DETECTION OF ROTAVIRUS ANTIGEN FROM CALVES IN RURAL AREAS OF THE NORTH-WEST PROVINCE, SOUTH AFRICA

Malatji KK

orcid.org 0000-0002-8913-3189

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

Supervisor: Prof NP Sithebe

Graduation ceremony July 2018

Student number: 22649069

DEDICATION

I dedicate this to my understanding and caring family and friends; I thank them for their support throughout the years.

Proverbs 22:6 (KJV)

6 Train up a child in the way he should go: and when he is old, he will not depart from it.

Philippians 4:13 (KJV)

13 I can do all things through Christ who strengthens me.

1 Corinthians 2:9

9 But as it is written: "Eye has not seen, nor ear heard, Nor have entered into the heart of man The things which God has prepared for those who love Him."

ACKNOWLEDGEMENTS

First and foremost, I would like to thank God, Heavenly Father for His protection and guidance throughout this study. I could not have made it without Him.

I would like to convey my profound gratitude and sincere appreciation to my supervisor, Prof N.P Sithebe for her supervision and support during this research. I give thanks to: Ms LP Kgosana, Mr KV Marope, Ms Rorisang Thebyane, Ms Lorato Modise, Ms Keletso Sebogodi, Mr Daniel Matlou and Mr J Morapedi for their continual support.

My sincere appreciation also goes to Prof Raymond Motadi for providing us with some of the reagents to complete the study, Dr A Jordaan (NWU-PC) for assisting with TEM work, Mrs Ina Peenze (WHO Rotavirus Research Unit –SMU) and Dr Mathew Nyirenda, a qualified veterinary surgeon, for assisting with stool collection.

I would also like to thank the officers in the Post Graduate Office, the Department of Biological Science, and the North-West University Mafikeng Campus for helping in various ways related to my academic work. I also wish to thank the Food and Beverages, the National Research Foundation (NRF) for financial support through their internship programme and also the North West University Mafikeng – Campus postgraduate merit bursary. Thank you to my family, colleagues and friends whom I have not mentioned their names for their encouragement towards the completion of this research.

Finally, I would like to convey my heartfelt gratitude to my parents for their reassurance and confidence in me.

ABSTRACT

Diarrhoea remains a major problem in animals worldwide with rotavirus being one of the most common pathogens associated with its pathology. The clinical signs and outcomes of the diseases are not different in most species and severity may range from an asymptomatic or subclinical condition to severe enteritis. Meanwhile in domestic animals and humans, rotavirus diarrhoea is a major cause of death to millions of infants in developing countries and severe losses to livestock. Substantial economic loss occurs due to increased mortality, treatment costs and reduced growth rates of animals as a result of rotavirus infection. The purpose of the present study was to detect the presence of rotavirus in diarrhoeal stools collected from calves below the age of 3 months in the rural areas of the North-West Province of South Africa.

In this study 200 diarrhoeal samples were randomly collected from calves below the age of 3 months from the rural areas of Mafikeng, between 2015 and 2017. Collection was done in different seasons i.e. winter and summer. Out of the 200 diarrhoeal samples collected, 108 (54%) samples were from male calves and the remaining 92 (46%) were from female calves. The screening methods used included the Vikia Rota-Adeno test kit, enzyme immunoassay (EIA), electron microscopy and the polyacrylamide gel electrophoreses (PAGE). Polymerase Chain Reaction (PCR) and the Sequencing (Sanger) methods were applied for confirmation purposes.

The Vikia Rota-Adeno kit was able to detect 36/200 (18%) positive samples, indicating rotavirus exposure. EIA was applied only on 50 samples (36 from Vikia positive and 14 equivocal samples) and none were positive. From the 50 samples only 2/50 (4%) were confirmed positive by RT-PCR i.e. samples C6 and 101, and showed the presence of the 11 segmented genome (characteristics of rotavirus) when tested by PAGE. Morphological studies conducted using Transmission Electron Microscopy yielded rotaviral-like particles. The 2 samples confirmed positive by RT-PCR, were genotyped using multiplex RT-PCR and sample C6 was identified as genotype G10P11 while sample101 could not be typed. Samples C6 and 101 were also taken for sequencing

and only sample 101 was successfully sequenced and confirmed to be G12P8 genotype. These results show the importance of using more than one method for genotyping lest you miss some types. From the 200 samples that were subjected to lab techniques, we report an overall presence of 1% (2/200). The conclusion from this study might be two fold, viz., a larger sample size might have yielded a different result or that the current prevalence status of rotavirus amongst calves in the rural areas around Mafikeng is low and might not be a cause for concern when compared with various studies conducted globally, especially considering the fact that rotavirus is also self-limiting.

Table of Contents

DECLARATION i
DEDICATION ii
ACKNOWLEDGEMENTS iii
ABSTRACT iv
Table of Contents vi
LIST OF TABLES ix
LIST OF FIGURES x
LIST OF APPENDICES xii
LIST OF ABBREVIATIONS AND SYMBOLS xiii
DEFINITION OF CONCEPTS xiv
CHAPTER 1 1
1.0 INTRODUCTION AND PROBLEM STATEMENT 1
1.1 Introduction 1
1.2 Problem Statement 3
1.3 Research Aim and Objectives of the Study 3
1.3.1 Aim 3
1.3.2 Objective of the Study 3
1.4 Literature Review 5
1.4.1 The History of Rotavirus 5
1.4.2 Virology of Rotavirus 5
1.4.3 Disease Transmission 12
1.4.4 Clinical Signs 12
1.4.5 Rotavirus Replication Cycle 13
1.4.6 Pathogenesis 14
1.4.7 Diagnosis 15
1.4.8 Treatment and Prevention 19
1.4.9 Rotavirus Vaccinations 20
CHAPTER 2 23
2.0 METHODS AND MATERIALS 23
2.1 Ethical Considerations 23
2.2 Study Design 23

2.3 Study Sites 23
2.3.1 Study Site and Sample Collection 23
2.4 Collection Method of Samples 24
2.5 Detection of the Rotavirus Antigen in Samples 25
2.5.1 Screening Processes 25
2.6 Extraction of RNA 30
2.7 MOLECULAR TECHNIQUES 33
2.7.1 RT-PCR 33
CHAPTER 3 38
3.0 RESULTS 38
3.1 Demographics 38
3.2 Overview of results 38
3.3 Screening Results 38
3.3.1 Vikia Rota-Adeno Kit 38
3.3.2 Enzyme Immunoassay (EIA 39
3.3.3 Transmission Electron Micrcoscopy (TEM) 39
3.3.4 Polyacrilamide Gel Electrophoresis (PAGE) 40
3.4 Polymerase Chain Reaction 41
3.4.1 Amplification using Reverse Transcriptase-Polymerase Chain Reaction (VP4 and VP7) 41
3.4.2 Genotyping using Multiplex PCR (Rotavirus P and G Genotypes) 41
3.4.3 Genotyping by Sequence Analysis of VP4 and VP7 42
CHAPTER 4 43
4.0 DISCUSSION AND CONCLUSION 43
4.1 Discussion 43
4.1.1 Overview of the Study 43
4.1.2 Screening Processes 44
4.1.2.1 Vikia Rota-Adeno 44
4.1.2.2 Enzyme Immunoassay 44
4.1.2.3 Electron Microscopy 45
4.1.2.4 Polyacrylamide Gel Electrophoresis (PAGE) 45
4.1.3.1 Amplification using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) 45
4.1.3.2 VP4 and VP7 Genotyping by Polymerase Chain Reaction (RT-PCR) 46

4.1.3.3 Sequencing 46
4.1.3.3.1 Phylogenetic Analysis 47
4.2 Conclusion 48
4.2.1 Recommendations 48
4.2.2 The Limitations of this study 49
REFERENCES 50
APPENDICES 61
APPENDIX 3: Ethical clearance letter 63
Appendix 4 64

LIST OF TABLES

Title	Page
Table 1.1: *Genome size and selected characteristics of group A rotavirus ....*	8
Table 1.2 *Known serogroups and RNA electrophoretic patterns of rotaviruses affecting animals and human.....*	18
Table 2.1: *Oligonucleotide primers used to amplify the fragment of VP4 gene and human [P]-genotyping with primer sequences, primer positions on the respective genes and expected amplicon size.....*	35
Table 2.2: *Oligonucleotide primers used to amplify the fragment of VP7 gene and human [G]-genotyping with primer sequences, primer positions on the respective genes and expected amplicon size.....*	36
Table 3.1: *Total number of samples collected and tested for the presence of Rotavirus using the above techniques.....*	38

LIST OF FIGURES

Title	Page
Figure 1.1 *Rotavirus structure showing protein coding assignments of 11 genome RNA segments*.....	6
Figure 1.2 *The rotavirus replication cycle*	13
***Figure 2.1**: Map of the North West Province*.....	23
Figure 2.2: *Flow diagrams of laboratory procedures undertaken during screening and characterization of rotavirus strains*.....	24
Figure 3.1: *Examples of used Vikia Rota-Adeno test kit*.....	39
Figure 3.2 *Electron micrographs of viral particles-like particles from diarrheic samples of calves*.....	40
Figure 3.3 *Electropherotypic pattern of representative bovine rotavirus isolates from faecal samples of the diarrhoeic calves*	40
***Figure 3.4** The amplification product of rotavirus VP4 RT-PCR (876 bp)*	41
***Figure 3.5** The amplification product of rotavirus VP7 RT-PCR (1061bp)*	41
***Figure 3.6** The amplification product of rotavirus VP7 genotyping*	42
***Figure 3.7** The amplification product of rotavirus VP4 (515bp) genotyping*.....	42
***Figure 4.1** Rotavirus VP4 G12 strains phylogenetic tree displaying the relationship*	47

1. **Manuscripts from this work**

   i. Bovine Rotavirus: A Review

   K K Malatji, LP Kgosana I Du Preeze and NP Sithebe

   J of Veterinary Studies

   ii. Canine rotavirus infection: Interrelationship with human

   LP Kgosana, I Du Preez. K Malatji and NP Sithebe

   Indian of Virology

   iii. 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. **The work was presented at the following conferences**

   i. PHASA Conference 2015

   ii. Virology Africa 2015 (Cape Town)

   iii. NWU Science Day 2016 (Mafikeng)

   iv. 11th African Rotavirus Symposium 2017 (Malawi)

LIST OF APPENDICES

TITLE	Page
EIA worksheet	64
PAGE worksheet	65
Ethical clearance letter	66
Sample C6 (VP4) blasted sequences	67

LIST OF ABBREVIATIONS AND SYMBOLS

EIA : Enzyme Immunoassay

ELISA : Enzyme-linked immunosorbent assay

EM : Electron microscope

dsRNA : Double stranded ribonucleic acid

G : Glycoprotein

mRNA : Messenger ribonucleic acid

NSP : Nonstructural protein

P : Protease-cleaved

PAGE : Polyacrylamide gel electrophoresis

RT-PCR : Reverse Transcriptase Polymerase chain reaction

SG : Serogroup

ITC : Immunochromatography technique

VP : Viral protein

DEFINITION OF CONCEPTS

Antibodies: a blood protein produced in response to and counteracting a specific antigen. Antibodies combine chemically with substances which the body recognizes as alien, such as bacteria, viruses, and foreign substances in the blood.

Colostrum: the first secretion from the mammary glands after giving birth, rich in antibodies

Dehydration: is the excessive loss of body water, with an accompanying disruption of metabolic processes.

Deproteinize: remove protein from (a substance), usually as a stage in chemical purification.

Diarrhoea: a condition in which faeces are discharged from the bowels frequently and in a liquid form.

Electrophoresis: the movement of charged particles in a fluid or gel under the influence of an electric field.

Enterocytes: a cell of the intestinal lining.

Eppendorf Tubes: Is the benchmark covered tubes for simple and safe sample preparation from 0.5 to 2.0 mL.

Fomites: objects or materials which are likely to carry infection, such as clothes, utensils, and furniture.

Gastroenteritis: inflammation of the stomach and intestines, typically resulting from bacterial toxins or viral infection and causing vomiting and diarrhoea.

Glycoprotein: any of a class of proteins which have carbohydrate groups attached to the polypeptide chain.

Immunofluorescence: is a technique used for light microscopy with a fluorescence microscope and is used primarily on microbiological samples.

Self-limiting: a condition that is ultimately resolving itself without treatment.

Serotype: a serologically distinguishable strain of a microorganism

RNA extraction: is the purification of RNA from biological samples. This procedure is complicated by the ubiquitous presence of ribonuclease enzymes in cells and tissues, which can rapidly degrade RNA

Vaccines: an antigenic substance prepared from the causative agent of a disease or a synthetic substitute, used to provide immunity against one or several diseases.

CHAPTER 1

1.0 INTRODUCTION AND PROBLEM STATEMENT

1.1 Introduction

Diarrhoeal disease has been recognized in humans over many millennia. Until the early 1970s, a bacterial, viral, or parasitic aetiology of diarrheal disease in children could be detected in fewer than 30% of cases (Estes and Greenberg, 2013). In 1973, Bishop and partners detected a virus particle in the intestinal tissue of kids with diarrhoea by utilizing electron microscopy. This virus was later called "rotavirus" in view of its closeness in appearance to a wheel (Rota is Latin for wheel). By 1980, rotavirus was perceived as the most common cause of serious gastroenteritis in young children in the United States (Estes and Greenberg, 2013). It is presently realized that infection with rotavirus is about all inclusive, with all kids infected by 5 years old. Rotavirus is responsible for 20–60 deaths annually in the United States and up to 213,000 deaths from diarrhoea around the world (Tate *et al.*, 2013). The group A rotavirus is the most important aetiological agent that causes diarrhoea in humans and animals (Bernstein, 2009).

Rotaviruses belong to the Reoviridae family and have a genome comprising of 11 segments of double-stranded RNA encased in a triple-layered capsid. Rotaviruses are classified into eight groups (A to H) based on the VP6 capsid protein or on the migration pattern of genomic segments in polyacrylamide gel. Groups A, B, and C were discovered either in people or in animals, while groups D to G were discovered in animals (Holland, 1990).

Several countries that have implemented routine childhood vaccination against rotavirus have documented a tremendous impact on severe diarrhea and rotavirus disease requiring hospitalization (Manish *et al.,* 2012). Additionally, some countries, including Mexico, Brazil, and Panama, have documented substantial decreases of 22%–50% in diarrhea mortality among children <5 years of age following vaccine introduction (Bayard *et al.,* 2012). However, rotavirus vaccine implementation in settings of high child mortality in Africa and Asia is just beginning to occur, and the real lifesaving potential of vaccination has yet to be realized. To facilitate decision

making on rotavirus vaccine adoption by countries and to help donors prioritize investments in health interventions, up to-date estimates of childhood mortality from rotavirus are needed. Furthermore, baseline estimates of rotavirus mortality are required to measure the impact of vaccination.

Since earlier days, it has been observed that young ones of animals surrender to infectious agents at neonatal period, hence unfavourably affecting the economic stability of many animal farming ventures. Among the infectious diseases of calves, neonatal diarrhoea is a major distress, and multiple etiological agents that incorporate *Escherichia coli*, *Salmonella* species, *Clostridium perfringens*, rotavirus, coronavirus, *Cryptosporidium* and *Coccidia* have been suggested (Holland, 1990; Steele *et al.*, 2004; Gumusova *et al.*, 2007). Among these agents, bovine rotaviruses (BRV) contribute essentially to enteritis and diarrhoea in well raised neonatal calves (Malik *et al.*, 2005, Chauhan and Singh, 1996). The disease is mostly seen in young calves 2–8 weeks of age and the susceptibility decreases as the age of the animal increases.

Rotaviruses, being mostly species-specific and present as geno-groups, have various gene segments of double-stranded-RNA, and display considerable genetic diversity as a result of genetic shift, gene reorganizations or interchange of segments (Schroeder *et al.*, 1982, Shen *et al.*, 1994, Murphy *et al.*, 1999, Steele *et al.*, 2004). Besides, inter-species transmission and close relationship between human and animal rotaviruses have been reported (Nakagomi and Nakagomi, 1991, Adah *et al.*, 2003).

Extensive studies about human rotavirus have been done in South Africa. Nevertheless, few rotavirus studies in animals have been carried out in South Africa. Monitoring rotaviruses in animals is equally important, for the control of rotavirus infection in both humans and animals. Significantly, research is required in the North-West Province of South Africa, an area where there is the possibility of interspecies transmission more especially in the rural areas where animals are nurtured in home premises and there is close association and sharing of common source of drinking water with livestock. Rotavirus infection manifest in different forms and the forms of this infection differ from calf to calf, some suffering acute dehydration and death while others suffer from sub-acute forms with malnutrition that lasts for several days

(Steele *et al.,*2004). Neonatal diarrhoea is a global problem and is seen as one of the biggest challenges for cattle. Rotavirus is an important infection and causes a lot of economic damage to farmers through the need for increased management, veterinary treatment, reduced growth and death of the calves.

1.2 Problem Statement

Bovine rotavirus is a common cause of diarrhoea in calves which negative effects the economic growth of the country considering the role cattle play in the industry be it for food, clothes and art (Donovan *et al.*, 1998). The world's population is growing at an alarming rate. Due to this, quality food production needs to be directly proportional to the growth observed. It is of vital importance that we secure and ensure the health of our livestock especially cattle. The prevention of disease amongst neonatal calves is important and better understanding of the genetics and molecular epidemiology of the causative pathogens is crucial. Rotaviruses are the most common viral pathogens causing diarrhea in young cattle. Molecular epidemiology is an important tool for tracing the transmission of the diseases and helps in gaining knowledge about the viruses in order to produce effective vaccines. Rotavirus studies have been done adequately worldwide in human beings and animals but fewer studies have been conducted in South Africa about rotavirus in animals. So, there is a knowledge gap that needs to be filled on animal rotavirus research. Therefore, the insufficiency of studies that had been done locally have prompted us to carry out this study.

1.3 Research Aim and Objectives of the Study

1.3.1 Aim

The aim of the study was to detect the presence of bovine rotavirus antigens from diarrhoeal stools collected from calves under the age of 3 months in the rural areas of Mafikeng, North-West Province.

1.3.2 Objective of the Study

I. To screen diarrhoeal stools from calves less than 3 months old for the presence of rotavirus antigens using Vikia Rota-Adeno test kit and EIA and EM
II. To screen for the viral particles of rotavirus with electron microscopy
III. To determine the electrophoretic pattern of rotavirus strains using PAGE

IV. To confirm the presence of rotavirus using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

V. To genotype the circulating rotavirus strains in the rural areas of Mafikeng by multiplex PCR and sequencing

1.4 Literature Review

1.4.1 The History of Rotavirus

It has been years since rotavirus was perceived as a causative agent of gastroenteritis in calves and now it has been separated from a variety of species of animals and human. General data is accessible on pathogenesis, modes of virus transmission, finding of rotaviral infection, and molecular genetics of rotavirus (Estes and Cohen, 1989). However, rotavirus infection still remains an essential wellbeing issue in animals and in people. Vaccines are available in a few species. Mechanisms of mucosal immunity to rotavirus and other enteric pathogens needs better elucidation. Despite the fact that rotavirus immunogens have been identified (Lyoo, 1995) their part in protection against calf diarrhoea is not really understood. Serotypic diversity further complicates prevention and control of rotavirus associated gastroenteritis.

Rotavirus was initially isolated from calves with diarrhoea by Mebus *et al.* (1969), and subsequently this virus was identified in most animal species of veterinary significance including cattle, pigs, sheep, horse, dogs, felines, chickens and turkeys (Estes *et al.*, 1983, Snodgrass *et al.*, 1984, Estes and Cohen, 1989, Lyoo, 1995). Rotavirus infection in humans was initially depicted in 1973 by electron microscopy of intestinal substance of hospitalized youthful kids with acute gastro-intestinal infection (Bishop *et al.*, 1973).

1.4.2 Virology of Rotavirus

1.4.2.1 Morphology

Rotaviruses have a "wheel-like" appearance which relates to its name. The virion comprises of a triple layered capsid covering a genome of double-stranded RNA. The mature infectious virion has a diameter of 100nm (Estes and Cohen, 1989). The envelope is without lipid and comprises of three concentric layers of protein. The different layers are comprised of three of the 11 proteins that the rotavirus genome encodes. VP2 forms the deepest layer and encompasses the viral dsRNA. The centre layer is made out of VP6 and the peripheral layer of VP4 and VP7. The centre and peripheral layer have 132 large channels that link the outside of the virion to the VP2 layer. VP7 makes up the base of the furthest layer while VP4 forms spike-like extensions reaching out of the virion (Estes and Cohen, 1989). VP4 additionally

stretches out in through the two outer layers, and perhaps at the same time have some interaction with the VP6 layer. Both have important parts in the infectivity of the virion. Infectivity is rapidly lost with disinfectants like chlorine and 95% ethanol. These destroy the outer shell and thus make the virus unable to infect cells. The virus is stable and infectious in pH range between 3-9 and may under the right concentration of Calcium chloride remain infectious for months at 4°C and even up to 20°C (King, 2012).

1.4.2.2 Structure of the Virion

Rotavirus is a genus in the Reoviridae family. The viral genome comprises of 11 segments of double-stranded ribonucleic acid (dsRNA). The genome codes for six structural viral proteins (VP1-VP4, VP6 and VP7) and six or five non-structural proteins (NSP1-NSP6) contingent upon the strains (Gray *et al.*, 2008). The mature particle is made up of a triple shelled capsid consists of the external, intermediate, and inner layers. The external capsid contains two proteins VP4 and VP7, while the middle layer is formed by VP6 and the inner layer by VP2 which encases two different proteins VP1 and VP3 and the genome Figure 1.1. Because of the segmented nature of the rotavirus genome, genetic re-assortment happens at high frequency (Gray *et al.*, 2008)

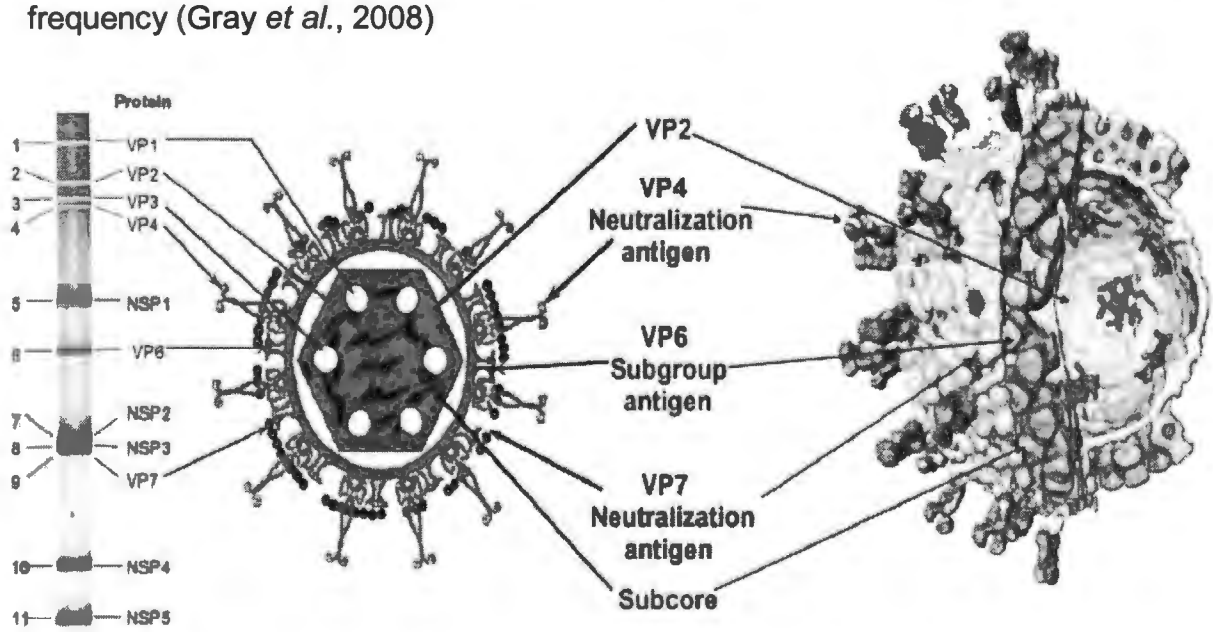

Figure 1.1: Rotavirus structure showing protein coding assignments of 11 genome RNA segments (left). Schematic diagram (middle) and cryoelectron microscopic reproduction of a virion (right) show the location of major structural proteins. NSP, nonstructural protein. Reproduced from (Estes, 2001).

1.4.2.3 Classification of Rotaviruses

Rotavirus has been initially classified into seven serogroups (A through H) based on the inner capsid protein VP6, this is the protein oligomerize to trimmers which constitutes the intermediate capsid. Furthermore, monoclonal antibodies to VP6 epitopes have been used to classify group A rotaviruses according to subgroups (SG) specificities SG I, SG II, SGI+II and SG non-I-non-II (Bresee *et al.*, 2005). Most human rotaviruses belong to either SGI or SG II (Estes and Cohen, 1989). In addition, rotaviruses are classified based on the molecular properties of the two outer-capsid proteins VP4 and VP7 that independently elicit protective neutralizing antibodies (Greenberg *et al.*, 1983).

There are 11 rotavirus gene segments, six are structural proteins (SP) and five are nonstructural proteins (NSP) (Table 1.1). Each is encoded in a unique genome segment except for proteins NSP5 and NSP6 which are encoded in overlapping reading frames of a single segment (Bresee *et al.*, 2005). According to Greenberg *et al.* (1983) the 11 genes of most human rotavirus exhibits significant homology to the corresponding genes of either Wa- or DS-1-like viruses or mixed genotypes with some genes being DS-1-like and some more Wa-like. The appearance of the Wa- and DS-1-like rotaviruses were evidence of re-assortment that occur during co-infection *in vivo* (Greenberg *et al.*, 1983).

However, due to re-assortment of all the 11 rotavirus gene segments playing a role in generating rotavirus diversity, a new classification system that is based on the entire rotavirus gene segments has been established to determine which genes influence rotavirus host range restriction, replication, and virulence and also for studying rotavirus epidemiology and evolution (Matthijnssens *et al.*, 2008). In 2008 Matthijnssens and his colleagues proposed a classification system of rotavirus strains for both human and animals. The new classification system enables identification of the different genotypes based on the nucleotide identity percentage of the 11 gene segments. The rotavirus 11 gene segments; VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6 (Table 1.1) are represented as Gx-Px-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (Matthijnssens *et al.*, 2008). To date, there are 35 type Gs and 50 types Ps that have been identified (Matthijnssens *et al.*, 2008, Maes *et al.*, 2009, Matthijnssens *et al.*, 2011).

Table 1.1: *Genome size and selected characteristics of group A rotavirus (Mattion et al., 1994)*

RNA Segment	**RNA Size (bp)**	**Encoded Protein**	**Location in Virion**	**Function**
1	3 302	VP1	Inner capsid	RdRp; ssRNA binding; complex with VP3
2	2 690	VP2	Inner capsid	Core shell; RNA binding; required for RdRp activity
3	2591	VP3	Inner capsid	Guanylyltransferase; methyltransferase; 2 ,5 -phosphodiesterase; ssRNA binding; complex with VP1
4	2362	VP4	Outer layer	Homotrimer; P type neutralization antigen; attachment protein; protease enhanced infectivity; virulence; fusion with cell membrane
		VP5		Permeabilizes membranes [
		VP8		
5	1611	NSP1	Nonstructural	Interferon antagonist; E3 ligase; RNA binding

6	1 356	VP6	Middle layer	Homotrimer, species determinant; protection (intracellular neutralization); required for Transcription
7	1 105	VP7	Outer later	Homotrimer; glycoprotein; G type neutralization antigen; Ca2+ dependent
8	1 059	NSP2	Nonstructural	Octamer; binds RNA, NTPase; NDP kinase; helix destabilizing; essential for viroplasm Formation
9	1 062	NSP3	Nonstructural	Nonstructural Dimer; binds to: 3 terminus of viral ss (+) RNA, cellular eIF4G, Hsp90; displaces PABP; inhibits host protein translation
10	751	NSP4	Mainly Nonstructural	Very few RER transmembrane glycoprotein; viroporin; intracellular receptor for DLPs; interacts with viroplasms and autophagy pathway; modulates intracellular Ca2+ and RNA replication; enterotoxin (secreted); virulence
11		NSP5	Nonstructural	Dimer; phospho- and O-glycosylated protein; RNA binding; kinase; essential for viroplasm

1.4.2.4 Molecular Epidemiology

Rotavirus is distributed evenly across the globe. Regardless of hygiene practices or access to clean water, nearly every child in the world will be infected with rotavirus before age five (Parashar *et al.,* 2003). However, the consequences of infection are markedly severe depending on where the child lives and the majority of deaths due to rotavirus diarrhoea occur in the developing countries of the Indian subcontinent and sub-Saharan Africa due to limited access to medical intervention (Parashar *et al.,* 2006; Cunliffe *et al.,* 2005). Humans of all ages are susceptible to rotavirus infection, but children aged 6 months to 2 years, premature infants, and the elderly and immuno-compromised individuals are particularly prone to more severe symptoms. Children become most susceptible after 6 months of age when the 8 protection afforded by maternal antibodies wanes. The median age of children hospitalized with rotavirus diarrhoea in many African and Asian countries is 6-9 months, and up to 80% are less than 1 year old (Cunliffe et al., 1998). In contrast, the median age in developed countries is 13-16 months and the highest proportion of cases occurs in the second year of life (Nakagomi *et al.,* 2005). By 15 months of age many have developed some protection after primary infection (O'Ryan *et al.,* 2009). Nevertheless, in both developing and developed countries, rotavirus is the major cause of severe gastroenteritis and is associated with approximately 40% of hospitalizations worldwide (CDC, 2008).

In virtually all studies investigating rotavirus as an aetiological agent of diarrhoeal disease in South Africa, the virus has been identified as the single most important pathogen associated with acute infantile gastroenteritis. A review of the epidemiology of rotavirus infection in South Africa was previously published (Steel *et al.*, 2003) and is only summarised here. In studies that included age- and sex-matched controls, rotavirus was recovered significantly more often from patients with diarrhoea (median 20%, range 13-26%) than from controls (3.25%). In a more recent and thorough analysisIn outpatient studies, children with diarrhoea had a lower level of rotavirus excretion (median 15%), suggesting that rotavirus was more commonly identified in children with more severe disease (Steel *et al.*, 2003).

Of all the different genotypes of bovine rotavirus some combinations of genes seem to be more common than others. Several different studies have been made all over

the world describing the prevalence of genotypes with quite similar results but also with some exceptions (Jakobsson, 2013).

1.4.2.4.1 G-Genotypes

In a study of the molecular characterization of the rotavirus' G-genotypes, it was found that the G6 was the most prevalent followed by the G10. During a cross-country study in Europe six different G-genotypes were identified in cattle: G4, G6, G8, G10, G11 and G12 where the most well-known type was G6 (in 80% of the samples) followed by G10 (13%). While G6 was the predominant genotype in all countries included in the study the commonness of G10 differed from 0-28% (Midgley *et al.*, 2012). In Ireland the most common was G6 (80.6%) followed by a combination of G6,G10 (9.7%) and G10 (6.5%) (Reidy *et al.*, 2006). That G6 is usually predominant was also observed by Monini *et al.* (2008) who found it to be present in 78.5% of positive rotavirus. It was followed by G10 (9.9%), G8 (4.7%) and combined types in 3.3% of samples. In this study they also watched a distinction in commonness of various genotypes from year to year where for instance G10 carried in prevalence from 3, 4-21, 3%. Recently a study in India noticed a new predominant G-genotype. In this study G3 was found in 39.4% of samples gathered from a few unique parts of the country. This was followed by the combinations of G3G8 (27.3%) and G3G10 (33.3%) (Malik *et al.*, 2012).

1.4.2.4.2 P-Genotypes

In Ireland, P[5] was observed to be the most commonly occurring P-genotype in 77.8% of the specimens; P[11] was the second most common with a predominance of 9.3% and in 1.9% of samples P[1] was found. A combination of P[5]P[11] was also seen in 11% of samples (Reidy *et al.*, 2006). Caruzo *et al.* (2010) found that 32.2% of the samples gathered in Goiás, Brazil was of P-genotype P[11]. But, they also found a high predominance of the more extraordinary P[1] (9.7%). In Italy P[11] was the most common (65.1%) followed by P[5] (25%) during the time period 2004-2005. The variation of genotypes from year to year seemed to be less for the P-genotype than for the G-genotype (Monini *et al.*, 2008). In Europe Midgley *et al.* (2012) found that the three genotypes P[1], P[5] and P[11] were predominant. While P[5] and P[11] were the most common ones but the predominance differed from country to country (Jakobsson, 2013).

1.4.2.5 Seasonality

Some studies showed dissemination of rotavirus year-round, and no clear relationship between the planning of the peak in rotavirus movement with either season (cooler versus hotter months) was seen between nations. However, in tropics the pattern is less defined and autumn/spring peak is more normal (Cook *et al.*, 1990). A recent survey considered the tropical areas particularly and related monthly disease incidence with meteorological factors for that month. The investigation found that a one degree centigrade increment in mean temperature brought about a 10% decrease in rotavirus incidence and a one centimetre increment in mean monthly rainfall was associated with a 1% diminishing in rotavirus occurrence (Levy *et al.*, 2009). Recent information indicates that the seasonality of rotavirus could be changed by the presentation of rotavirus antibodies (Cook *et al.*, 1990).

1.4.3 Disease Transmission

Bovine rotavirus can be transmitted mainly via oral-faecal route meaning when an unaffected cow has oral contact with contaminated material; this material can include the faeces from an infected cow and contaminated feed. If there is poor hygiene in the pens then this also acts as a source of infection. Some cows become re-infected and shed the virus throughout the whole of their life whilst remaining asymptomatic. In cows showing signs and symptoms, it is estimated that they are able to shed the virus for as long as a week. (Butz *et al.*, 1993).

The bovine rotavirus is a hardy virus which can survive for many months outside of the host and it cannot be destroyed by a large number of disinfectants. Therefore, due to the difficulty in the eradication of the virus and due to its rapid transmission, the disease can spread very quickly throughout the herd (Butz *et al.*, 1993)

1.4.4 Clinical Signs

Rotavirus infected calves present with watery diarrhoea; the diarrhoea can differ in colour from yellow to green (Estes and Greenberg, 2013). Calves that are infected become severely depressed and dehydrated. Decreased appetite and drooling may be seen in most of the infected calves. Hypovolemic shock can follow with tachycardia and cold extremities. If proper action is not taken against the infected calves, the death rate can be as high as 50% (Estes and Greenberg, 2013).

1.4.5 Rotavirus Replication Cycle

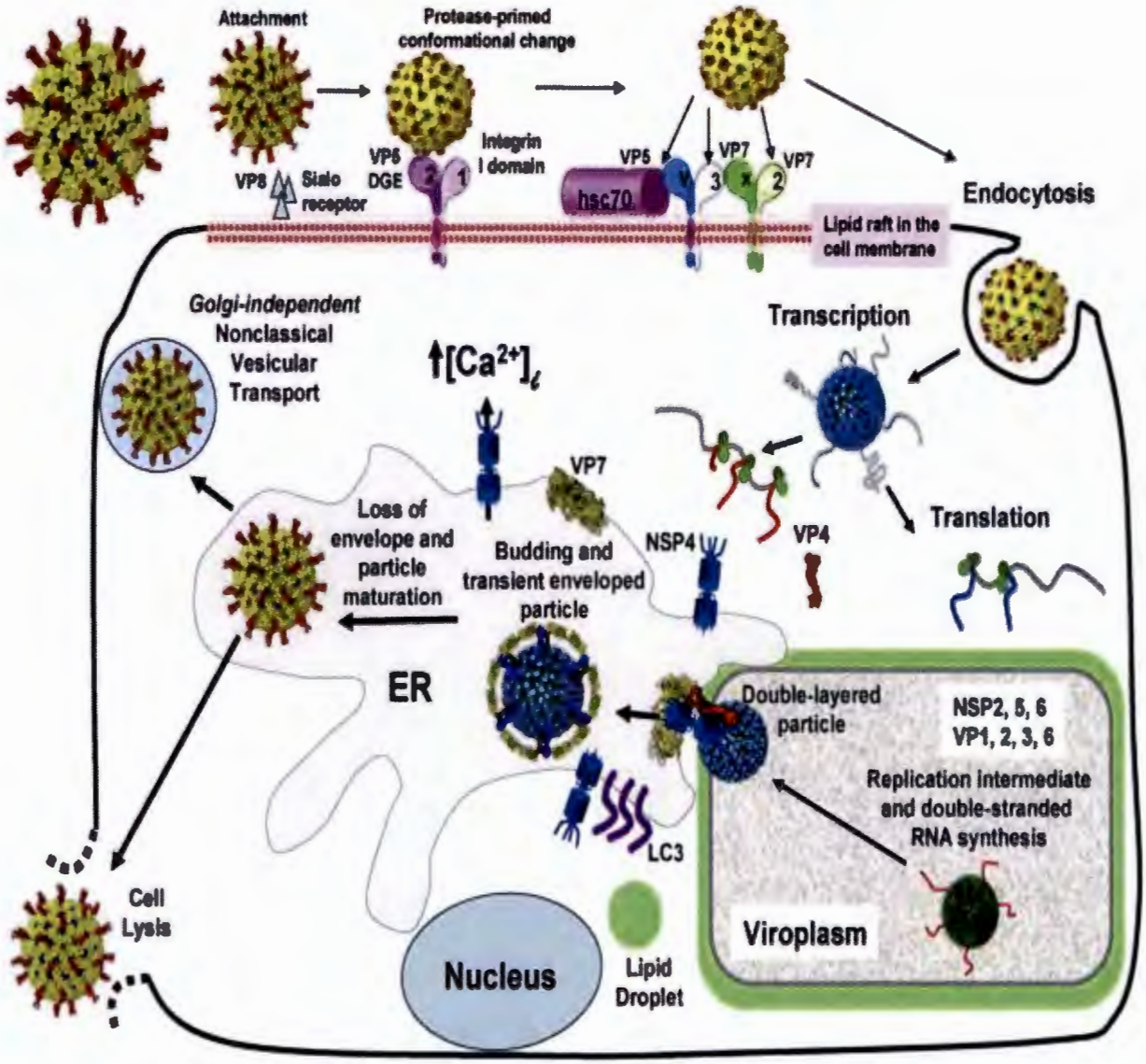

Figure 1.2: *The rotavirus replication cycle adopted from (Estes and Greenberg, 2013).*

Rotavirus has a cycle of replication at 10-12 hours of 37°C. Little is known about the early steps of replication. VP4 is known to bind to a mysterious receptor on the host cell. There are two suggested routes of entry into the cell. One is by endocytosis and the other by direct access by the virus. At the point when the virion enters the cell it loses its outside shell and the double layered transcriptionally active particle is released into the cytoplasm, mRNA (King, 2012). Two transcripts, the full length of the diverse segments, are delivered by DLP3-related enzymes from the dsRNA minus strand. The mRNA serves two needs. They are utilized in the synthesis of viral

proteins and control expression of the discrete genes making some genes more transcriptionally active than others. The other motif serves as layout for genome replication (Jakobsson, 2013). Minus strand synthesis is achieved after gathering of all the necessary mRNA and happens in an intermediate viral core. The intermediate core then shifts to the inside of the endoplasmic reticulum (ER) by actions of NSP4. The virion enters the ER by budding in and accepting a temporary envelope. Inside the temporary envelope is lost and VP7 and VP4 form the outermost shell of the virion (King, 2012).

1.4.6 Pathogenesis

Rotavirus that infects calves frequently causes extreme and life-threating diarrhoea. The diarrhoea is created by a few components. The virus replicates in mature enterocytes on the villi of the small intestines (Jakobsson, 2013). The replication ultimately causes lysis of the cells. The developed enterocytes are then substituted by juvenile enterocytes from the crypts of the villi. The balance between absorption and secretion of liquid is then changed bringing about a build-up of liquid in the small intestine. Loss of developed enterocytes also adds to a systemic insufficiency of bicarbonates, sodium, potassium, chloride and water causing acidosis. The loss of enterocytes decreases the ability to digest milk and the undigested milk is further fermented by microorganisms, which also contributes to causing acidosis. Low lactase, due to the loss of enterocytes, in the intestinal lumen further contributes to fluid gathering by a failed osmotic regulation (Jakobsson, 2013).

The histological picture displays short and blunt villi in the small intestine. The columnar epithelial cells are substituted by cuboidal or squamous cells from the crypts and infiltration of inflammatory cells in the lamina propria is seen (Dhama *et al.*, 2009). Latest studies have also revealed that the viral protein NSP4 may act like an enterotoxin. After cell lysis the protein binds to cells and causes emission of chloride into the intestinal lumen, causing osmotic diarrhoea (Dhama *et al.*, 2009). This is reinforced by the fact that diarrhoea often sets in even before histological changes are visible (King, 2012). On top of that, inflammatory changes in the small intestine caused by the infection provide the intestine a hypermobility that results in less absorption of fluid. The end result of all the factors is watery and most often blood- and mucus-free diarrhoea. If mucus and blood are present it probably

originates from secondary bacterial infection (Dhama *et al.*, 2009). The incubation period of the virus is 12-24 hours and the diarrhoeic calves are most often feverless. The disease may result in severe dehydration and mortality rates differ, but reports have projected it to be on average between 5 and 20%. The diarrhoea caused by rotavirus is self-limiting and animals most often recover if no severe dehydration has occurred. Recovered animals usually return to normal bodyweight within 10-28 days post infection (Dhama *et al.*, 2009).

1.4.7 Diagnosis

1.4.7.1 Laboratory Methods for the Diagnosis of Rotavirus

A variety of detection methods exist for the diagnosis of RV infections. Since both bacterial and viral pathogens can cause clinical signs in cows, diarrhoea alone does not indicate a RV infection and RV infections in older cows can be asymptomatic.

1.4.7.2 Electron Microscopy

Electron microscopy (EM) was initially used to diagnose a rotavirus infection (Bishop *et al.*, 1973). The EM assay uses the rotavirus morphology, the "wheel-like" structure, to identify an infection. An advantage of EM is the capacity to detect all rotavirus species (Bishop *et al.*, 1973). However, this strength is a restriction since it does not separate the different rotavirus species to execute suitable control and prevention strategies in cows. With a detection limit of 105 to 106 rotavirus particles per millilitre, EM lacks the sensitivity to detect a rotavirus infection. In addition, EM is very time consuming and labour intensive (Estes and Greenberg, 2013).

1.4.7.3 Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) is routinely used to detect rotavirus antigens (Estes and Greenberg, 2013). The strategy is generally straight forward. The plate is coated with antibodies against group A rotavirus, focusing on the VP6 protein. The sample is added to the plate, and the antigen, if present in the sample, attaches to the coating antibodies. The plate is washed away while the antigen remains bound to the coating antibodies. At that point, the rotavirus antigen is identified by adding an enzyme-conjugated anti-rotavirus antibody. This is also known as a direct test. On the other hand, a secondary antibody to detect RV particle is added and as enzyme-conjugated detecting the secondary antibody is applied, known as an indirect test (Gouvea *et al.*, 1990). A chromogenic substance is

added, which responds with the enzyme-conjugated antibody, resulting in a colour formation, if the sample is positive while no colour reaction will occur if rotavirus is not present. The reactions are read and documented on a commercial ELISA plate reader. The ELISA assay is a quick and reliable method to detect RVA antigens. A commercial ELISA assay is accessible to identify anti-RVA antibodies in human serum (Estes and Greenberg, 2013).

1.4.7.4 Polyacrylamide Gel Electrophoresis (PAGE)

The principle of PAGE is to isolate the 11 segments of the rotavirus using the polyacrylamide gel in order to show clearly dsRNA migration patterns (Table 1.2). The dsRNA is extracted using an equivalent measure of phenol and chloroform followed by PAGE. PAGE might be utilized to decide the genomic differing qualities of rotavirus in the populace (Bishop, 1996). PAGE permits identification and classification of rotavirus into two main groups, "Long" and "Short" patterns in view of the migration rates of the RNA segment gene 11 as shown in Figure 1.1 page 5 (Herring *et al.*, 1982). Electropherotypes can be utilized to follow the spread of rotavirus through a population group however cannot be used to foresee virulence or classification of rotavirus (Bishop, 1996).

1.4.7.5 Reverse Transcription-Polymerase Chain Reaction and Real-Time PCR

Reverse transcription-polymerase chain reaction (RT-PCR), is presently broadly used to detect rotavirus infections. The rotavirus dsRNA is extracted from the faecal or intestinal homogenate, amplified using rotavirus specific primers, and after that, the RT-PCR product is visualised on an agarose gel, which is referred to as conventional RT-PCR (Gouvea *et al.*, 1990). Real time RT-PCR is quicker than conventional RT-PCR because of the addition of a hydrolysis probes. The hydrolysis probes contain a fluorescent dye, which is released during the amplification of the rotavirus RNA. A real time thermal cycler documents the fluorescent signal and hence the rotavirus amplification, which generates a sigmoidal curve. Real time RT-PCR is extremely quick and accurate. In addition, by using specific primers and probes with various fluorescent dyes, multiple rotavirus species can be identified within a single reaction. Since real time RT-PCR is extremely sensitive, precautionary steps must be taken to lessen cross contamination and false positives. Also, if single nucleotide polymorphisms (SNPs) occur in the dsRNA corresponding

to the primer and probe sequences, the real time RT-PCR will not identify the rotavirus dsRNA. The real time RT-PCR must be designed to recognize a preserved region of the rotavirus genome to guarantee accurate detection (Gouvea *et al.*, 1990, Lyoo, 1995).

1.4.7.6 Genotyping of Rotavirus Strains

Genotyping allows the characterisation of both G and P types. The RT-PCR product is used for genotyping using a combination of primers described by Gentsch *et al.* (1992) and Gouvea *et al.* (1990) to genotype both VP4 and VP7, respectively. Genotyping of strains can display single or multiple infections (single cell being infected with different virus strains) (Iturriza-Gómara *et al.*, 2001).

Table 1.2: *Known serogroups and RNA electrophoretic patterns of rotaviruses affecting animals and human (Lyoo, 1995).*

Serogroups	**Electrophoretic pattern of RNA segments**	**Animal Species Affected**	**References**
A	4:2:3:2	Cattle (NCDV) Pigs, Sheep, Foals, Dogs, Cats, Rabbits, Man	(Mebus *et al.*, 1969, Estes *et al.*, 1983)
B		Avian	(Todd *et al.*, 1980)
B	4:2:2:3	Pigs	(Pedley *et al.*, 1983, Theil *et al.*, 1985, Snodgrass *et al.*, 1984)
		Cattle	
		Man	(Chen *et al.*, 1985)
C	4:3:2:2	Pigs	(Saif *et al.*, 1980, Bohl *et al.*, 1982, Tsunemitsu *et al.*, 1991)
		Cattle	
		Man	(Rodger *et al.*, 1982)
D	5:2:2:2	Chicken	(Mcnulty *et al.*, 1981, Mcnulty *et al.*, 1984, Bridger, 1987, Snodgrass *et al.*, 1984)
E	4:2:2:3	Pigs	(Pedley *et al.*, 1986)
F	4:3:2:2	Chicken and Turkeys	(Mcnulty *et al.*, 1984, Bridger, 1987)
G	4:2:2:3	Chickens	(Mcnulty *et al.*, 1984, Bridger, 1987)

1.4.8 Treatment and Prevention

There is no cure for calves and cows infected with the bovine rotavirus although supportive therapy is usually effective. For calves it is suggested that they be fed diluted formula and lactose free carbohydrate rich foods as early as the symptoms are observed (Bernstein, 2009). The treatment is by means of replacing lost fluids and restoring the balance of the body's lost electrolytes. These may be administered intravenously, the infected animal must be kept in a warm and dry area, and sufficient amounts of colostrum may be given to young calves to encourage recovery (Jakobsson, 2013). The use of antibiotics is not relevant in this case since they do not treat viruses. However, if there is a presence of a secondary bacterial infection the antibiotics may be administered (Ye *et al.*, 2013). This is because once a secondary infection comes into play, the severity and risk of the diseases to the animal's health significantly increases (Bernstein, 2009).

Preventing rotavirus disease can meaningfully reduce economic loss and stress for the cattle farmers. The prevention methods are very similar to the majority of measures used to treat viral diarrhoea (Bresee *et al., 1999*). The most important thing is to keep are high hygiene standards, ensuring the calves have acceptable amounts of colostrum, and a good vaccination programme (Bernstein, 2009).

Good cleanliness can prevent the transmission of bovine rotavirus. Calves and cows living indoors should be provided with clean and dry bedding and should have good ventilation. Infected animals should be isolated and restricted from having contact with the other members of the herd in order to reduce the possibility of rapid transmission. It has also been suggested that animals of different ages should be separated (Bresee *et al., 1999*)

In terms of keeping calves healthy, the calves should be given adequate amount of colostrum. This is especially true for calves under the age of five days old (Jakobsson, 2013). Calves should be kept free of stress at the age of below five days as this can also contribute to infection (Bresee *et al., 1999*).

1.4.9 Rotavirus Vaccinations

1.4.9.1 History and Development of Rotavirus Vaccines

The development of a live oral rotavirus vaccine started in the mid-1970s, when researchers found that previous infection with animal rotavirus strain protected laboratory animals from human rotavirus infection (Bresee *et al., 2005.* Although humans can be infected by animal strains, interspecies transmission of animal rotavirus to humans is relatively uncommon. The first vaccines were based on a Jennerian approach (pioneered by Jenner in 1798 for human smallpox vaccination); when antigenically related non-human rotavirus strains were given orally, they acted as immunogens for VP6, but not for VP7 or VP4, inducing a similar immune response to that caused by the natural rotavirus infection (Bresee *et al., 2005)*

1.4.10 Inter-Species Transmission of Rotaviruses

The inter-species transmission abilities of mammalian rotaviruses have been demonstrated by assessing the presence of non-genogroup-specific antibodies or by challenge studies with different rotaviruses (Sato *et al.*, 1981, Heinrich *et al.*, 1983, Castrucci *et al.*, 1984, Castrucci *et al.*, 1985). Calves inoculated with equine or human rotaviruses have been protected against BRV challenge, showing their close relationship (Woode *et al.*, 1978). It has also been suggested that porcine, murine, simian and equine rotaviruses are antigenically related (Mebus *et al.*, 1977, Castrucci *et al.*, 1985). The capability of human rotaviruses (HRV) to infect calves or piglets has been reported, and it has been found that piglets excreted the virus

without clinical signs while calves produced intestinal lesions (Bridger *et al.*, 1975, Mebus *et al.*, 1977). Further, the presence of HRV antibodies has been detected in milk of cows (Yolken *et al.*, 1985). In another study, calves were reported to be susceptible to rabbit rotaviruses and the rabbits also became infected when inoculated with a BRV strain (Castrucci *et al.*, 1984). Also, it has been suggested that infection can occur in calves from rotaviruses of simian, porcine or lapine origin (Castrucci *et al.*, 1985). Similarly, pet animals like cats and dogs may also excrete BRV, and are thought of having a role in the propagation of BRV (Schwers *et al.*, 1982). In an interesting observation, a BRV isolate has been noticed to differ from other mammalian isolates during probe analysis, but got hybridized to the genome of an rotavirus A strain, thus representing a classical candidate for a natural interspecies transmission between different classes of vertebrates (Brüssow *et al.*,

1992). Apart from bovines and human, interspecies transmission of ARVs to experimental animals has also been reported (Rohwedder *et al.*, 1995, Mori *et al.*, 2001). Similarly, instances of transmission of mammalian rotaviruses to avian species have also been documented (Wani *et al.*, 2003).

1.4.11 Zoonotic Significance of Animal Rotaviruses

Animal rotaviruses could be considered as a potential threat to humans due to the possibility of genetic re-assortment coming about due to the exchange of gene segments. Infections by bovine-human reassortants and the presence of several uncommon strains in cases of new-born diarrhoea suggest that animal rotaviruses could be considered to be having important zoonotic impact (Ramani and Kang, 2007). Increasing evidence has been found regarding direct transmission; or animal rotaviruses contributing one or several genes to make animal-human reassortant viruses (Nakagomi and Nakagomi, 1991, Adah *et al.*, 2003, Malik *et al.*, 2005, Müller and Johne, 2006, Ramani and Kang, 2007). Additionally, the surveillance of circulating rotaviruses in the human population has exposed the presence of uncommon serotypes that are commonly found in domestic animals (Cook *et al.*, 2004, Malik *et al.*, 2005)

In new-born animals, generally the rotavirus infection is characterized by watery diarrhoea and severe dehydration while the infection in adults is often subclinical (Malik *et al.*, 2005, Ramani and Kang, 2007) The main purpose for a zoonotic transmission is the close contact between humans and domestic animals, promoting exposure to rotaviruses, especially in geographical regions where there may be intermittent floods or torrential rains. Potential also exists for contamination of water bodies and food crops with animal rotaviruses, via animal excreta. Likewise, animal rotaviruses could also be spread by food materials which are eaten uncooked, especially vegetables (Svensson, 2000, Malik *et al.*, 2005). Further, rotavirus strains such as G3 (commonly seen in cats, dogs, pigs and horse), G5 (pigs and horses), G6 and G8 (cattle), G9 (pigs and lambs) and G10 (cattle) have been isolated from the human population from various parts of the world (Desselberger *et al.*, 2001, Malik *et al.*, 2005, Ramani and Kang, 2007).

Studies have also given many indications showing human rotaviruses (HRV) deriving their surface proteins from animal rotaviruses. To further understand the

epidemiological and genetic basis for the origin of human rotavirus (HRV) strains, relative frequencies of different serotypes of bovine rotaviruses have been analyzed (Varshney *et al.*, 2002). Based on the sequence analysis of VP4 and VP7 genes, it is presumed that there is predominant association of bovine rotavirus G10 serotype-derived reassortant strains causing asymptomatic infections in new-borns. It has now been well proven that the HRV can acquire genomic segments from bovine rotavirus strains by the phenomenon of gene re-assortment (Adah *et al.*, 2003). Similarly, hybridization experiments with HRV have provided the evidence for the close relationship with feline and canine rotaviruses (Nakagomi *et al.*, 1990). In Italy, the sequence analysis of VP6, VP7, VP4 and NSP4 genes of human rotaviruses has given vital suspicions concerning the role of canine rotaviruses in contributing gene segments for reassortment with human viruses (De Grazia *et al.*, 2007). Furthermore, existence of relatedness has been identified in case of porcine rotaviruses, when VP1, VP2, VP3, VP4, VP7 and NSP4 genes were studied with those of recent HRV strains (Teodoroff *et al.*, 2005, Varghese *et al.*, 2006, Mascarenhas *et al.*, 2007). Cumulatively, all these investigations are pointing to the fact that such events may lead to development of novel reassortant human viruses during mixed infections that could further complicate infection in infants. To conclude, the rapid evolution often seen with rotaviruses and the emergence of novel strains warrants an intensive serotype-specific surveillance before implementing any vaccination program to control the infection in human beings (Ramani and Kang, 2007).

CHAPTER 2

2.0 METHODS AND MATERIALS

2.1 Ethical Considerations

Ethical approval for this study was obtained from the North-West University Research and Ethical clearance Committee (S9/NWU-00217-15-S9). All samples were collected according to good clinical practices (animal). Farmers/ owners of the livestock were presented with a letter describing the aim and the significance of the study in order to solicit for their permission and assistance.

2.2 Study Design

This was a laboratory-based exploratory study.

2.3 Study Sites

2.3.1 Study Site and Sample Collection

Samples were collected, between 2015 and 2017 from randomly selected rural areas/farms including Molelwane farm, Rooigrond Prison Farm, Majemantsho Village, Disaneng village, Ramatlabama, Motlhabeng and Lotlhokane village (Figure 2.1). Two hundred (200) diarrhoeal samples were collected from calves below the age of 3 months, from the samples collected, 108 (54%) were male calves and 92 (46%) were female calves.

***Figure 2.1**: Map showing area of collection around Mafikeng, North West Province (https://en.wikipedia.org/wiki/Mahikeng)*

2.4 Collection Method of Samples

Samples were randomly collected depending on the availability and the presence of calves that had diarrhoea in the particular area. Diarrhoeal samples were collected from animals using the free flow method, meaning that fresh samples were picked from the ground immediately after the calves defecated and other samples were collected directly from rectum. These methods were safely practiced under the supervision of a veterinarian and I am also a qualified Animal Health Technician (BSc in Agriculture Animal Health) and therefore, am qualified to practice such procedures. Samples were transported on ice to the Virology Laboratory. Upon arrival at NWU- Mafikeng Campus, the samples were recorded in the laboratory register book. All diarrhoeal samples received were given a unique laboratory number for identification purposes according to good laboratory practices (GLP) and Good Animal Handling Procedures and they were stored at 4°C until immediate use or at minus 80°C for later use. Samples were analyzed using the following laboratory procedures (Figure 2.2).

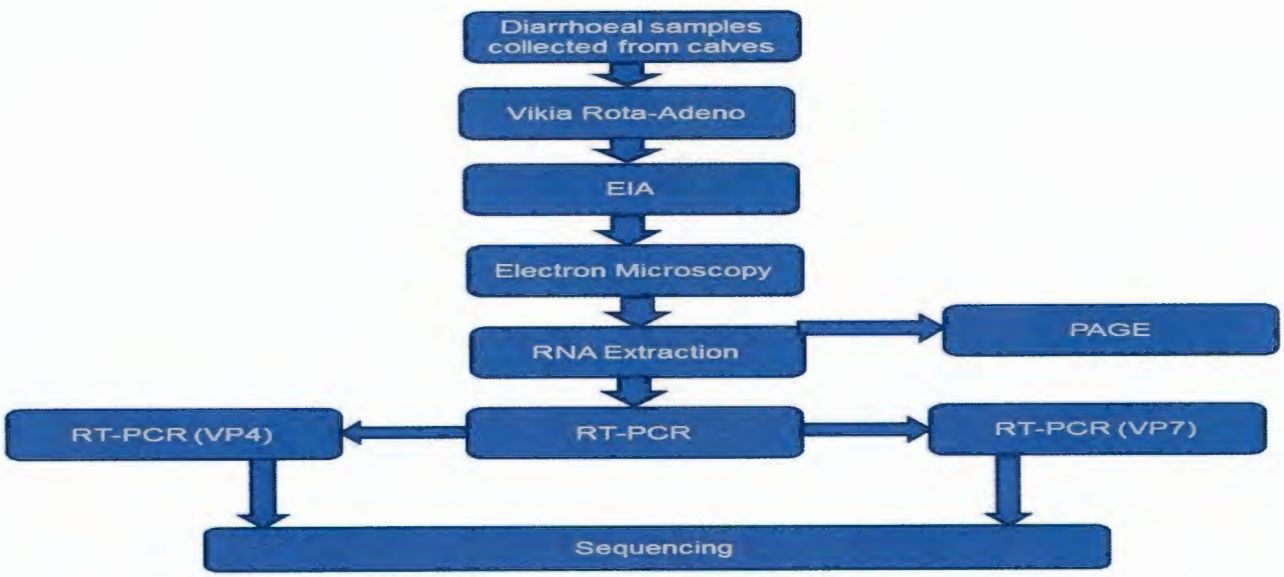

Figure 2.2: *Flow diagrams of laboratory procedures followed during screening and characterization of rotavirus strains*

2.5 Detection of the Rotavirus Antigen in Samples

2.5.1 Screening Processes

2.5.1.1 Vikia-Rota Adeno test

1.5.1.1.1 Principle

VIKIA Rota-Adeno is a qualitative test based on the association of monoclonal antibodies specific to rotavirus and adenovrius respectively. This test uses immunological reactions performed on a test strip by migration (ICT or lateral flow format). The test consists of a plastic device containing: A chromatographic membrane to which are fixed: - in the test region, an anti-rotavirus monoclonal antibody (test region "R") and an anti-adenovirus monoclonal antibody (test region "A"), - in the control region, an anti-mouse IgG polyclonal antibody (control region "C") and a test strip impregnated with a conjugate consisting of a mixture of monoclonal anti-rotavirus antibody coupled to blue dyed polystyrene microspheres and monoclonal anti-adenovirus antibody coupled to red dyed polystyrene microspheres. The sample is added to the sample well and migrates by capillarity along the membrane. If the sample contains rotaviruses, they form an antigenantibody complex with the antibodies specific to this virus present on the blue dyed polystyrene microspheres. If the sample contains adenoviruses, they form an antigen-antibody complex with the antibodies specific to this virus present on the red dyed polystyrene microspheres. The antigen-antibody complexes migrate along the membrane and bind to the anti-rotavirus and/or antiadenovirus antibodies, forming complexes revealed by a blue and/or red line in the "R" or "A" test line regions of the membrane Absence of this/these colored line(s) suggests a negative result. To serve as a procedural control, a colored line will always appear in the control line region "C" if the test has been performed correctly.

2.5.1.1.2 Method

The Rota-Adeno test kit (Figure 2.3) is a rapid test, based on the immunochromatography technique, for the dual detection of rotaviruses and adenoviruses in a single sample. The test was conducted following the manufacturer's instructions. The samples were taken out of the -80°C freezer a night

before use to allow them to defrost to room temperature (15-30°C) before performing the test. The cap was unscrewed on the R2 vial and removed the applicator stick required to collect the specimen. Approximately 50mg of samples (equivalent to ¼ of a pea) was collected using the applicator stick in the R2 vial. The samples were inserted into the R2 vial containing the dilution buffer. The cap was screwed back onto the R2 vial and was shaken vigorously by votexing (Intercal, G-560E, Vortex – 2 Gene, USA) to homogenize.

The test kit was removed from the sealed pouch and was used as soon as possible, and then the test device was then placed on a clean, level surface. The tip of the R2 vial containing the diluted sample was broken off, turned the R2 vial upside down and held vertically. Two (2) drops of diluted sample (approximately 80µl) were transferred to the sample well (S) of the test device and the timer was set. The trapping of air bubbles in the sample well (S) was avoided. Then waited for the control line "C" to appear and possibly lines "R" and/or "A". Read the results 10 minutes after having dispensed the sample.

2.5.1.2 10% Stool dilution

In order to perform further rotavirus analysis 10% stool dilution was prepared. A 10% faecal suspension of pea-sized stool in 10 ml of distilled water was made and stored at 4°C until analysed.

2.5.1.3.1 Principle

The general principle of this technique is based on the binding of conjugated enzyme molecule with specific antibodies to detect and quantify the presence of either antigens or antibodies in the test sample. This is followed by adding appropriate colourless substrate which catalyses the interaction complex to produce a visible coloured product. There are many chromogenic substrates used in ELISA technique but the most common are alkaline phosphatase (AP) and horseradish peroxidase (HRP). The end product can be determined by using spectrophotometer and the intensity of colour is directly proportional to the presence of either antigens or antibodies in the test samples.

2.5.1.3.2 Method

The samples were screened for the presence of group A rotavirus antigen using a commercially available ProSpecT™ Rotavirus enzyme immunoassay kit according to

the manufacturer's instructions. Briefly, 100 µl of a positive control, a negative control (Tris buffer saline solution, antimicrobial agent and red dye) and 100 µl each of the 10% stool dilution were added into separate microwell plates. The controls were included for quality control purposes and were supplied with the EIA kit. Two drops (100 µl) of conjugate antibody (rotavirus specific rabbit polyclonal antibody conjugate to horseradish peroxidise) in a buffered protein solution containing antimicrobial agent and blue dye supplied with the kit was added to each well, including the control wells. The microwell plates were then incubated on the bench at room temperature for 60 minutes. While incubating the specimens 1x wash buffer solution was freshly prepared using 120 ml phosphate buffer solution containing antimicrobial agent and detergent from a stock solution at 10x concentration supplied with the kit, by adding 1 part concentrate to 9 parts distilled water. Once the hour for incubation had elapsed, the microwell plates were washed 6 times using the freshly prepared 1x wash buffer. The wells were blotted dry to remove excess water. Two drops of substrate solution (stabilized peroxide and 3.3'-5.5'tetramethylbenzidine in a dilute buffer solution) were added to each well and incubated for 10 minutes in the dark. After 10 minutes incubation two drops of stop solution (0.45 M sulphuric acid) supplied with the kit was added in each well to stop the reaction. After stopping the reaction, the presence of rotavirus antigen was confirmed by reading the plate at a wavelength of 450 nm using a spectrophotometer.

2.5.1.4 Interpretation of results

The positive control was considered positive if the absorbance value was above 0.500. The cut off was calculated by adding 0.200 to the negative control absorbance. All the samples with absorbance below the cut-off were considered negative. Absorbance above the cut-off value was considered positive. The results were recorded based on the range of the absorbance values as shown below:

Negative results

Absorbance value ≤ cut off - negative

Positive results

Absorbance

Above cut off < 0.500 - -/+

0.500- 1.000 - 1+

1.100 – 2.000 - 2+

2. 100 - 3.000 - 3+

> 3.000 - 4+

2.5.1.5 Electron Microscopy

2.5.1.5.1 Principle

Electrons are made to pass through the specimen and the image is formed on the fluorescent screen, either by using the transmitted beam or by using the diffracted beam.

2.5.1.5.2 Method

A total of 20 samples which were randomly selected were sent to North-West University Potchefstroom Campus for electron microscopy (EM) courtesy of Dr A Jordaan EM Unit. However only 6 samples were prepared and viewed under the TEM due to time constraints.

2.5.2.1 Polyacrylamide Gel Electrophoresis (PAGE)

2.5.2.1.1 Principle

PAGE (Polyacrylamide Gel Electrophoresis), is an analytical method used to separate components of a protein mixture based on their size. The technique is based upon the principle that a charged molecule will migrate in an electric field towards an electrode with opposite sign.The general electrophoresis techniques cannot be used to determine the molecular weight of biological molecules because the mobility of a substance in the gel depends on both charge and size. To overcome this, the biological samples needs to be treated so that they acquire uniform charge, then the electrophoretic mobility depends primarily on size. For this different protein molecules with different shapes and sizes, needs to be denatured (done with the aid of SDS) so that the proteins lost their secondary, tertiary or quaternary structure .The proteins being covered by SDS are negatively charged and when loaded onto a gel and placed in an electric field, it will migrate towards the anode (positively charged electrode) are separated by a molecular sieving effect based on size. After the visualization by a staining (protein-specific) technique, the

size of a protein can be calculated by comparing its migration distance with that of a known molecular weight ladder(marker).

2.5.2.1.2 Extraction of dsRNA from stools for page analysis

Briefly, viral RNA was extracted from all 50 samples. In a clearly labelled sterile Eppendorf tube, 450 µl of a 10% stool suspension was mixed with 50 µl of a pre-warmed 1M-sodium acetate (NaAc) solution containing 1% Sodium Dodecyl Sulphate (SDS) with a pH 5.0. SDS is an ionic detergent used to denature secondary and tertiary structure of the virus. The mixture was incubated at 37°C for 15 minutes in a water bath. An amount of 500 µl phenol / chloroform (1:1) was added to each Eppendorf tube to denature the proteins. The phenol / chloroform mixture increases the efficiency of nucleic acid extraction. The tubes were incubated for 15 minutes at 56°C in a water bath after vortexing for 1 minute. The mixture was then centrifuged (Intercal, eppendorf 5415 R, Germany) for 3 minutes at 12,000 rpm to separate the phases. Three phases were observed, the upper phase was a colourless aqueous phase containing dsRNA, the interphase (middle layer) and lower organic phase. The upper phase containing dsRNA was carefully transferred into a clearly marked sterile Eppendorf tube. The viral dsRNA was then precipitated with 1/10 volume (approximately 40 µl) of 3M NaAC buffer pH 5.0 and two volumes of ice cold absolute ethanol were added before incubating at - 20°C overnight. The Eppendorf tube containing the dsRNA was centrifuged at 4°C for 15 minutes at 12,000 rpm to pellet the dsRNA. The supernatant was carefully removed by pouring off the ethanol from the tubes and the pellet was allowed to air-dry before re-suspending in 30 µl prepared gel loading dye buffer as indicated in Appendix 4.2.

2.5.2.1.3 Preparation of 10% resolving gel

The electrophoretic apparatus which included glass plates, glass stands and clamps was assembled. The PAGE glass plates were first cleaned using absolute ethanol (96%) then paired together and two horizontal sides separated with a 0.75 mm rubber gasket, clamped and locked the two glasses together and placed on the electrophoretic apparatus. The glasses were marked to indicate the level of the resolving gel. Reagents were prepared to make two gels by adding 7.9 ml of dH_2O, 5.0 ml 30% acrylamide stock solution, 1.875 ml resolving buffer (pH 8.9), 7.75 µl of TEMED and 225µl of freshly prepared 10% ammonium persulphate (APS) (Cleaveland, USA) into the glass beaker. The mixture was poured into the assembled

glass plates. Approximately 1ml of dH_2O was added on top of the resolving gel. The gel was left to polymerize for 2 hours on the bench. The ammonium persulphate solution is an initiator for gel formation and TEMED (Cleaveland, USA) used a catalyst for polymerization of the gel.

2.5.2.2 Preparation of 3% spacer gel

A mixture of a stacking solution was prepared by adding 3.4 ml of dH_2O, 0.8 ml 30% acrylamide, 0.625 ml spacer (pH 6,7), 2.5 µl of TEMED and 75 µl of 10% APS. After removing the layer of dH_2O, the mixture was added on to the resolving gel in between the glass plates. A 12-well plastic comb was inserted between the glass plates immediately so as to guide the samples into the 12 wells.

A solution of 5x Tris-glycine running buffer was prepared by weighing 15.1 g of 25 mM Tris base and 94 g of glycine 250 mM and mixed in 1000 ml distilled water. A

working solution of 1x Tris-glycine buffer was prepared from the prepared 5x Tris-glycine by adding 800 ml distilled water to 200 ml of 5x Tris-glycine. The gels were then placed in an electrophoretic current through 700 ml of 1x Tris buffer at the bottom tank and 200 ml 1x Tris buffer was poured on the top of the tank. A total volume of 20 µl loading dye was mixed with the extracted dsRNA sample in 2 ml Eppendorf tubes then loaded into the wells. Electrophoresis utilizes a 10% polyacrylamide gel overlaid with a 3% stacking gel, using a discontinuous Tris-glycine buffer system at 100 volts at room temperature for 5 hours (Theil *et al.*, 1981). The gels were stained using ethidium bromide (Lauretti *et al.*, 2003).

2.6 Extraction of RNA

2.6.1 10% Stool dilution

In order to perform further rotavirus analysis 10% stool dilution was prepared. Ten percent faecal suspension of pea-sized stool in 10 ml of distilled water was made and stored at 4°C until analysed.

2.6.2 Viral RNA QIAGEN QIAamp

2.6.2.1 Principle

QIAamp Viral RNA Mini Kits (QIAamp, Hamberg, Germany) provide the fastest and easiest way to purify viral RNA for reliable use in amplification technologies. Viral

RNA can be purified from plasma (treated with anticoagulants other than heparin), serum, and other cell-free body fluids. Samples may be fresh or frozen, but if frozen, should not be thawed more than once. Repeated freeze–thawing of plasma samples will lead to reduced viral titers and should be avoided for optimal sensitivity. Cryoprecipitates accumulate when samples are subjected to repeated freeze–thaw cycles. This may lead to clogging of the QIAamp membrane when using the vacuum protocol. QIAamp Viral RNA Mini Kits are for general use and can be used for isolation of viral RNA from a wide variety of viruses, but performance cannot be guaranteed for every virus.

2.6.2.2 Method

The viral RNA was extracted from the stool using Viral RNA QIAGEN QIAamp mini kit (Qiagen, Hamberg, Germany) following the manufacturer's protocol, with modifications. About 560µl of the AVL-RNA complex was mixed with a 140µl of stools in a 2ml micro centrifuge tube. After 10 minutes of incubation at room temperature, 560µl of ethanol was added to the mixture. The mixture was then vortexed and 630µl of it was pipetted into another 2ml centrifuge tube that contains a filter. For a brief period of time, the mixture was centrifuged at a speed of 8000rpm. The filtrate was then discarded and the mini column containing the filter was placed in a new collection tube. Some 500µl of washing buffer was used to wash the filter. AW1 washing buffer was used first, followed by AW2 buffer. The mini columns were centrifuged at a speed of 8 000 rpm (1min) and 14 000 rpm (3min) respectively. Once again, the mini column was placed in a clean collection tube and the viral RNA was eluted with 60ul eluting buffer (AVE). The tube was centrifuged for a minute at a speed of 8 000 rpm to collect the viral RNA from the mini column. This time the mini column was disposed off and the collection tube containing the viral RNA was stored at -20°C until further use.

2.6.3 Trizol extraction

2.6.3.1 Principle

RNA (Ribonucleic acid) is a polymeric substance present in living cells and many viruses, consisting of a long single-stranded chain of phosphate and ribose units with the nitrogen bases adenine, guanine, cytosine, and uracil, which are bonded to the

ribose sugar. RNA is used in all the steps of protein synthesis in all living cells and carries the genetic information for many viruses. The isolation of RNA with high quality is a crucial step required to perform various molecular biology experiment. TRIzol Reagent is a ready-to-use reagent used for RNA isolation from cells and tissues.TRIzol works by maintaining RNA integrity during tissue homogenization, while at the same time disrupting and breaking down cells and cell components.

2.6.3.2 Method

A previously prepared 10% sample suspension was left to settle at room temperature for 30 minutes before use. About 250µl of the sample's supernatant was mixed with 750µl TRIzol in a 1.5 ml Eppendorf tube then vortexed for 30 seconds. After 5 minutes of incubation at room temperature 200µl of chloroform was added to the mixture then vortexed for 30 seconds. After 3 minutes of incubation the mixture was centrifuged at 12 000 rpm for 5 minutes at 4°C to separate the phases. The clear, upper aqueous phase (containing the RNA) was transferred into a new sterile Eppendorf tube and 650-700µl of ice cold isopropanol was added to the mixture. The mixture was gently mixed by turning the tube 4-6 times then incubated at room temperature for 20 minutes for precipitation of RNA. The mixture was centrifuged at 12 000rpm at 4°C for 15 minutes to pellet the RNA. The supernatant was discard immediately to allow the RNA pellets to air dry at room temperature, then suspend the pellet in 70-950µl sterile RNAse/ DNAse free deionised water and store samples at -20°C until further use.

2.6.4 Gel Electrophoresis

2.6.4.1 Principle

Gel electrophoresis separates DNA fragments by size in a solid support medium (an agarose gel). DNA samples are pipetted into the sample wells, seen as dark slots at the top of the picture. Application of an electric current at the top (anodal, negative) end causes the negatively-charged DNA [remember it's an acid] to migrate (electrophorese) towards the bottom (cathodal, positive) end. The rate of migration is proportional to size: smaller fragments move more quickly, and wind up at the bottom of the gel. DNA is visualized by including in the gel an intercalating dye, ethidium bromide. DNA fragments take up the dye as they migrate through the gel. Illumination with ultraviolet light causes the intercalated dye to fluoresce with a pale pink colour.

2.6.4.2 Method

The extracted materials from both methods were run through a 1% agarose gel which was stained with ethidium bromide to enable fluorescence under ultraviolet light. The gel was run for 120 minutes at 100V to determine the presence of any RNA from the samples.

2.7 MOLECULAR TECHNIQUES

2.7.1 RT-PCR

2.7.1.1 Principle

As the name implies, it is a chain reaction, a small fragment of the DNA section of interest needs to be identified which serves as the template for producing the primers that initiate the reaction. One DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e., the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates.

2.7.1.2 Method

The PCR technique is more sensitive and specific and may be used to establish the genetic diversity of VP4 and VP7 genes. PCR has a sensitivity of 93% and compared to 82% observed in EIA techniques (Gouvea et al., 1990), thus it is a method of choice. The RT-PCR assay enables amplification via reverse transcription polymerase chain reaction where by a very low copy number of dsRNA molecule can be detected. A total 50 samples were analysed for P and G types using specific primer sets as indicated in Table 2.1 and Table 2.2 below. During PCR both positive and negative controls of rotavirus were included and treated similarly as the samples. The negative controls were prepared using nuclease free water and the positive control was from a known rotavirus-positive specimen.

2.7.2 Detailed procedure

The genomic segment 4 (VP4) gene was amplified using primers Con 2 and Con 3 to generate an 876 bp amplicon according to (Gentsch *et al.*, 1992) as shown in Table 2.1. In cases where the P type was not determined, inner VP4F and VP4R were used to amplifying a 663 bp fragment according to (Simmonds *et al.*, 2008). These primers were used for first round PCR. This enables specimens with mutations at the terminal sites to be eliminated. Primers sBeg9 and EndA (Gouvea *et al.*, 1990) were used to amplify the VP7 gene to generate an amplicon of 1062 bp as shown in Table 2.2 below. Briefly, an amount of 8 µl of dsRNA extracted using QIAamp was added into a clearly labelled sterile 0.2 ml PCR tube (Axygen, California, USA) containing 1 l of each of the two terminal primers 5' (10 pmol/µl sBeg9) and 3' (10 pmol/µl EndA) or 3' (10 pmol/µl Con 2) and 5' (10 pmol/µl Con 3). The tubes with the mixture were incubated on a heating block at 95°C for 5 min to denature the dsRNA strands. Reverse Transcription (RT) master mix containing 0.25 µl of 10 mM of each of dCTP, dTTP, dGTP, dATP (Bioline, USA), 0.2 of 5 U Avian Myeloblastosis Virus (AMV) reverse transcriptase and 2 µl of 5x AMV Buffer was prepared. An amount of 3.2 µl of the master mix was added to each PCR tube and incubated in the heating block at 42°C for 30 min.

A PCR master mix of 40 µl was prepared by adding 1 µl of 10 Mm of each of dCTP, dTTP, dGTP, dATP, 5µl of 10x *Taq* Buffer, 1.4 µl 50 mM magnesium chloride (Bioline ,USA), 30 µl dH_2O and 0.3 µl of 5 U/ µl x BIOTAQ™ DNA polymerase enzyme (Bioline USA). Then 40µl of master mix was added to each PCR tube. The reaction was then placed in a Biorad thermocycler with 30 cycles for about 3 hours. The 30 cycling conditions comprised of a denaturation step at 95°C for 1 min to separate of dsRNA strands), an annealing step at 42°C for 1 and an extension step to generate newly formed strands at 72°C for 1 min and followed by final extension at 72°C for 7 min.

Table 2.1: *Oligonucleotide primers used to amplify the fragment of VP4 gene and human [P]-genotyping with primer sequences, primer positions on the respective genes and expected amplicon size.*

Primer	Sequence (5'-3')	Position (nt)	Strain Genotype	Reference
Con 2	ATT TCG GAC CAT TTA TAA CC	868-887	3'	(Gentsch *et al.*, 1992)
Con3	TGGCTTCGCTCATTTATAGACA	11-32	5'	(Gentsch *et al.*, 1992)
VP4F	TATGCTCCAGTNAATTGG	132-149	5'	(Gentsch *et al.*, 1992)
VP4R	ATTGCATTTCTTTCCATAATG	775-795	3'	(Gentsch *et al.*, 1992)
PGott	GCTTCAACGTCCTTTAACATCAG	465-487	Gott P[6]	(Freeman et. al.,2008)
pOSU	CTTTATCGGTGGAGAATACGTCAC	389-412	OSU P[7]	(Gentsch *et al.*, 1992)
pUK	GCCAGGTGTCGCATCAGAG	336-354	UK P[5]	(Gentsch *et al.*, 1992)
pNCDV	CGAACGCGGGGGTGGTAGTTG	269-289	NCDV P[1]	(Gentsch *et al.*, 1992))
pB223	GGAACGTATTCTAATCCGGTG	574-594	B223 P[11]	(Gentsch *et al.*, 1992)

Table 2.2: *Oligonucleotide primers used to amplify the fragment of VP7 gene and human [G]-genotyping with primer sequences, primer positions on the respective genes and expected amplicon size*

Primer	Sequence (5'-3')	Position (nt)	Strain (genotype)	References
SBeg9	GGCTTTAAAAGAGAGAATTTC	1-21	5'	(Gouvea *et al.*, 1990)
EndA	ATAGTATAAAATACTTGCCACCA	922-944	3'	(Gouvea *et al.*, 1990)
Sb-2 Pig	GACGTAACAACGAGTACATG	779-760	OSU (G5)	(Gouvea *et al.*, 1990)
NCDV/UK	GATTCTACACAGGAACTAG	499-481	NCDV/UK (G6)	(Gouvea *et al.*, 1990)
A5 Cow	GTGTCTAATCCGGAACCG	273-256	B37 (G8)	(Gouvea *et al.*, 1990)
B233 Cow	GAAGTCGCAACGGCTGAA	714-697	B233 (G10)	(Gouvea *et al.*, 1990)
YM Pig	GCAACTCAGATTGCTGATGAC	336-316	YM (G11)	(Gouvea *et al.*, 1990)

2.7.3 Agarose gel electrophoresis

Resulting PCR products were analysed using a 1% agarose gel electrophoresis prepared as following;- The Gel is prepared transferring 100ml of TAE buffer to a conical flask then 1grams of agarose is weighed and added to the 100ml buffer solution. The solution is then heated in a microwave and then ethidium bromide is added the solution.

The PCR produ+cts were loaded on to the gel together with a lane for 100 bp molecular weight marker. The gel electrophoresis was carried out in 1x TBE for 45-60 min at 90V. The amplicons were analysed and visualised under UVP transilluminator. Results were first interpreted based on the molecular weight (size) of cDNA for each specimen that was obtained by aligning against the position of each amplicon the 100 bp marker on the agarose gel.

4.7.4 Sequence alignment and phylogenetic analysis

The consensus sequences of the VP4 and VP7 genes were cleaned and the nucleotide sequences were submitted to GenBank for a BLASTN search (http://blast.ncbi.nlm.nih.gov) on the National Center for Biological Information website. They were accurately aligned and analysed manually using ChromasPro (School of Health Science, Griffith University Australia) version 1.49. This created alignments of the edited nucleotides and deduced amino acid sequences and compared them with a selection of reference strains from the GenBank database using BioEdit software packages (Hall, 1999). The genetic relatedness of the VP4 and VP7 sequences of rotavirus strains were determined by constructing a phylogenetic tree using the neighbour joining and clustering methods that was performed with MEGA software version 7 (Kamura et al., 2011). The phylogenetic genetic distances between genotypes were measured by the Kimura two-parameter model and the phylogenetic trees were statistically supported by bootstrapping using replicate data sets of 1000.

CHAPTER 3

3.0 RESULTS

3.1 Demographics

Two hundred (200) diarrhoeal samples were collected between 2015 and 2017 during the winter and the summer seasons. The collection areas were randomly selected around the rural areas/ farms of Mafikeng and its surroundings. Samples were collected from calves of three (3) months or less presenting with diarrhoea.

3.2 Overview of results

Table 3.1: Total number of samples collected and tested for the presence of Rotavirus using the above techniques.

Methods	Positive	Negative	Total	%
Vikia Rota-Adeno	36	164	200	18
EIA	0	50	50	0
EM	1	5	6	16
PAGE	2	48	50	4
RT-PCR	2	48	50	4
Genotype	1	1	2	50%
Sequencing	1	1	2	50%

3.3 Screening Results

3.3.1 Vikia Rota-Adeno Kit

200 samples were screened for the presence of rotavirus antigen using the Vikia Rota-Adeno test kit (Figure 3.1). From the 200 samples, 36 (18%) samples were positive and 164/200 (82%) samples tested negative (Table 3.1). However, 14 samples from the 164 negative samples were slightly positive/equivocal (very faint reaction).

Figure 3.1: *Examples of used Vikia Rota-Adeno test kit*

3.3.2 Enzyme Immunoassay (EIA)

From the 200 screened samples, only 50 (i.e. 36 Rotavirus Vikia positive and 14 equivocal) samples were further subjected to the EIA testing. Out of the total of 54 samples including 4 control samples (2 negative and 2 positive controls), 50/50 (100%) samples were found negative for rotavirus (Table 3.3).

3.3.3 Transmission Electron Micrcoscopy (TEM)

A total of 20 samples which were randomly selected from positive Vikia Rota-Adeno test were sent to North-West University Potchefstroom Campus for electron microscopy (EM) courtesy of Dr A Jordaan EM Unit. However only 6 samples were prepared and viewed under the TEM due to time constraint4s. Only 1/6 (16%) samples showed rotaviral-like particles.

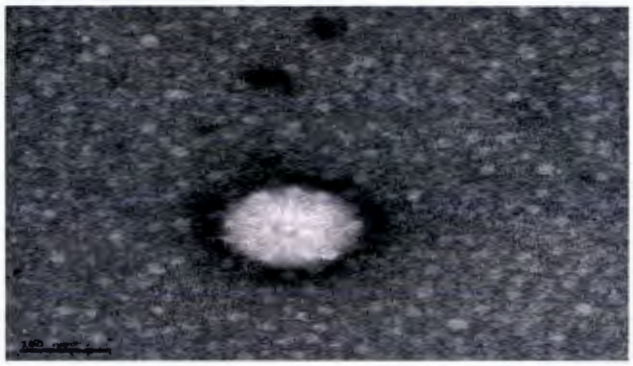

Figure 3.2 *Electron micrographs of viral particles in diarrheic samples from calves*

3.3.4 Polyacrilamide Gel Electrophoresis (PAGE)

A total of 50 (36 vikia positive and 14 slightly positive) samples were subjected to PAGE analysis. Two electropherotypes were detected in this study. Two (4%) similar electropherotypes observed showing RNA migration patterns labeled as C6 and 101 (well 7 and 12). However, 96% (48/50) of the samples had no visible RNA segments by PAGE (Figure 3.4).

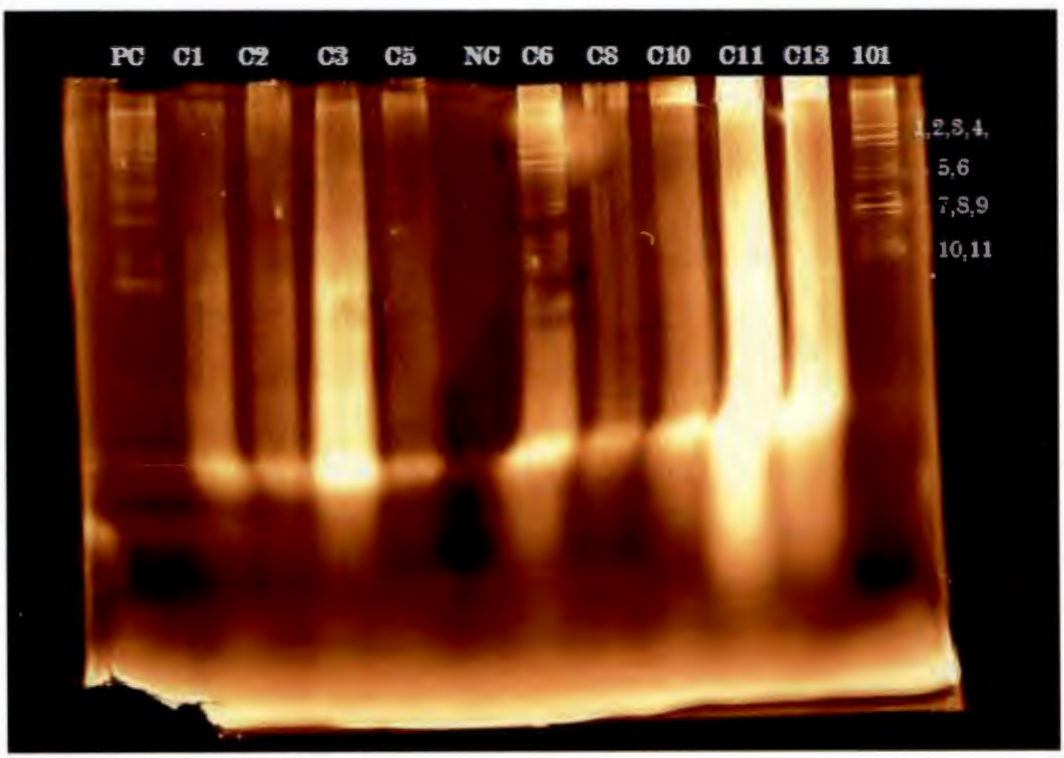

Figure 3.3 *Electropherotypic pattern of representative bovine rotavirus isolates from faecal samples of the diarrhoeic calves*

3.4 Polymerase Chain Reaction

3.4.1 Amplification using Reverse Transcriptase-Polymerase Chain Reaction (VP4 and VP7)

A total of 2/50 (4%) specimens were positive for a gene encoding VP4 protein and 48/50 (96%) could not be amplified following RT-PCR assay. Two (2) out 50 (4%) samples were positive for the VP7 gene and 48/50 (96%) samples could not be amplified. This could have been as a result of low viral load as was indicated in the electrophoresis gel for extracted material (Figure 3.3). The VP4 and VP7 results are shown in figure 3.4 and figure 3.6.

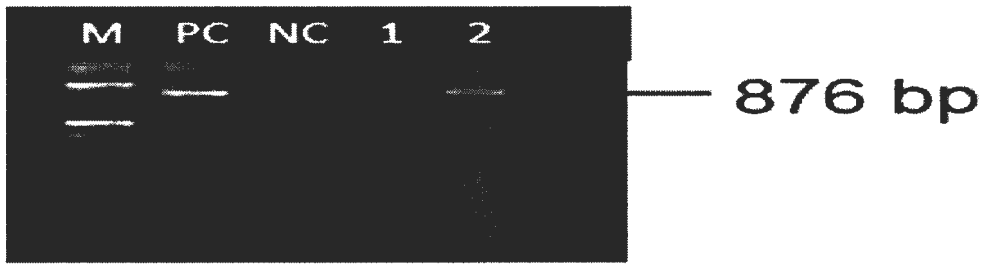

Figure 3.4 *The amplification product of rotavirus VP4 RT-PCR (876 bp) on 1% agarose gel electrophoresis with ethidium bromide staining, molecular weight size base pair marker (100bp).*

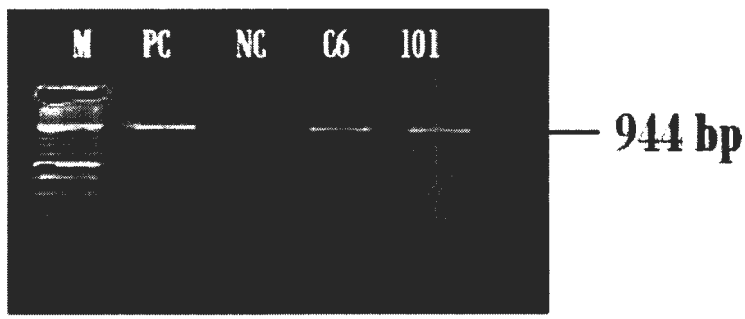

Figure 3.5 *The amplification product of rotavirus VP7 RT-PCR (944 bp) on 1% agarose gel electrophoresis with ethidium bromide staining, molecular weight size base pair marker (100bp)*

3.4.2 Genotyping using Multiplex PCR (Rotavirus P and G Genotypes)

RT-PCR was performed to amplify genes encoding the outer capsid VP4 and VP7 proteins of group A rotavirus. A multiplex PCR was performed to determine the rotavirus G and P genotypes. Only C6 was successfully genotyped for both VP4 and VP7 genes. Sample 101 was blank (figure 3.7 and figure 3.8).

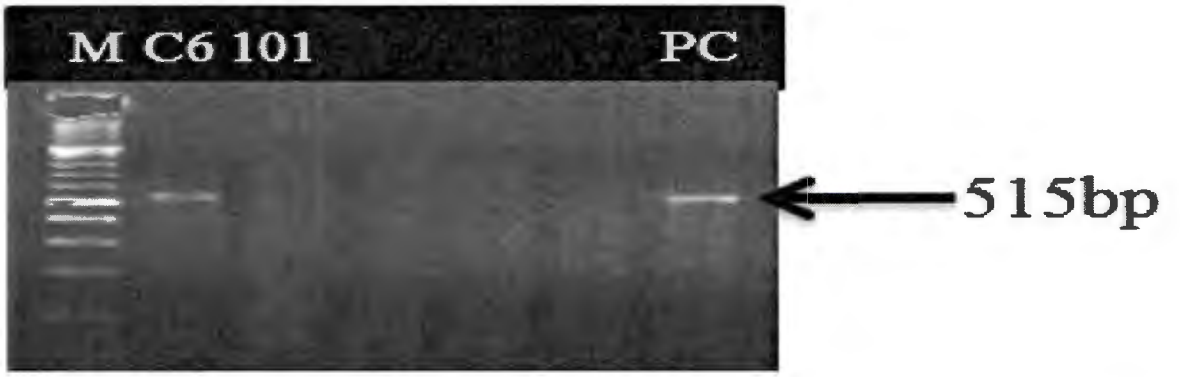

Figure 3.6 *The amplification product of rotavirus VP4 (515 bp) genotyping on 2% agarose gel electrophoresis with ethidium bromide staining, molecular weight size base pair marker (100bp). PCR product of 515 bp size was observed as P11 genotype only for the C6 sample and sample 101 was blank.*

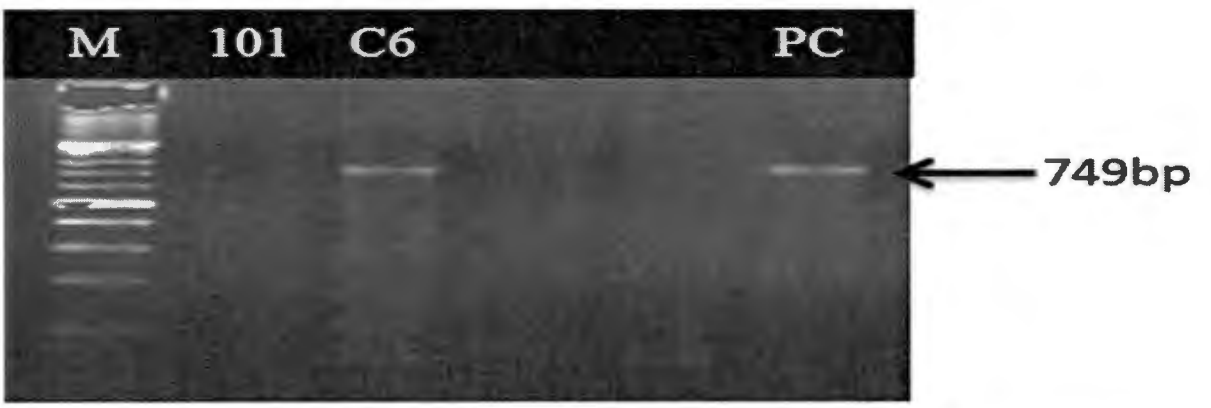

Figure 3.7 *The amplification product of rotavirus VP7 genotyping on 2% agarose gel electrophoresis with ethidium bromide staining, molecular weight (100bp). PCR product of 749 bp size was observed as G10 genotype only for the C6 sample and sample 101 was blank.*

3.4.3 Genotyping by Sequence Analysis of VP4 and VP7

The 2 samples, C6 and 101 which were RT-PCR positive (first round) were sent to Inqaba Biotech for sequencing. Of the 2 samples, only one sample was successfully sequenced namely 101-VP4. The 101 sample was further confirmed to be 99% similar to G12P8 strain through the National Centre for Biological Information (NCBI).

CHAPTER 4

4.0 DISCUSSION AND CONCLUSION

4.1 Discussion

4.1.1 Overview of the Study

Rotavirus is zoonotic, and therefore there are some strains that affect both humans and animals. This poses a serious health risk to humans and severe gastroenteritis in young animals. Infection of calves with this virus results in substantial economic loss due to increased mortality, treatment costs and reduced. It is therefore important that the health of livestock remains good and positive especially in cattle as the livelihood of both the commercial and subsistence farmers depends on cattle farming. There is a need to investigate the presence of rotavirus from diarrhoeal stools of calves from rural areas. There is thus this gap in knowledge, identified in most of the rural areas of South Africa.

In this study 200 diarrhoeal samples were collected from calves below the age of three (3) months in the rural areas of Mafikeng. Collection was done in different seasons i.e. in winter and summer. Out of the 200 diarrhoeal samples colle.lcted, 108 (54%) samples were from male calves and the remaining 92 (46%) were from female calves. According to Dash *et al.* (2011) male bovines are more susceptible to rotavirus compared to female bovines.

The screening methods used included the Vikia Rota-Adeno test kit, enzyme immunoassay, electron microscopy and the polyacrylamide gel electrophoreses (PAGE). While the Polymerase Chain Reaction (PCR) and the Sequencing (Sanger) methods were applied for confirmation and for typing purposes.

The Vikia Rota-Adeno kit was able to detect 36/200 (18%) positive samples and 14 of the 200 samples were equivocal. EIA was carried out on the 50 samples (36 from Vikia positive and 14 slightly positive samples) and there were no positive samples detected. From the same 50 positive and slightly positive samples only 2/50 (4%) were positive and showed the presence of the 11 segmented genome (characteristics of rotavirus) when tested by PAGE (figure 3.4). The 50 samples were further subjected to RT-PCR and only 2 (4%) were confirmed positive (samples C6 and 101). The two samples were genotyped using multiplex RT-PCR and genotype G10P11 was identified only for the C6 sample. The 2 samples were also taken for sequencing and only sample 101 was successfully sequenced and the sequence data obtained in the present study revealed 99% similarity to G12P[8].

From the 50 samples that were subjected to molecular techniques, the study reports an overall of 1% (2/200) samples confirmed for rotavirus infection, however further studies using a larger sample size are recommended in order to compare well with other studies conducted in different parts of the world and thus be able to come up with an informed conclusion. Studies conducted globally have shown a varied rotavirus prevalence among calves e.g. a study done in Netherlands in 2007 reported a 30.9% rotavirus infection (Bartels *et al.*, 2010), while Australia reported 79.9% (Izzo *et al.*, 2011), Sweden reported 47% rotavirus infection (De Verd Er, 2006) and Switzerland reported a 58.7% prevalence rate (Uhde *et al.*, 2008) (fig 3.1)

4.1.2 Screening Processes

4.1.2.1 Vikia Rota-Adeno

In this present study the first objective was to screen for the presence of rotavirus from diarrhoeal stools of calves collected in rural areas using Vikia Rota-adeno test kit and Enzyme immunoassay. The Vikia Rota-Adeno test kit is an immuno-chromatograph test for detecting fast and simultaneously rotavirus and adenovirus infections from the samples of stools (De Rougemont *et al.*, 2009). Out of the 200, only 36 (18%) samples were presumptively positive for rotavirus according to the kit's description (Figure 3.1). According to studies conducted by De Rougemont *et al.* (2009) and Bon *et al.* (2006) it was shown that the Vikia Rota-Adeno test kit is reliable as shown by its 100% sensitivity and specificity for rotavirus detection. From the 36 samples that tested positive using Vikia Rota-Adeno plus another 14 randomly selected samples to make 50 were subjected to EIA and were all negative and were also subjected to PAGE and RT-PCR to check the validity of the Vikia Rota-Adeno test. Only 2 samples (C6 and 101) were positive in both techniques. However a study conducted by Ye *et al.* (2013) raised concerns about the specificity of the Vikia Rota-Adeno test were many samples were positive with this test but few remain positive when using other molecular techniques, that's why it is important not to rely one technique.

4.1.2.2 Enzyme Immunoassay

EIA is widely used because it provides for the rapid detection of rotavirus antigen in a relatively short period of time and it is also sensitive and specific for the detection of rotavirus antigen in faeces (Knisley *et al.*, 1986). From the 200 Vikia screened samples, only 50 (i.e. 36 Vikia positive and 14 randomly selected slightly positive) samples were further subjected to the EIA testing. All the 50 samples tested negative (Table 3.2). A sample was diagnosed negative EIA while it was positive on Vikia Rota-Adeno test, which could be explained by the presence of virus particles in very small quantities in the sample

or by the freeze-thaw cycle of the stools that can affect the samples by degrading or interfering with the reactivity of the antigens. The samples were further taken for PAGE and RT-PCR which proved to be more sensitive and accurate.

4.1.2.3 Electron Microscopy

The second objective was to screen samples using Electron Microscopy. The electron microscope is valuable due to the fact that it gives rapid and definitive results and it also has the advantage of being able to detect other viruses present in the stools that may have a causal role in producing gastroenteritis. There is a necessity for rapid and simple diagnostic techniques for the viral agents of gastroenteritis e.g. the rotavirus (De Beer *et al.*, 1997). Therefore in our study we used the transmission electron microscope (TEM) to study the morphology of rotavirus. Twenty (20) diarrhoeal samples, screened positive for the rotavirus using the Vikia-Rota-Adeno kit, were sent to the Electron Microscope Unit, North-West University, Potchefstroom Campus. However, only 6 samples, randomly selected, were prepared for TEM analysis (courtesy of Dr A Jordaan). Of the 6 samples analyzed only one (sample 101) showed rotaviral-like particles (Figure 3.2). The picture of sample 101 shows the circle shape of the virus which was named rotavirus which was translated from a Latin word "wheel" because of its closeness appearance of a wheel and the structure of the virion has a diameter of 100nm. The structure is spiral-like outside although it's not very clear. Sample 101 was further taken for other technique to prove if it's rotavirus and it was proven to be end.

4.1.2.4 Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis proved in previous studies to be a practical and highly sensitive technique for rapid diagnosis of rotavirus infections and distribution of strains for different genome profile. According to Tewari *et al.* (2012) It has some advantages over other diagnostic techniques. Therefore our third objective of the study was to determine the rotavirus electropherotypes. The 50 samples were analysed using polyacrylamide gel electrophoresis. 2/50 (i.e, C6 and sample 101) samples tested positive with a migration pattern of 4-2-3-2 suggesting group A rotavirus (Tewari *et al.*, 2012). The migration patterns were classified as short electrophotypes which means there was a slow migration of segment 10 and 11 (Basera *et al.*, 2010).

4.1.3.1 Amplification using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

PCR is a more sensitive and specific technique used to establish the genetic diversity of VP4 and VP7. It also serves as a reliable confirmatory tool in providing information of circulating rotavirus strains. PCR sensitivity is about 93% as compared to 82% observed

in EIA techniques (Gouvea *et al.*, 1990), and therefore the fourth objective of this study was to confirm screened positive samples using RT-PCR. In our investigation, out of 200 samples amplified using specific primers, con2, Con3/, sBeg9 and EndA, only 2 (1%) samples were positive for the VP4 and VP7 gene (Figure 3.4 and 3.5). Therefore, the 2 samples (C6 and sample 101) in this present study have been confirmed as true positives.

4.1.3.2 VP4 and VP7 Genotyping by Polymerase Chain Reaction (RT-PCR)

Two samples (C6 and sample 101) were genotyped using nested-Multiplex PCR with type-specific primers (Table 2.1 and 2.2). Only one sample, namely, C6 was successfully genotyped as G10 and P11 type using Multiplex PCR (Figure 3.7 and 3.8). Therefore, we can conclude, though with caution, that is the presence of rotavirus strain P11[G10]. However, a bigger study is required to confirm or refute the findings. The P11[G10] genotype strain is an important genotype of group A *BRV* because of its zoonotic transmission from humans to cattle as well as from cattle to humans, as reported by (Iturriza-Gómara *et al.*, 2004).

4.1.3.3 Sequencing

The final objective of the study was to genotype positive samples using sequencing (Sanger) method. Two rotavirus RT-PCR positive samples i.e. C6 and sample 101, were sent to Inqaba Biotec for sequencing mainly to confirm the C6 sample genotype and to type sample 101 which we could not type using nested-Multiplex PCR and to construct phylogenetic trees in order to study strain relatedness. From the sequences obtained we determined that sample 101 is a G12[P8] strain. This particular strain is common in humans and therefore also an indication for zoonosis.

4.1.3.3.1 Phylogenetic Analysis

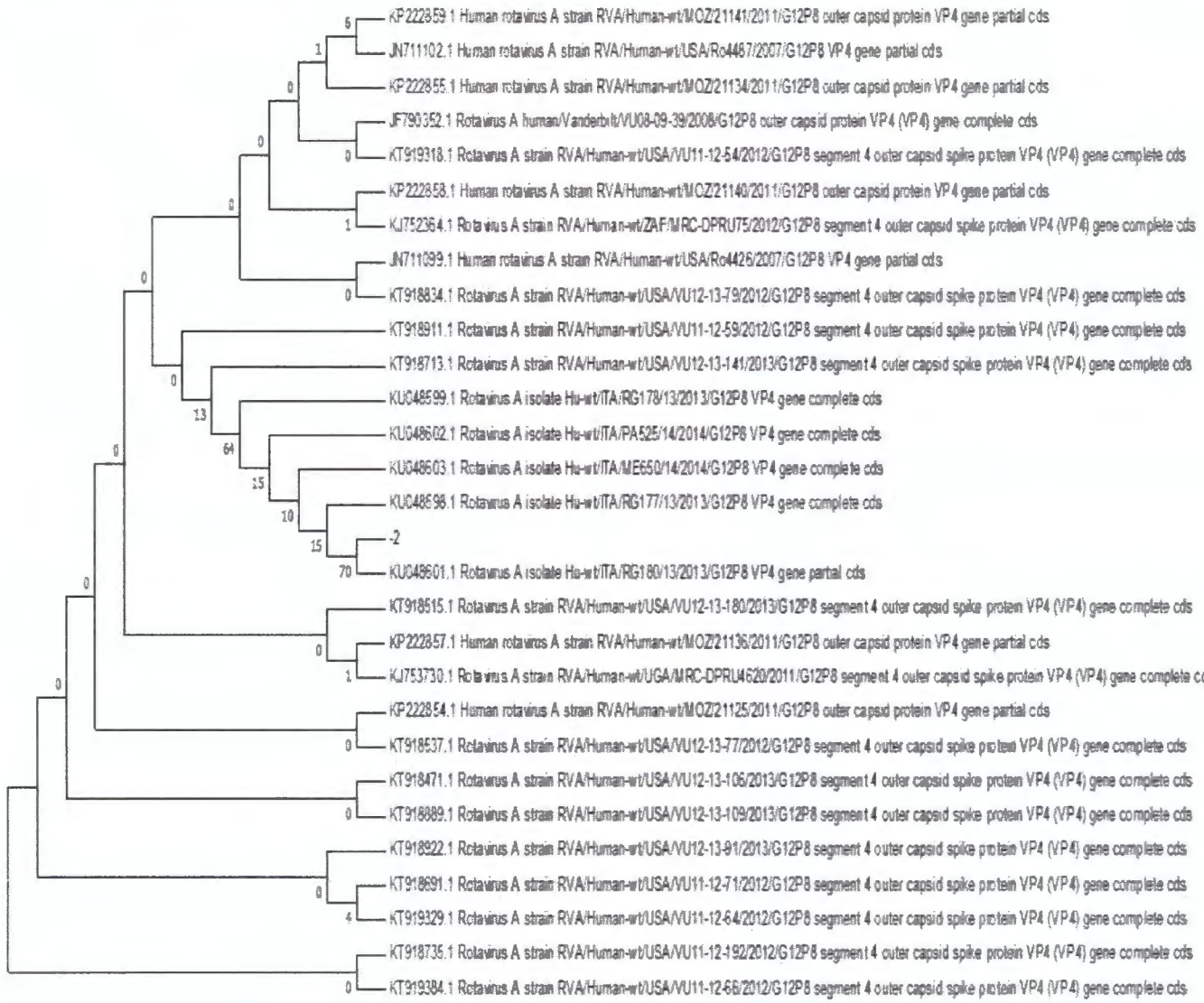

Figure 4.1: *Rotavirus VP4 G12 strains phylogenetic tree displaying the relationship between strains detected in the study and the global reference strains. The strains found in Mafikeng is the one in the tree (101-VP4)*

Sample 101 was accurately aligned and analysed manually using ChromasPro (School of Health Science, Griffith University Australia) version 1.49. This created alignments of the edited nucleotides and deduced amino acid sequences and compared them with a selection of reference strains from the GenBank database using BioEdit software packages (Hall, 1999). The genetic relatedness of the VP4 and VP7 sequences of rotavirus strains were determined by constructing a phylogenetic tree using the neighbour joining and clustering methods that was performed with MEGA software version 7 (Kamura et al., 2011). The phylogenetic genetic distances between genotypes were measured by the Kimura two-parameter model and the phylogenetic trees were statistically supported by bootstrapping using replicate data sets of 1000.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou *et al,.*1987). The bootstrap consensus tree inferred from 1000 replicates [2] is taken to represent the evolutionary history of the taxa analyzed (Felsenstein *et al.*, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Saitou *et al,.*1987). The evolutionary distances were computed using the Kimura 2-parameter method [3] and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 29 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 317 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.,* 2016).

4.2 Conclusion

In conclusion, the objectives of the study have been fully met and we can conclude, however, with caution, there is the presence of rotavirus in the rural areas of Mafikeng. This is a very small percentage so a larger research scale needs to be done in order to know the status of rotavirus in Mafikeng. Vikia Rota-Adeno test detected 18% of rotavirus and the percentage was narrowed down when coming to other techniques. Only 2 samples were found positive on PAGE and RT-PCR might mean that the results found on Vikia Rota-Adeno might be false positive or might be the presence of virus particles in very small quantities in the sample or by the freeze-thaw cycle of the stools that can affect the samples by degrading or interfering with the reactivity of the antigens. Genotyping and sequencing was performed on the 2 samples to identify the strains. Sample C6 proved to be P11G[10] and sequence data obtained for sample 101 in the present study revealed 99% similarity to strain G12P[8] in the Genbank.

4.2.1 Recommendations

Rural farmers especially in remote areas around Mafikeng are mostly with limited education and with limted resources, thus managing their livestock properly, maintaining good hygienic standards or even vaccinating their animals is quite a challenge. There is also an under reporting of diarrhoeal cases among calves in these areas and therefore rotavirus cases might be missed. The results emanating from this study might not be alarming, but there is therefore a great need to educate rural farmers about this pathogen and its implications to their livestock and also to educate them about the importance of reporting any diarrhoeal cases from their calves to the nearest veterinary stations.

Secondly, there is a need for mobile Veterinary Clinics to reach out to the most remote rural areas in order to assist with good animal health management to avoid transmission of zoonotic diseases.

Finally, there is currently very few or no studies published on molecular prevalence of rotavirus among calves in the country to refer to, and therefore there is a need to conduct cross sectional studies for surveillance purposes.

4.2.2 The Limitations of this study

- Limited number of diarrheoal samples from calves
- Time constraint as optimizing techniques took a long time and
- Study proved to be very expensive and difficult.
- Difficult to convince farmers to collect samples from their livestock
- Optimization of the methods because of the low detection rate

REFERENCES

Adah, M. I., Nagashima, S., Wakuda, M. and Taniguchi, K. 2003. Close relationship between G8-serotype bovine and human rotaviruses isolated in Nigeria. *Journal of clinical microbiology,* 41, 3945-3950.

Bartels, C. J., Holzhauer, M., Jorritsma, R., Swart, W. A. and Lam, T. J. 2010. Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. *Preventive veterinary medicine,* 93, 162-169.

Basera, S. S., Singh, R., Vaid, N., Sharma, K., Chakravarti, S. and Malik, Y. 2010. Detection of rotavirus infection in bovine calves by RNA-PAGE and RT-PCR. *Indian Journal of Virology,* 21, 144-147.

Bayard V, DeAntonio R, Contreras R, Tinajero O, Castrejon MM, Ortega-Barrı R,

Colindres. 2012. Impact of rotavirus vaccination on childhood gastroenteritis-related mortality and hospital discharges in Panama. International Journal of Infectious Disease; 16:e94–8

Bernstein, D. I. 2009. Rotavirus overview. *The Pediatric infectious disease journal,* 28, S50-S53.

Bishop, R. 1996. Natural history of human rotavirus infection. *Viral Gastroenteritis.* Springer.

Bishop, R., Davidson, G., Holmes, I. and Ruck, B. 1973. Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. *The Lancet,* 302, 1281-1283.

Bohl, E. H., Saif, L. J., Theil, K., Agnes, A. and Cross, R. 1982. Porcine pararotavirus: detection, differentiation from rotavirus, and pathogenesis in gnotobiotic pigs. *Journal of clinical microbiology,* 15, 312-319.

Bon, F., Kaplon, J., Metzger, M.-H. and Pothier, P. 2006. Evaluation of seven immunochromatographic assays for the rapid detection of human rotaviruses in fecal specimens. *Pathologie Biologie,* 55, 149-53.

Bresee JS, Glass RI, Iyanoff B, Gentsch JR. (1999). Current status and future priorities for rotavirus vaccine development, evaluation and implementation in developing countries. *Vaccine.* **17:** 2207-2222.

Bresee, J. S., Hummelman, E., Nelson, E. and Glass, R. I. 2005. Rotavirus in Asia: the value of surveillance for informing decisions about the introduction of new vaccines. *Journal of Infectious Diseases,* 192, S1-S5.

Bridger, J. C. 1987. Novel rotaviruses in animals and man. *Novel Diarrhoea Viruses, Ciba Found. Sympos,* 128, 5-23.

Bridger, J. C., Woode, G., Jones, J. M., Flewett, T., Bryden, A. and Davies, H. 1975. Transmission of human rotaviruses to gnotobiotic piglets. *Journal of medical microbiology,* 8, 565-569.

Brüssow, H., Nakagomi, O., Gerna, G. and Eichhorn, W. 1992. Isolation of an avianlike group A rotavirus from a calf with diarrhea. *Journal of clinical microbiology,* 30, 67-73.

Butz, A. M., Fosarelli, P., Dick, J., Cusack, T. and Yolken, R. 1993. Prevalence of rotavirus on high-risk fomites in day-care facilities. *Pediatrics,* 92, 202-205.

Caruzo, T. a. R., Brito, W. M. E. D. D., Munford, V. and Rácz, M. L. 2010. Molecular characterization of G and P-types bovine rotavirus strains from Goiás, Brazil: high frequency of mixed P-type infections. *Memórias do Instituto Oswaldo Cruz,* 105, 1040-1043.

Castrucci, G., Frigeri, F., Ferrari, M., Cilli, V., Aldrovandi, V., Caleffi, F. and Gatti, R. 1984. Comparative study of rotavirus strains of bovine and rabbit origin. *Comparative immunology, microbiology and infectious diseases,* 7, 171-178.

Castrucci, G., Frigeri, F., Ferrari, M., Cilli, V., Aldrovandi, V., Gatti, R. and Rampichini, L. 1985. A comparison of rotavirijs strains of bovine, simian and porcine origin. *European journal of epidemiology,* 1, 274-280.

CDC. 2008. Rotavirus surveillance: worldwide, 2001–2008. Morbidity and Mortality WeeklyReport, 57, 1255–1257.

Chauhan, R. and Singh, N. 1996. Epidemiology of rotavirus infection in calves in India. *International Journal of Animal Sciences,* 11, 221-223.

Chen, G.-M., Hung, T., Bridger, J. and Mccrae, M. 1985. Chinese adult rotavirus is a group B rotavirus. *The Lancet,* 326, 1123-1124.

Cook, N., Bridger, J., Kendall, K., Gomara, M. I., El-Attar, L. and Gray, J. 2004. The zoonotic potential of rotavirus. *Journal of Infection,* 48, 289-302.

Cook, S., Glass, R., Lebaron, C. and Ho, M.-S. 1990. Global seasonality of rotavirus infections. *Bulletin of the World Health Organization,* 68, 171.

Cunliffe, N. A., Kilgore, P. E. and Breese, J. S. 1998. Epidemiology of rotavirus diarrhoea in Africa: a review to assess the need for rotavirus immunization. Bulletin of the World HealthOrganization, 525-537.

Dash, S., Tewari, A., Kumar, K., Goel, A. and Bhatia, A. 2011. Detection of Rotavirus from diarrhoeic cow calves in Mathura, India. *Veterinary World,* 4, 554-556.

De Beer, M., Peenze, I., Mendes, V. D. C. and Steele, A. 1997. Comparison of electron microscopy, enzyme-linked immunosorbent assay and latex

agglutination for the detection of bovine rotavirus in faeces. *Journal of the South African Veterinary Association,* 68, 93-96.

De Grazia, S., Martella, V., Giammanco, G. M., Gòmara, M. I., Ramirez, S., Cascio, A., Colomba, C. and Arista, S. 2007. Canine-origin G3P [3] rotavirus strain in child with acute gastroenteritis. *Emerg Infect Dis,* 13, 1091-1093.

De Rougemont, A., Kaplon, J., Billaud, G., Lina, B., Pinchinat, S., Derrough, T., Caulin, E., Pothier, P. and Floret, D. 2009. Sensitivity and specificity of the VIKIA Rota-Adeno immuno-chromatographic test (bioMerieux) and the ELISA IDEIA Rotavirus kit (Dako) compared to genotyping. *Pathologie-biologie,* 57, 86-89.

De Verd Er, K. 2006. Infektionspanoramat vid diarreer hos svenska kalvar. *SVENSK VETERINARTIDNING,* 58, 29.

Desselberger, U., Iturriza-Gómara, M. and Gray, J. J. Rotavirus epidemiology and surveillance. Gastroenteritis Viruses: Novartis Foundation Symposium 238, 2001. Wiley Online Library, 125-152.

Dhama, K., Chauhan, R., Mahendran, M. and Malik, S. 2009. Rotavirus diarrhea in bovines and other domestic animals. *Veterinary research communications,* 33, 1-23.

Donovan, G. A., Dohoo, I. R., Montgomery, D. M. and Bennett, F. L. 1998. Calf and disease factors affecting growth in female Holstein calves in Florida, USA. *Preventive veterinary medicine,* 33, 1-10.

Estes, M. K., Palmer, E. L. and Obijeski, J. F. 1983. Rotaviruses: a review. *Current topics in microbiology and immunology.* Springer.

Estes, M. K. and Cohen, J. 1989. Rotavirus gene structure and function. *Microbiological reviews,* 53, 410-449.

Estes MK. Rotaviruses and their replication. 2001. In: Howley PM, ed. Fields virology. 4th ed. Vol. 2. Philadelphia: Lippincott, Williams & Wilkins,:1747–86.

Estes, M. and Greenberg, H. 2013. Rotaviruses In: Knipe DM, Howley PM, editors.

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Fields Virology. Philadelphia: Lippincott Williams & Wilkins.

Gentsch, J., Glass, R., Woods, P., Gouvea, V., Gorziglia, M., Flores, J., Das, B. and Bhan, M. 1992. Identification of group A rotavirus gene 4 types by polymerase chain reaction. *Journal of Clinical Microbiology,* 30, 1365-1373.

Gentsch, J. R., Laird, A. R., Bielfelt, B., Griffin, D. D., Bányai, K., Ramachandran, M., Jain, V., Cunliffe, N. A., Nakagomi, O. and Kirkwood, C. D. 2005. Serotype diversity and

reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs. *Journal of Infectious Diseases,* 192, S146-S159.

Iturriza-Gómara, M, Cubitt, D., Desselberger, U. and Gray, J. 2001. Amino acid substitution within the VP7 protein of G2 rotavirus strains associated with failure to serotype. *Journal of clinical microbiology,* 39, 3796-3798.

Iturriza-Gómara, M, Kang, G., Mammen, A., Jana, A. K., Abraham, M., Desselberger, U., Brown, D. and Gray, J. 2004. Characterization of G10P [11] rotaviruses causing acute gastroenteritis in neonates and infants in Vellore, India. *Journal of clinical microbiology,* 42, 2541-2547.

Gouvea, V., Glass, R. I., Woods, P., Taniguchi, K., Clark, H. F., Forrester, B. and Fang, Z. 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *Journal of clinical microbiology,* 28, 276-282.

Gray J, Vesikari T, Van Damme P, C, G., Mrukowicz J, Guariano A, Dagan R, Szajewska H and V., U. 2008. Rotavirus. *Journal pediatric gastroenterology nutrition.*, 2: 24-31.

Greenberg, H. B., Flores, J., Kalica, A. R., Wyatt, R. G. and Jones, R. 1983. Gene coding assignments for growth restriction, neutralization and subgroup specificities of the W and DS-1 strains of human rotavirus. *Journal of General Virology,* 64, 313-320.

Gumusova, S. O., Yazici, Z., Albayrak, H. and Meral, Y. 2007. Rotavirus and coronavirus prevalence in healthy calves and calves with diarrhea. *Medycnya Weterinaria,* 63, 62-64.

Heinrich, H., Schirrmeier, H., Lange, E. and Granzow, H. 1983. Cross infection with rotavirus of different species. *Archiv für experimentelle Veterinärmedizin,* 37, 55.

Herring, A., Inglis, N., Ojeh, C., Snodgrass, D. A. and Menzies, J. 1982. Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. *Journal of Clinical Microbiology,* 16, 473-477.

Holland, R. E. 1990. Some infectious causes of diarrhea in young farm animals. *Clinical microbiology reviews,* 3, 345-375.

Hoshino, Y. and Kapikian, A. Z. 1994. Rotavirus vaccine development for the prevention of severe diarrhea in infants and young children. *Trends in microbiology,* 2, 242-249.

Hull, J. J., Teel, E. N., Kerin, T. K., Freeman, M. M., Esona, M. D., Gentsch, J. R., Cortese, M. M., Parashar, U. D., Glass, R. I. and Bowen, M. D. 2011. United States rotavirus strain surveillance from 2005 to 2008: genotype prevalence before and after vaccine introduction. *The Pediatric infectious disease journal,* 30, S42-S47.

Izzo, M., Kirkland, P., Mohler, V., Perkins, N., Gunn, A. and House, J. 2011. Prevalence of major enteric pathogens in Australian dairy calves with diarrhoea. *Australian veterinary journal,* 89, 167-173.

Jakobsson, S. 2013. Diagnosis and molecular epidemiology of bovine rotavirus and coronarvirus in Brazil.

King, A. M. 2012. *Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses*, Elsevier.

Knisley, C. V., Bednarz-Prashad, A. and Pickering, L. 1986. Detection of rotavirus in stool specimens with monoclonal and polyclonal antibody-based assay systems. *Journal of clinical microbiology,* 23, 897-900.

Kumar S., Stecher G., and Tamura K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets.Molecular Biology and Evolution 33:1870-1874.

Kumar S, Tamura K, Peterson D, Peterson N, , Nei M and Stecher G (2011) MEGA 5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol Biol Evol.* 28: 2731-2739.

Langa, J. S., Thompson, R., Arnaldo, P., Resque, H. R., Rose, T., Enosse, S. M., Fialho, A., De Assis, R. M. S., Da Silva, M. F. M. and Leite, J. P. G. 2016. Epidemiology of rotavirus A diarrhea in Chókwè, Southern Mozambique, from February to September, 2011. *Journal of medical virology,* 88, 1751-1758.

Lauretti, F., De Melo, F. L., De Mello Volotão, E., Santos, N., Linhares, R. E. C. and Nozawa, C. 2003. Use of acridine orange staining for the detection of rotavirus RNA in polyacrylamide gels. *Journal of virological methods,* 114, 29-35.

Levy, K., Hubbard, A. E. and Eisenberg, J. N. 2009. Seasonality of rotavirus disease in the tropics: a systematic review and meta-analysis. *International journal of epidemiology,* 38, 1487-1496.

Lyoo, Y. S. 1995. Expression and characterization of bovine rotavirus proteins in the baculovirus system.

Maes, P., Matthijnssens, J., Rahman, M. and Van Ranst, M. 2009. RotaC: a web-based tool for the complete genome classification of group A rotaviruses. *BMC microbiology,* 9, 1.

Malik, S., Barbuddhe, S., Rawool, D., Vaidya, V. and Sahare, A. 2005. Data sheet on Rotaviruses (Global status of Rotavirus infections in man and animals). *Animal Health and Production Compendium, CAB International, Wallingford, UK.*

Malik, Y. S., Sharma, K., Vaid, N., Chakravarti, S., Chandrashekar, K., Basera, S. S., Singh, R., Prasad, G., Gulati, B. R. and Bhilegaonkar, K. N. 2012. Frequency of

group A rotavirus with mixed G and P genotypes in bovines: predominance of G3 genotype and its emergence in combination with G8/G10 types. *Journal of veterinary science,* 13, 271-278.

Manish M Patel MD Roger Glass PhD, Rishi Desai MD, Jacqueline E, Tate PhD, Umesh D, Parashar MBBS. 2012. Fulfilling the promise of rotavirus vaccines: how far have we come since licensure. Infectious Disease Volume 12, Issue 7, July 2012, Pages 561-570

Mascarenhas, J. D. a. P., Leite, J. P. G., Lima, J. C., Heinemann, M. B., Oliveira, D. S., Araujo, I. T., Soares, L. S., Gusmao, R. H. P., Gabbay, Y. B. and Linhares, A. C. 2007. Detection of a neonatal human rotavirus strain with VP4 and NSP4 genes of porcine origin. *Journal of medical microbiology,* 56, 524-532.

Matthijnssens, J., Ciarlet, M., Heiman, E., Arijs, I., Delbeke, T., Mcdonald, S. M., Palombo, E. A., Iturriza-Gómara, M., Maes, P. and Patton, J. T. 2008. Full genome-based classification of rotaviruses reveals a common origin between human Wa-Like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. *Journal of virology,* 82, 3204-3219.

Matthijnssens, J., Ciarlet, M., Mcdonald, S. M., Attoui, H., Bányai, K., Brister, J. R., Buesa, J., Esona, M. D., Estes, M. K. and Gentsch, J. R. 2011. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). *Archives of virology,* 156, 1397-1413.

Mattion, N., Cohen, J. and Estes, M. 1994. The rotavirus proteins. *Viral infections of the gastrointestinal tract,* 169-249.

Mcnulty, M., Allan, G., Todd, D., Mcferran, J. and Mccracken, R. 1981. Isolation from chickens of a rotavirus lacking the rotavirus group antigen. *Journal of General Virology,* 55, 405-413.

Mcnulty, M., Todd, D., Allan, G., Mcferran, J. and Greene, J. 1984. Epidemiology of rotavirus infection in broiler chickens: recognition of four serogroups. *Archives of virology,* 81, 113-121.

Mebus, C., Underdahl, N., Rhodes, M. and Twiehaus, M. 1969. *Calf diarrhea (scours): reproduced with a virus from a field outbreak*, University of Nebraska USA.

Mebus, C., Wyatt, R. and Kapikian, A. 1977. Intestinal lesions induced in gnotobiotic calves by the virus of human infantile gastroenteritis. *Veterinary Pathology Online,* 14, 273-282.

Midgley, S. E., Bányai, K., Buesa, J., Halaihel, N., Hjulsager, C. K., Jakab, F., Kaplon, J., Larsen, L. E., Monini, M. and Poljšak-Prijatelj, M. 2012. Diversity and zoonotic potential of rotaviruses in swine and cattle across Europe. *Veterinary microbiology,* 156, 238-245.

Monini, M., Cappuccini, F., Battista, P., Falcone, E., Lavazza, A. and Ruggeri, F. 2008. Molecular characterization of bovine rotavirus strains circulating in northern Italy, 2003–2005. *Veterinary microbiology,* 129, 384-389.

Mori, Y., Sugiyama, M., Takayama, M., Atoji, Y., Masegi, T. and Minamoto, N. 2001. Avian-to-mammal transmission of an avian rotavirus: analysis of its pathogenicity in a heterologous mouse model. *Virology,* 288, 63-70.

Müller, H. and Johne, R. 2006. Rotaviruses: diversity and zoonotic potential--a brief review. *Berliner und Munchener tierarztliche Wochenschrift,* 120, 108-112.

Murphy, F. A., Gibbs, E. P. J., Horzinek, M. C. and Studdert, M. J. 1999. Reoviridae. In: F.A. Murphy, E.P.J.

Nakagomi, O. and Nakagomi, T. 1991. Genetic diversity and similarity among mammalian rotaviruses in relation to interspecies transmission of rotavirus. *Archives of virology,* 120, 43-55.

Nakagomi, O. and Nakagomi, T. 1993. Interspecies transmission of rotaviruses studied from the perspective of genogroup. *Microbiology and immunology,* 37, 337-348.

Nakagomi, O., Ohshima, A., Aboudy, Y., Shif, I., Mochizuki, M., Nakagomi, T. and Gotlieb-Stematsky, T. 1990. Molecular identification by RNA-RNA hybridization of a human rotavirus that is closely related to rotaviruses of feline and canine origin. *Journal of clinical microbiology,* 28, 1198-1203.

Nakagomi, T, Nakagomi, O. and Takahashi, Y. 2005. Incidence and burden of rotavirus gastroenteritis in Japan as estimated from a prospective sentinel hospital study. Journal ofInfectious Diseases, 106-110.

O'Ryan, M. L., Hermosilla, G. and Osorio, G. 2009. Rotavirus vaccines for the developing world. Current Opinion in Infectious Diseases, 22, 483-489.

Parashar, U. D., Gibson, C. J., Bresse, J. S. and Glass, R. I. 2006. Rotavirus and severe childhood diarrhea. Emerging Infectious Diseases, 12, 304-6.

Parashar, U. D., Hummelman, E. G., Bresee, J. S., Miller, M. A. and Glass, R. I. 2003. Global illness and deaths caused by rotavirus disease in children. Emerging Infectious Diseases, 9, 565- 72.

Pedley, S., Bridger, J., Brown, J. and Mccrae, M. 1983. Molecular characterization of rotaviruses with distinct group antigens. *Journal of general virology,* 64, 2093-2101.

Pedley, S., Bridger, J., Chasey, D. and Mccrae, M. 1986. Definition of two new groups of atypical rotaviruses. *Journal of general virology,* 67, 131-137.

Ramani, S. and Kang, G. 2007. Burden of disease & molecular epidemiology of group A rotavirus infections in India. *The Indian journal of medical research,* 125, 619.

Reidy, N., Lennon, G., Fanning, S., Power, E. and O'shea, H. 2006. Molecular characterisation and analysis of bovine rotavirus strains circulating in Ireland 2002–2004. *Veterinary microbiology,* 117, 242-247.

Rodger, S. M., Bishop, R. F. and Holmes, I. H. 1982. Detection of a rotavirus-like agent associated with diarrhea in an infant. *Journal of clinical microbiology,* 16, 724-726.

Rohwedder, A., Schütz, K. I., Minamoto, N. and Brüssow, H. 1995. Sequence analysis of pigeon, turkey, and chicken rotavirus VP8* identifies rotavirus 993/83, isolated from calf feces, as a pigeon rotavirus. *Virology,* 210, 231-235.

Saif, L. J., Bohl, E. H., Theil, K. W., Cross, R. F. and House, J. A. 1980. Rotavirus-like, calicivirus-like, and 23-nm virus-like particles associated with diarrhea in young pigs. *Journal of Clinical Microbiology,* 12, 105-111.

Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Sato, K., Inaba, Y., Shinozaki, T. and Matumoto, M. 1981. Neutralizing antibody to bovine rotavirus in various animal species. *Veterinary Microbiology,* 6, 259-261.

Schroeder, B., Street, J., Kalmakoff, J. and Bellamy, A. 1982. Sequence relationships between the genome segments of human and animal rotavirus strains. *Journal of virology,* 43, 379-385.

Schwers, A., Hoyois, P., Chappuis, G., Dagenais, L. and Pastoret, P. Propagation of bovine rotavirus by cats and dogs. Annales de Rechérches Vétérinaires, 1982. 303-308.

Shen, S., Burke, B. and Desselberger, U. 1994. Rearrangement of the VP6 gene of a group A rotavirus in combination with a point mutation affecting trimer stability. *Journal of virology,* 68, 1682-1688.

Simmonds, M. K., Armah, G., Asmah, R., Banerjee, I., Damanka, S., Esona, M., Gentsch, J. R., Gray, J. J., Kirkwood, C. and Page, N. 2008. New oligonucleotide primers for P-typing of rotavirus strains: Strategies for typing previously untypeable strains. *Journal of Clinical Virology,* 42, 368-373.

Snodgrass, D., Herring, A., Campbell, I., Inglis, J. and Hargreaves, F. 1984. Comparison of atypical rotaviruses from calves, piglets, lambs and man. *Journal of general virology,* 65, 909-914.

Steele A.D, Peenze I, De Beer MC, et al. Anticipating rotavirus vaccines: epidemiology and surveillance of rotavirus infection in South Africa. Vaccine 2003; 21: 354-360

Steele, A. D., Geyer, A. and Gerdes, G. H. 2004. Rotavirus infections. In: Coetzer, J.A.W. and Tustin, R.C.(eds), Infectious diseases of Livestock, Oxford University Press, Southern Africa,.1256–1264.

Stupka, J. A., Degiuseppe, J. I. and Parra, G. I. 2012. Increased frequency of rotavirus G3P [8] and G12P [8] in Argentina during 2008–2009: whole-genome characterization of emerging G12P [8] strains. *Journal of Clinical Virology,* 54, 162-167.

Svensson, L. 2000. Diagnosis of foodborne viral infections in patients. *International journal of food microbiology,* 59, 117-126.

Tate, J. E., Mutuc, J. D., Panozzo, C. A., Payne, D. C., Cortese, M. M., Cortes, J. E., Yen, C., Esposito, D. H., Lopman, B. A. and Patel, M. M. 2011. Sustained decline in rotavirus detections in the United States following the introduction of rotavirus vaccine in 2006. *The Pediatric infectious disease journal,* 30, S30-S34.

Tate J.E, Haynes A, Payne DC, Cortese MM, Lopman BA, Patel MM, Parashar UD. 2013. Trends in national rotavirus activity before and after introduction of rotavirus vaccine into the national immunization program in the United States, 2000 to 2012. Pediatr Infect Dis J. 2013 Jul;32(7):741-4. doi: 10.1097/INF.0b013e31828d639c.

Teodoroff, T. A., Tsunemitsu, H., Okamoto, K., Katsuda, K., Kohmoto, M., Kawashima, K., Nakagomi, T. and Nakagomi, O. 2005. Predominance of porcine rotavirus G9 in Japanese piglets with diarrhea: close relationship of their VP7 genes with those of recent human G9 strains. *Journal of clinical microbiology,* 43, 1377-1384.

Tewari, A., Dash, S. K., Jain, B. and Bhatia, A. K. 2012. Electrophoretic detection of bovine. *Journal of Animal Research: v,* 2, 67-71.

Theil, K., Mccloskey, C., Saif, L. J., Redman, D., Bohl, E., Hancock, D., Kohler, E. and Moorhead, P. 1981. Rapid, simple method of preparing rotaviral double-stranded ribonucleic acid for analysis by polyacrylamide gel electrophoresis. *Journal of clinical microbiology,* 14, 273-280.

Theil, K., Saif, L. J., Moorhead, P. and Whitmoyer, R. 1985. Porcine rotavirus-like virus (group B rotavirus): characterization and pathogenicity for gnotobiotic pigs. *Journal of clinical microbiology,* 21, 340-345.

Todd, D., Mcnulty, M. and Allan, G. 1980. Polyacrylamide gel electrophoresis of avian rotavirus RNA. *Archives of virology,* 63, 87-97.

Tsunemitsu, H., Saif, L. J., Jiang, B., Shimizu, M., Hiro, M., Yamaguchi, H., Ishiyama, T. and Hirai, T. 1991. Isolation, characterization, and serial

propagation of a bovine group C rotavirus in a monkey kidney cell line (MA104). *Journal of clinical microbiology,* 29, 2609-2613.

Uhde, F. L., Kaufmann, T., Sager, H., Albini, S., Zanoni, R., Schelling, E. and Meylan, M. 2008. Prevalence of four enteropathogens in the faeces of young diarrhoeic dairy calves in Switzerland. *The Veterinary Record,* 163, 362-366.

Varghese, V., Ghosh, S., Das, S., Bhattacharya, S. K., Krishnan, T., Karmakar, P., Kobayashi, N. and Naik, T. N. 2006. Characterization of VP1, VP2 and VP3 gene segments of a human rotavirus closely related to porcine strains. *Virus Genes,* 32, 241-247.

Varshney, B., Jagannath, M., Vethanayagam, R. R., Kodhandharaman, S., Jagannath, H., Gowda, K., Singh, D. and Rao, C. D. 2002. Prevalence of, and antigenic variation in, serotype G10 rotaviruses and detection of serotype G3 strains in diarrheic calves: implications for the origin of G10P11 or P11 type reassortant asymptomatic strains in newborn children in India. *Archives of virology,* 147, 143-165.

Wani, S., Bhat, M., Ishaq, S., Ashrafi, M., Buchh, A. and Haq, M. 2003. Detection of a mammalian-like group A rotavirus in diarrhoeic chicken. *Veterinary microbiology,* 94, 13-18.

Woode, G., Bew, M. and Dennis, M. 1978. Studies on cross protection induced in calves by rotaviruses of calves, children and foals. *The Veterinary record,* 103, 32-34.

Ye, S., Roczo-Farkas, S., Whiley, D., Lambert, S., Robson, J., Heney, C., Nimmo, G., Grimwood, K., Sloots, T. and Kirkwood, C. 2013. Evidence of false-positive results in a commercially available rotavirus assay in the vaccine era, Australia, 2011 to 2012. *Eurosurveillance,* 18, 14-18.

Yolken, R. H., Losonsky, G. A., Vonderfecht, S., Leister, F. and Wee, S.-B. 1985. Antibody to human rotavirus in cow's milk. *New England Journal of Medicine,* 312, 605-610.

Zeller, M., Donato, C., Trovão, N. S., Cowley, D., Heylen, E., Donker, N. C., Mcallen, J. K., Akopov, A., Kirkness, E. F. and Lemey, P. 2015. Genome-wide evolutionary analyses of G1P [8] strains isolated before and after rotavirus vaccine introduction. *Genome biology and evolution,* 7, 2473-2483.

Zeller, M., Patton, J. T., Heylen, E., De Coster, S., Ciarlet, M., Van Ranst, M. and Matthijnssens, J. 2011. Genetic analyses reveal differences in the VP7 and VP4 antigenic epitopes between human rotaviruses circulating in Belgium and rotaviruses in rotarix™ and RotaTeq™. *Journal of clinical microbiology,* JCM. 05590-11.

http://www.localgovernment.co.za/locals/view/203/Mahikeng-Local-

https://en.wikipedia.org/wiki/Mahikeng

APPENDICES

APPENDIX 1: ELISA Worksheet

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

Samples .....

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												

APPENDIX 2: PAGE Worksheet

Date:.....

Name:.....

Volts:.....

Gel Number.....		Electropherotype
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		

APPENDIX 3: Ethical clearance letter

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
MAFIKENG CAMPUS

Faculty of Agriculture, Science and Technology

School of Research and Postgraduate Studies

Private Bag X2046, Mmabatho
South Africa, 2735

Tel: 018 389-2531/2319
Fax: 018 389-2052
Web: http://www.nwu.ac.za

To: Prof Sithebe,

BIOLOGY

FAST

Date: 14 August 2015

Dear Prof Sithebe,

Feedback from Ethics committee on submission titled: Investigation of inter/intraspecies-transmission of rotaviruses between human and cows, pigs, sheep, goats in Mahikeng, North West Province

---

Please find below feedback from reviewers for ethics application reference: NWU-00174-15-S9/NWU-00217-15-S9

General Feedback

1. No risk to humans
2. This research shall not involve any invasive procedures. Stools will be collected from farms, vet clinics and SPCA by either the animal keepers or vets.

Decision: Approved

Best Wishes

UUseh

Prof Ushotanefe Useh PhD

Chairperson Health Ethics

Appendix 4: Results obtained from enzyme immunoassay where 50 samples (with 2 positive control and 2 negative controls) were screened for EIA.

Appendix 4

	1	2	3	4	5	6	7
A	0.052 SMP1 **CONTROL** **Negative**	0.062 SMP9 C1 Results: negative	0.053 SMP17 C2 Results: negative	0.057 SMP25 C3 Results: negative	0.052 SMP33 C4 Results: Negative	0.062 SMP41 C5 Results: negative	0.057 SMP49 C6 Results: negative
B	0.054 SMP2 **CONTROL** **Negative**	0.048 SMP10 C7 Results: negative	0.058 SMP18 C8 Results: negative	0.053 SMP26 C9 Results: negative	0.051 SMP34 C10 Results: Negative	0.048 SMP42 C11 Results: negative	OUT SMP50 **CONTROL** Results: positive(++++)
C	OUT SMP54 **CONTROL** Results: positive(++++)	0.051 SMP11 C12 Results: negative	0.059 SMP19 C13 Results: negative	0.054 SMP27 C14 Results: negative	0.048 SMP35 C115 Results: Negative	0.060 SMP43 C16 Results: negative	0.070 SMP51 C17 Results: negative
D	0.059 SMP4 C18 Results: negative	0.051 SMP12 C19 Results: negative	0.051 SMP20 C20 Results: negative	0.054 SMP28 C21 Results: negative	0.052 SMP36 C17 Results: Negative	0.062 SMP44 C22 Results: negative	0.055 SMP52 C23 Results: negative
E	0.064 SMP5 C24 Results: negative	0.054 SMP13 C25 Results: negative	0.064 SMP21 C26 Results: negative	0.062 SMP29 C27 Results: negative	0.059 SMP37 C28 Results: Negative	0.066 SMP45 C29 Results: negative	0.070 SMP53 C30 Results: negative
F	0.056 SMP6 C31 Results:	0.060 SMP14 C32 Results:	0.068 SMP22 C33 Results:	0.056 SMP30 C34 Results:	0.054 SMP38 C35 Results:	0.059 SMP46 C36 Results:	2.384 SMP54 **(CONTROL)** Results:

	negative	negative	negative	negative	Negative	negative	positive(++)
G	0.065 SMP7 C37 Results: negative	0.056 SMP15 C38 Results: negative	0.062 SMP23 C39 Results: negative	0.062 SMP31 C40 Results: negative	0.057 SMP39 C41 Results: Negative	0.067 SMP47 C42 Results: negative	2.050 SMP55 2758 **(CONTROL)** Results: positive(++)
H	0.064 SMP8 C43 Results: negative	0.064 SMP16 C44 Results: negative	0.061 SMP24 C45 Results: negative	0.058 SMP32 C46 Results: negative	0.056 SMP40 C47 Results: Negative	0.060 SMP48 C48 Results: negative	0.067 SMP56 C50 Results: negative