding is supported by Madhi *et al.,* (2010) who reported that rotavirus has high peak in spring-winter peak as opposed to Malawi which has an all year round of peak infection. Based on this outcome, we conclude that rotavirus in human and dogs is most prevalent during winter season than it is Summer.

4.1.3 Rotavirus Antibody Detection by VIKIA Rota/Adeno kit

The presence of rotavirus antibodies in this study was found to be 9.76% i.e. 24 samples tested positive for rotavirus antibodies out of the 245 samples screened using the VIKIA Rota/Adenovirus Kit. The test is not a qualitative test indicating only an exposure to the virus and confirmation of current viral infection still needed to be done. In a study conducted by Van Maarseveen *et al.,* (2010) in the Netherlands, the presence of rotavirus antibodies was 10.0% where 24/239 samples tested positive for the rotavirus antibodies. From these 24 samples, 19 samples were confirmed positive by RT-PCR. In our study RT-PCR was also conducted but only 2 samples out the 24 antibody positive samples were confirmed as RT-PCR positive from the overall collection of 245 samples (Figures 3.8-3.10). Our results might indicate that the low confirmation rate might be as a result of a very low concentration of the virus and therefore not detected or the antibody prevalence might be only an indication of previous exposure.

4.1.3 Polyacrylamide Gel Electrophoresis (PAGE)

The dsRNA of rotavirus can be detected by electrophoretic gel separation and staining (Attoui *et al.*, 2000). In this study, the presence of rotavirus was screened and confirmed through Polyacrylamide Gel Electrophoresis. PAGE is the most commonly used test to study the migration pattern of viral samples. Médici *et al.*, (2011) were able to detect group A, B and C rotaviruses by silver-stained polyacrylamide gel electrophoresis and found that there was no relationship in the prevalence of certain groups with respect to other animals.

4.1.4. Viral-Like Particle Detection by Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy works by transmitting beams of electrons through an ultra-thin specimen, cooperating with the sample as it passes through. TEM studies in our study shows the morphology of a viral-like particle with size 50nm. Gabby *et al.*, 2003 observed 70nm rotavirus particles from dogs' faeces using TEM, however similarly to this study not all positive samples were successfully studied. The Image on Figure 3.4 shows the 3 layers of rotavirus-like particles.

4.1.6. Amplification by Real Time Reverse Transcriptase Polymerase Chain Reaction

The principle of Real Time RT-PCR tests is that following the reverse transcription of RNA into cDNA, it entails an appropriate detection chemistry to report the presence of PCR products, an instrument to observe the amplification in real-time and suitable software for analysis (Bustin and Mueller., 2005). The real time RT-PCR is 100-fold extra sensitive than the conventional nested PCR that has a longer amplicon (Pang *et al.*, 2004).

Real Time RT-PCR was run to confirm the presence of rotavirus on the PAGE positive samples using Syber green. Syber green binds to all double stranded DNA, the possibility of non-specific binding and primer dimer can cause an amplification curve. For this reason other analyses need to be carried out to establish if the amplicon observed were true amplicons. The validity of the run was established by the one peak on the melt peak (Figure 3.5 and Figure 3.6). An agarose gel electrophoresis also confirmed that amplification was a true amplicon by the bands observed with the expected size (Figure 3.7)

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In a study conducted by Pang *et al.,* (2004), 25% of the samples which amplified by Real Time RT-PCT could not be amplified by nested RT-PCR, suggesting that the sensitivity of Real Time RT-PCR was much higher than that of conventional RT-PCR. For this reason we believe that the conventional RT-PCR run in this study was not sensitive enough to detect the 3 samples which were positive by Real Time RT-PCR. But also we do not rule out the fact that the DNA might have been of minute quantity and therefore, below detection.

4.1.7. Genotyping by Conventional Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR is regarded as a suitable high throughput and cost-effective method for typing rotavirus strains (McKell *et al.,* 2013). Three samples were successfully typed for rotavirus (Figures 3.8-3.10). Similar to our study, real time RT-PCR, however, was found to be more sensitive in detecting the presence of rotavirus than conventional RT-PCR, detecting 20% rotavirus while RT-PCR detected only 18% (Pang *et al.,* 2004). The difference however, might have been as a result of human error. Conversely,

McDermid *et al.,* (2012) reported that 38.2% of the samples that tested negative by an immunoassay were successfully typed with RT-PCR.

The positive samples from our study, were subjected to genotyping; however the reaction was not successful. In a study conducted by Gasparinho *et al.,* (2017) 16.3% of the RT-PCR positive could not be further analysed by genotyping. In addition, McDermid *et al.,* (2012) could only identify 74% of the positive sample through genotyping. For this reason we believe that genotyping may occasionally be unsuccessful on positive samples.

4.1.8. Sequencing of PCR Products and Phylogenic Analyses

The successfully amplified PCR products (VP4 and NSP5) were sequenced and a phylogenetic analysis was conducted. The result revealed that the isolated strains were not of canine origin but showed major similarities with pig and human strains. The isolates were found to share some identity with a rotavirus strain characterized in India and the isolates were also found to share identity with a human strain from China. Based on this, we suspect a possible interspecies transmission between dogs and human (Figures 3.11 and 3.12).

The strains detected showed strong similarities with genotype G2 and P8, however the strains were found to share some identity with G9, G4 and P6. Seheri *et al.,* (2014) found that most prevalent human strains in South Africa were G1 with 28.8%, G2 was 16.8% and that the P8 genotype is the most prevalent human strain (40.6%) in South Africa (Seheri *et al.,* 2014).

4.2. Conclusion

All the objectives of the study were met under difficult conditions. To our knowledge, this is the first study in South Africa and we could not find any reports to refer to. However, several studies conducted abroad have reported high prevalence of rotavirus antigens in older dogs. The lack of regular surveillance of rotavirus infection amongst dogs in the country and that most of these infections possibly will go undetected hamper the capacity to establish firm epidemiologic relations. From this study, using just two samples we were able to detect between 93% to 99% identity between the isolated strains in dogs and human strains.

Although the difference in the occurrence of rotavirus between diarrhoeal and non-diarrhoeal dogs observed in this study is not plentiful, this demonstrates that most dogs may present asymptomatic infection. In spite of the prevalence of rotavirus antigen in dog populations, detection rates' using molecular techniques has been low. We therefore recommend more studies especially to optimize molecular techniques for the detection of animal rotaviruses.

4.3. Recommendations

The study results showed a low prevalence of rotavirus infection amongst dogs in this region; however, we cannot conclusively determine that because there are currently no other studies to compare with in the country. It is therefore recommended that further prevalence and characterization studies be done and perhaps also a constant surveillance of the infection be considered, especially in rural areas. Dogs are children's friends thus making zoonotic transmission easy and unnoticeable especially from asymptomatic dogs. We also strongly recommend that immunoassay negative samples

be subjected also to sensitive amplification methods like RT-PCR as some studies have shown that such samples are confirmed as positive when using sensitive techniques to avoid reporting inaccurate estimates.

4.4. Limitations of the study

Study proved to be very difficult, laborious and expensive than anticipated.

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APPENDICES

Reagents preparations

70% ethanol

Ethanol	70ml
distilled H2O	30ml

Preparation of RNase-free deionised water

Add 1 000l of diethylpyrocarbonate (DEPC) to 1 000ml of deionised water.

Incubate for 12hr at 37°C.

Autoclave for 15min

10% Sodium dodecyl sulphate (SDS) stock

1 g SDS in 10 ml distilled water.

Dissolve in a 65°C water bath.

1M Sodium acetate (NaAc) containing 1% SDS

8.2 g sodium acetate in

60 ml distilled water.

Add 10 ml 10% SDS stock and mix

pH to 5.0 was adjusted with glacial acetic acid and make up to 100 ml with sterile distilled water.

In case of precipitation is noticed, the mixture should be heat solution to 42°C

Phenol/chloroform (1:1)

Mix equal volumes of saturated phenol (pH 4.3) and chloroform.

Place in a dark or foil-covered bottle, as the solution is light sensitive.

Store at 4°C.

3M Sodium acetate (NaAc) pH 5.0

4.92 g sodium acetate in

10 ml distilled water.

Make up to 20 ml with distilled water.

30% Acrylamide stock

Acrylamide	30 g
N, N' methylene bis-acrylamide	0.8 g

Dissolve in 100 ml distilled water.

Place solution in a foil covered bottle and store at 4°C.

1N Hydrochloric acid (HCl)

Add 8.6 ml concentrated HCl to 91,4 ml sterile distilled water.

Resolving gel buffer - pH 8.9

Tris Base 36.3 g

1N HCl 4.8 ml

Dissolve and adjust the pH to 8.9 with HCl or 3M sodium hydroxide (NaOH).

Make up to 100 ml with distilled water.

Spacer gel buffer - pH 6.7

Tris Base 5.98g

Distilled water 50 ml

Adjust the pH to 6.7 with 1N HCl.

Make up to 100 ml with sterile distilled water.

NWU LIBRARY

10% Ammonium persulphate (APS)

0.1 g ammonium persulphate

1 ml distilled water just prior to use.

5 x Tris-glycine Running Buffer

25 mM Tris Base 15.1 g

250 mM Glycine 94 g

Dissolve and make up to 1000 ml with distilled water.

1 x Tris-glycine running buffer

Dilute 200 ml 5 x Tris-glycine buffer with 800 ml of sterile distilled water.

Use to run PAGE gels.

1X TBE (pH 7.9)

Add 100 ml of 10X TAE to 900 ml of distilled water.

1% Agarose gel

Mix 0.3 g agarose in 30 ml 1 x TBE buffer (pH 7.9).

Heat in the microwave until agarose is completely dissolved.

Make solution up to 30 ml with distilled water.

After cooling of the gel mixture using running tap water or ice, add 2 ul of ethidium bromide stock and pour gel into cassette tray.

Insert comb and allow gel to set for at least 30 minutes before use.

Agarose gels are electrophoresed at 90V in 1 X TBE.

Preparation of gel for PAGE

	RESOLVING GEL LAYER	STOCKING **GEL LAYER**
DPEC H2O	7 ml	7.5 ml
Tris PH 8.8	5 ml	1.25 ml (PH 6.8)
30% Acrylamide	7.75 ml	1 ml

10% SDS	200 µl	100 µl
10% APS	100µl	100 µl
TEMED	25 µl	10 µl

0.2M PB, pH 7.4:

Na_2HPO4 ---------- 21.8 g

NaH_2PO4 ---------- 6.4 g

Distilled water ---------- 1000 ml

4F1G Fixative (4% Formaldehyde & 1% Glutaraldehyde in 0.1 M PB, pH 7.4):

0.1M PB, pH 7.4 ---------- 88 ml

37-40% Formaldehyde ----- 10 ml

50% Glutaraldehyde --------- 2 ml

8% (0.2M) Sucrose in 0.1M PB:

Sucrose ---------- 8 g

0.1 M PB ---------- 100 ml

1% Osmium in 0.1M PB:

2% Osmium ---------- 5 ml

0.2M PB, pH7.4 ---------- 5 ml

5% Uranyl Acetate Solution:

To prepare 50 ml, add 2.5 g of uranyl acetate to 50 ml of distilled water. Cover with foil and stir overnight. Add 10 drops of glacial acetic acid. Store solution in 4 °C.

Reynold's Lead Citrate Solution:

To prepare 50 ml, add chemicals in distilled water in following order

Lead nitrate ---------- 1.33 g

Sodium citrate, dihydrate ---------- 1.76 g (solution becomes cloudy when sodium citrate is added)

1N NaOH ---------- 5 ml (solution becomes clear when NaOH is added)

Distilled water ---------- 30 ml

Stir for 10 minutes to dissolve and add additional 15 ml of distilled water. Store solution for 3-6 months at 4 °C. Note: the mount of NaOH is very important. Too much will make solution cloudy.

EMBed 812:

Final Volume	**EMBed-812**	**DDSA**	**NMA**	**DMP-30 (2%)**
20.91 ml	10 ml	4.5 ml	6 ml	0.41 ml
31.37 ml	15 ml	6.75 ml	9 ml	0.62 ml
41.82 ml	20 ml	9 ml	12 ml	0.82 ml
52.28 ml	25 ml	11.25 ml	15 ml	1.03 ml

Mix all four components in plastic beaker and stir with wood stick

ROTAVIRUS GROUP A ELISA Worksheet

Kit..... Exp date .....Lab. Signature .....

Date ..... Name .....

Samples ..... Country/Hosp .....

Cut off value .....

	1	2	3	4	5	6	7	8	9	10	11	12
A	**+ve Control**											
B	**-ve Control**											
C												
D												
E												
F												
G												
H												

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

PAGE Worksheet

Date:.....Name:.....

Country/Hosp:.....

Run started at:.....

Volts:..... Lab.Signature:.....

Gel Number.....		Electropherotype
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		

12		
13		

PCR Worksheets

RT-PCR amplification of Rotavirus (dsRNA)

dsRNA:_____ Country_____

_____

Method of Extraction:_____Date:_____

A: DENATURE (dsRNA)

_____µl dsRNA

_____µl primer 1 VP7____ VP4 _____ Other_____

_____µl primer 2 VP7____ VP4 _____ Other_____

6-10 µl Total

Denature for 5 minutes in boiling water bath.

Chill immediately in an ice bath (maximum 5 minutes).

B: REVERSE TRANSCRIPTION (dsRNA)

1x reaction		_____ x reactions
0.25µl	10mM dATP	_____µl
0.25µl	10mM dCTP	_____µl
0.25µl	10mM dGTP	_____µl
0.25µl	10mM dTTP	_____µl
0.2 µl	(AMV) RTase	_____µl Roche Boehr.(23U/µl)
2.0 µl	AMV Buffer	_____µl

Work on ice. Add 3.2 µl of RT- master mix to each tube and mix with pipette. Quick spin.

Incubate 25-30 minutes at 42°C.

APPENDICES Genotyping or Re-Amplification of cDNA

Samples:_____ Country:_____

_____

Primers:_____ Date:_____

1x Reaction _____xReactions _____ x Reactions

NMU LIBRARY

0.1-6 µl cDNA _____µl _____µl

1 µl 10mM dATP _____µl _____µl

1 µl 10mM dCTP _____µl _____µl

1 µl 10mM dGTP _____µl _____µl

1 µl 10mM dTTP _____µl _____µl

1 µl 10pmol Primer 1_____µl _____µl

1 µl 10pmol Primer 2_____µl _____µl

5 µl 10 x *Taq* Buffer _____µl _____µl (BIOLINE-Celtic)

1.2 µl 50 $MgCl_2$ _____µl _____µl

26 µl sterile dH_2O _____µl _____µl (depends on amount of primers used)

0.3 µl *Taq* Pol. (5U/µl)_____µl _____µl

Add 40 µl of PCR-MM to each tube and 2 drops of mineral oil if needed.

Keep on ice!

Genotyping of cDNA

1 x reaction ____ x reactions

0.1-6 µl cDNA _____µl

1 µl 10mM dATP _____µl

1 µl 10mM dCTP _____µl

1 µl 10mM dGTP _____µl

1 µl 10mM dTTP _____µl

1 µl 10pmol Primer 1 _____µl

1 µl 10pmol Primer 2 _____µl

5 µl 10 x Buffer _____µl

1.2 µl 50mM $MgCl_2$ _____µl

26 µl ddH_2O _____µl

0.3µl *Taq* Polymerase (5U/µl)_____µl

AMPLIFICATION CYCLES used:

……°C……min……°C……min……°C……..min

……..°C…….x40…….°C……..min

Nucleotide sequences for NSP5

290-NSP5:

TGCGGCGTGCTAGGGAGTCAACGGATACGGCGGCAGTAGCATGGTCACAAAACG

GGAGTGGGGAGCTCCCTAGTGTGCTCTCAGGTTAACATTACAAATCTTCGATCAAT

TGCATTGCGACTCGCTTCATCCTCATTCTTAGTGCGAAATATTTTTTCTTGTATTTAC

AATTCTTACATTTACCGTCATCACTATCTGAATCATCCAAAACATAATCTTCAGAATC

AGAATCTGCTTCAATTCTTGGGTAGTGTTTCCTACTTTTATCTTTCTTGGATTTCTCC

TTCTTATGATCA—GTTGAAATTGATATACATGATATCCAAGATTAGCA

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TTAACCTGAGAGCACACTAGGGAGCTCCCCACTCCCTTTTTGTGACC

JN706673:

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NWU LIBRARY

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KU048767:

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Nucleotide sequences for VP4

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