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ISOLATION AND CHARACTERIZATION OF ROTAVIRUS ASSOCIATED WITH CALVES, IN CENTRAL PART OF OROMIA, ETHIOPIA

MVSc Thesis

BY

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BISHOFTU, ETHIOPIA

ISOLATION AND CHARACTERIZATION OF ROTAVIRUS ASSOCIATED WITH CALVES, IN CENTRAL PART OF OROMIA, ETHIOPIA

A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Microbiology

By

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First, I declare that this thesis is my actual work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ACRONYMS

Ag	Antigen
Ag-ELISA	Antigen Capturing Eenzyme-Linked Immunosorbent Assay
B cell	B lymphocyte
BCV	Bovine Corona Virus
BRV	Bovine Rotavirus
BToV	Bovine Toroviruses
BVDV	Bovine Viral Diarrhea Virus
CD4+	Cluster of Differentiation four
CDC	Center for Disease Control and Prevention
cDNA	Complementary DNA
CPE	Cytopathic Effect
CSA	Central Statistical Agences
CSF	Cerebrospinal Fluid
CTL	Cytotoxic T-lymphocyte
DLP	Double-Layered Particles
DMEM	Dulbecco's Modified Eagle Medium
DNA	Dioxy Nucleotide Ribonucleic Acid
DNTPs	Dioxy nucleotide Triphosphates
dsRNA	double stranded RNA
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscopy
ER	Endoplasmic Reticulum
ETEC	Enterotoxigenic *E. Coli*
FAO	Food and Agriculture Organization
GDP	Gross Domestic Product
ICTV	International Committee on Taxonomy of Viruses
IEM	Immune-Electron Microscopy
Ig	Immunoglobulin
ILRI	International Livestock Research Institutes

LAT	Latex Agglutination Test
MA-104	Rhesus Monkey Kidney
MDBK	Madin Darby Bovine Kidney Cell
mRNA	Messenger RNA
NAHDIC	National Animal Health Diagnostic and Investigation Center
NCD	Neonatal Calf Diarrhea
NSP	Non Structural Proteins
NVD	No Virus Detected
OD	Optical Density
p.i	Post Infection
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
RE	Restriction Endonuclease
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rpm	Revolution Per Minute
RT-LAMP	Reverse Transcription Loop-Mediated Isothermal Amplification
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RVA	Rotavirus group A
T cells	Thymus cell
TLP	Triple Layered Particles
TLR	Toll-like Receptors
UK	United Kingdom
USA	United States of America
VP	Structural Proteins
WHO	World Health Organization

ABSTRACT

Rotavirus is a major pathogen responsible for diarrheal disease in calves resulting in loss of productivity and economy of farmers. However, various facets of diarrheal disease caused by rotavirus in calves in Ethiopia are inadequately understood. A cross sectional study was conducted with the aim of isolation and molecular characterization of rotavirus in calves from central part of Oromia (Bishoftu, Sebata, Holeta and Addis Ababa), Ethiopia from November 2018 to May 2019. The four study areas were purposively selected and fecal samples were collected by simple random sampling for diagnosis of rotavirus infection by using antigen detection Enzyme linked immunosorbent assay (Ag-ELISA) kit. In addition, this study was carried out to have insight in prevalence and associated risk factors of rotavirus infection in calves. Different farm levels (floor of the calf) and calf level (sex, age) factors were measured the level of association between variables. During the present study 83 diarrheic and 162 non-diarrheic fecal samples collected from calves less than 4 weeks of age were screened for rotavirus. Of the 83 diarrheic samples, 6 samples (7.2%) were found to be positive for rotavirus antigen (Ag) by Ag-ELISA. All the non-diarrheic samples were negative for rotavirus Ag. The overall prevalence of rotavirus infection in calves was estimated as 2.45% (6/245). All samples (6) of ELISA test positive were propagated in Madin Darby bovine kidney cells. After 3 subsequent passages, progressive cytopathic effect (CPE) i.e. rounding, detachment as well as destruction of mono-layer cell of four sample (66.7%) were observed. At the molecular stage, reverse transcriptase polymerase chain reaction (RT-PCR) technique was used to determine the presence of rotavirus nucleic acid by using specific generic primers for VP4 genetic regions in feces samples. The 4 samples that were rotavirus antigen positive by ELISA and develop CPE on cell culture were also positive on RT-PCR technique. Infection prevalence peaked have been obtained at 1st and 2nd weeks of age in male calves. Considering that diarrheal disease caused by rotavirus is a vital health problem in calves that interrupts production benefits with reduced weight gain and increased mortality, and its potential for zoonotic spread. So the present findings revealed rotavirus infection in calves in Ethiopia that needs to be addressed by practicing early colostrums feeding in newborn calves, using vaccine, or improving livestock management.

Key word: *Calf diarrhea, ELISA Test, Ethiopia, Rotavirus, Central Oromia.*

1. INTRODUCTION

Diarrhea in neonatal calves is one of the most challenging clinical syndromes encountered by the practicing large animal veterinarian worldwide. It is caused by multifactorial agents like viruses, bacteria, protozoa (de Graaf *et al.,* 1999; Lorenz *et al.,* 2011). Among these agents implicated in calf diarrhea, bovine rotavirus (BRV) group A and bovine corona virus (BCV) act as viral agents, *Salmonella* species, *E. coli* K99+ and *Clostridium* species act as bacterial agents and *Cryptosporidium* species as a protozoan agent (Bhat *et al.,* 2013, 2012; Singla *et al.,* 2013), which are collectively responsible for 75-95% of infection in neonatal calves worldwide. Among these etiological agents of calve diarrhea, rotavirus alone accounts for about 27- 36% (Cho and Yoon, 2014). According few reports have been made under local conditions, the incidence and risk of diarrhea in calves varies from 20.05% to 52.51% (Malik *et al.,* 2012; Megersa *et al.,* 2009; Samad, 2008; Wudu *et al.,* 2008). It has been estimated that 75% of early calf mortality in dairy herds is caused by acute diarrhea in the pre-weaning period (Bartels *et al.,* 2010; Uhde *et al.,* 2008).

Clinically calf diarrhea is diagnosed as infectious enteritis but it is very difficulties to diagnosis at clinical or laboratory level, because of frequent nonspecific clinical signs and lesions, the presence of asymptomatic infections, the involvement of multiple agents and other factors (Athanassious *et al.,* 1994). However, many laboratory diagnostic tests based on the different methods such as direct electron microscopic test, ELISA, latex agglutination, polyacrylamide gel electrophoresis, RT-PCR as well as immuno-electron microscopy. Among these methods, ELISA using monoclonal antibody as a sensitive, fast, and inexpensive method is used for the simultaneous detection of BRV in the feces of diarrheic calves (Mayameei *et al.,* 2010).

Bovine rotavirus is the most recognized pathogens causing acute diarrhea in cattle and buffalo calves under one month of age worldwide (Alfieri *et al.,* 2006; Barrington *et al.,* 2002). It has also been recognized as the major pathogens of acute diarrhea in both humans and animals. So it has the potential of zoonotic and economic impact (Cook *et al.,* 2004). Infection appears and spreads rapidly causing extensive damage to the intestinal lining which results in rapid fluid loss and dehydration (Foster and Smith, 2009). Genetic re-assortment is one of the important mechanisms for generating genetic diversity of rotaviruses and eventually for viral evolution (Martella *et al.,* 2010a; Zhou *et al.,*

2015). There is no treatment for BRV, but early and confirmatory diagnosis helps to make appropriate prevention and control measures, which could prevent the great economic losses to farmers and the livestock industry (Barua, 2019).

Rotavirus is environmentally distributed worldwide and was extensively studied (Straw *et al.,* 2006; Zimmerman, 2006). In different studies BRV infection rates of 20-60% in samples of diarrhea have been reported (Björkman *et al.,* 2003; Garaicoechea *et al.,* 2006; Uhde *et al.,* 2008). Prevalence of rotavirus was estimated ranging from 11.8% to 26.8% in India among diarrheic calves (Basera *et al.,* 2010; Jindal *et al.,* 2000; Malik *et al.,* 2013; Nataraju *et al.,* 2009). Also in European countries rotavirus infection was widely examined. In Sweden between 1993 and 2006 estimated prevalence was 24-47% (De Verd Er, 2006), 42% in diarrheal outbreak in the UK (Reynolds *et al.,* 1986), and 37 to 47.4% in France (Bendali *et al.,* 1999; De Rycke *et al.,* 1986). In Asian countries like Bangladesh, prevalence of rotavirus infection in calf feces varied from 0 to 7% (Alam *et al.,* 2011; Samad and Ahmed, 1990; Selim *et al.,* 1991). In developing country like Ethiopia the prevalence of rotavirus was 16.7% (Abraham *et al.,* 1992).

Rotavirus in calves is not well studied in Ethiopia. However, only one report by Abraham *et al.* (1992) indicated presence of 16.7% rotavirus in calves in central Ethiopia. But among children < 5 years of age rotavirus prevalence range from 18%-28% of diarrhea hospitalizations (Hagbom *et al.,* 2011) and such absence of the information could be the reason for lack of the Ethiopian government not to have any strategy for control of rotavirus infection of calves through vaccination. Absence of study conducted on isolation and molecular characterization of rotavirus in calves in Ethiopia may exacerbate the problem. Hence, detecting the circulating strains of rotavirus isolate and molecular evolution of the virus is needed for planning a proper control and preventive measure in the country.

Therefore, the objectives of current study were:

- ✓ To detect rotavirus from calves less than one month of age and
- ✓ To estimate the prevalence and identify the risk factors rotavirus in calves
- ✓ To Isolate and characterize the virus at molecular level in study area.

2. LITERATURE REVIEW

2.1. Calf Diarrhea

Neonatal calf diarrhea (NCD) is one of the major health challenges in beef and dairy cattle herds around the world. More than 20% of beef cattle owners consider that calf diarrhea has a significant impact on their economic productivity (Foster and Smith, 2009). In several countries, such as Belgium, Sweden, Italy, Switzerland and Brazil, the prevalence and incidence of risk factors for NCD in calves less than 30 days old, have been reported to be 19.1 and 33.7%, respectively (Collins *et al.,* 2014; Papp *et al.,* 2013). Bacteria, viruses and parasites, by attacking the lining of the calf's intestine, give rise to diarrhea. It is a commonly reported disease and a major cause of economic loss to cattle producers. The 2007 National Animal Health Monitoring System (NAHMS) for U.S. dairy (Mayameei *et al.,* 2010) reported that 57% of weaning calf mortality was due to diarrhea and occurred in calves less than 1 month old. A similar mortality rate of 53.4% for dairy calves due to calf diarrhea was reported in Korea (Hur *et al.,* 2013). Calf diarrhea is attributed to both infectious and non-infectious factors (Bartels *et al.,* 2010; Izzo *et al.,* 2011).

NCD is a pathological condition with complex multifactorial etiology that is influenced by infectious, nutritional and environmental factors, as well as by management practices (Blanchard, 2012; Izzo *et al.,* 2011; Martella *et al.,* 2010b). Some of studies show that the most important pathogen agents in calf diarrhea are rotavirus, coronavirus, enterotoxigenic *E. coli*, S*almonella* species and *cryptosporidium* (Reynolds *et al.,* 1986; Steiner *et al.,* 1997). Seventy five percent to ninety percent of calf diarrhea is related to rotavirus, coronavirus, enterotoxigenic *E. coli* and *cryptosporidium* (Radostits *et al.,* 2007). Currently, enterotoxigenic *E. coli* (ETEC), *Clostridium perfringens*, *Cryptosporidium parvum*, rotavirus and coronavirus appear to be the most significant infectious causes of calf diarrhea (Blanchard, 2012; Meganck *et al.,* 2015).

In some reports, rotavirus is the most prevalent pathogen in less than one month old diarrheic calves (Radostits *et al.,* 2007; Reynolds *et al.,* 1986; Snodgrass *et al.,* 1986). Bovine rotavirus usually causes diarrhea in calves at 1 to 2 weeks of age. The milk uptake by calves can provide a good environment

for rotavirus survival under a wide range of gastrointestinal pH levels and infection of the intestine epithelia cells. This may explain why calves are more susceptible to calf diarrhea. The virus has a very short incubation period (12 to 24h) and induces per acute diarrhea in affected calves (Izzo *et al.,* 2011).

2.2. Discovery of Rotavirus

Rotavirus was discovered in 1972 by an Australian research group led by Dr. Ruth Bishop (Bishop *et al.,* 1973). The virus was recognized by direct electron microscopy visualization in the duodenal biopsies of a child with acute diarrhea and named duovirus. The virus was subsequently observed in large numbers in feces as demonstrated by direct thin layer electron microscopy and significant antibody titre was shown between acute and convalescent sera from the children by immune electron microscopy (Bishop *et al.,* 1974). The virus was renamed rotavirus because of its characteristic wheel-shaped (rota is a latin word which means wheel) morphology when viewed under an electron microscope (Paredes *et al.,* 1993).

2.3. Epidemiology of Rotavirus and Geographical Distribution

2.3.1. Epidemiology of rotavirus in humans

Rotavirus is distributed evenly across the globe and is the leading cause of severe gastroenteritis in infants and young children worldwide; it was reported to be responsible for about 128,500 deaths in 2016, with over 70% of cases occurring in sub-Saharan Africa (Jonesteller *et al.,* 2017; Troeger *et al.,* 2018). However, the consequences of infection are markedly severe depending on where the child lives and the majority of deaths due to rotavirus diarrhea occur in the developing countries of the Indian subcontinent and sub-Saharan Africa due to limited access to medical intervention (Parashar *et al.,* 2006). Rotavirus causes approximately 258 million episodes of gastroenteritis requiring home care and about 24 million cases requiring medical attention (Troeger *et al.,* 2018). Six countries India, Nigeria, Congo, Ethiopia, China, and Pakistan account for more than half of the global mortality burden of rotavirus diarrhea (Payne *et al.,* 2016; Tate *et al.,* 2016). Table 1 is summarized some study of epidemiology of rotavirus in human.

Table 1: Seroprevalence of rotavirus in humans in different countries.

Country	Prevalence of Rotavirus	Reference
African	40 %	Mwenda *et al.* (2010)
Ethiopia	25%	Gelaw *et al.* (2018)
Uganda	37 %	Bwogi *et al* (2016)
Narobi Kenya	31.5%	Agutu *et al.* (2017)
Western Kenya	27%	Khagayi *et al.* (2014)
Brazil	33.3%	Carvalho-Costa *et al.* (2019)
Indian	35.5%	Giri *et al* (2019)
Vietnam	46.7%	Huyen *et al* (2018)
China	30%	Yu *et al.* (2019)
South India	40%	Rajendran and Kang (2014)

Molecular epidemiological studies of rotavirus have identified 5 common serotypes, including G1, G2, G3, G4, and G9, which tend to predominate globally (Desselberger *et al.,* 2003). G1 is the most prevalent strain worldwide whereas G9 is the fastest emerging worldwide (Kirkwood *et al.,* 2003; Nyangao *et al.,* 2010; Page *et al.,* 2010). However, in developing countries, additional serotypes may circulate and even predominate in some setting (eg, G5, G8, G10, and G12). Of the 27 VP4 genotypes identified, genotypes P[8], P[4] and P[6] are detected most frequently in children (Hoshino *et al.,* 2004; Hoshino and Kapikian, 2000). Analogously to VP7 epidemiology, supplementary P genotypes, including P[9] and P[10] may also predominate or circulate at lower levels in developing countries (Santos and Hoshino, 2005).

2.3.2. Epidemiology of rotavirus in animals

Diarrhea in the neonatal calf is a serious welfare problem and a cause of economic loss due to mortality, treatment costs and poor growth. Two facts are of which is importance in the epidemiology of rotavirus infections in calves. First, virus particles are present in very large numbers (10^{10} -10^{12} particles/ml) in infected feces. Second, the virus is resistant to inactivation. It has been shown that calf rotavirus can survive for 9 months at room temperature in fecal material, and can resist 60°C for one hour (Woode, 1978). Furthermore rotaviruses are not easily inactivated by the commonly used disinfectants (Snodgrass and Herring, 1977). This means that environmental contamination is both heavy and persistent.

Rotavirus surviving in a contaminated environment from one calving season to the next may therefore be the source of infection in an outbreak. However Woode (1978) has suggested that adults are the major source of infection for calves. Once an outbreak is underway it appears that the major source of infection is environmental rather than maternal. While it is known that rotaviruses have some ability to cross species barriers, it is not generally believed that cross-infection from other species is significant in the epidemiology of rotavirus infections in calves. Whatever the source of the virus, infection spreads predominantly by fecal-oral contact (Mcnulty, 1983).

Calves most often become infected with rotavirus during the first week of life. The corresponding figure in a Dutch survey was 7.7 days in six herds with over 60% of calves excreting the virus and with less than 40 % of the calves infected. Given that over 90% of adult bovines possess antibody against rotavirus (De Leeuw *et al.,* 1980), it is surprising that so many colostrum fed calves are susceptible to infection so soon after birth. Depending on the antibody titre of the colostrum and on the interval between birth and feeding, calves fed colostrum absorb a variable amount of passively derived maternal antibody against rotavirus into the blood. However circulating antibody is not protective. Instead protection is dependent on the presence of colostral antibody to rotavirus within the gut lumen (Snodgrass and Wells, 1978). Neutralizing antibody to rotavirus is present in cows' milk for only a few days after calving. The elimination of this antibody from the gut renders the calf totally susceptible to infection (Snodgrass *et al.,* 1986). The following (Table 2) is to summarized the some study of rotavirus in animals in different part of the world.

Table 2: Prevalence of rotavirus infection in animals.

Country	Prevalence Rotavirus	Reference
Western Algeria	14.63%	Ammar *et al.* 2014)
Northern India	26.8 %	Jindal *et al.* (2016)
Ethiopian	16.7%	Abraham *et al.* (1992)
Indian	15.68%	Rai *et al.* (2011)
Iraq	15.5%	Al-Robaiee and Al-Farwachi (2013)
Brazilian	20.2%	Alfieri *et al.* (2006)
Tunisia	22.8%	Zrelli *et al.* (1990)
Brazilian	25.1%	Langoni *et al.* (2004)
Algeria	21.84%	Kam *et al.* (2011)
England	42%	Reynolds *et al.* (1986)
Scotland	50%	Snodgrass *et al.* (1986)
Spain	42.7%	De la Fuente *et al.* (1998)
Australia	79.9%	Izzo *et al.* (2011)

2.3.3. The status of rotavirus in human and animals in Ethiopia

Ethiopia is one of the five countries with the greatest rotavirus burden worldwide and accounts for 6% of all rotavirus deaths globally (Tate *et al.,* 2012). It is estimated that 28 percent of all under-five diarrheal disease hospitalizations in Ethiopia are caused by rotavirus (WHO, 2013). Also some study said, among children < 5 years of age rotavirus prevalence range from 18%-28% of diarrhea hospitalizations (Hagbom *et al.,* 2011). In a cross-sectional study carried out in Jima Hospital, Ethiopia, to reveal the prevalence of rotavirus infection among 154 infants and young children, rotavirus was detected in 26.6 % of fecal specimens and 90.2% (37/41) occurred in children under 2 years. The highest rate of rotavirus antigen detection was observed among the 7-12 months of age group (34%) (Bizuneh *et al.,* 2004).

A study to see the epidemiology of rotavirus and norovirus in Awassa, southern Ethiopia from 200 under five children with diarrhea 2008-2009, the prevalence of rotavirus was 22% and the genotyping showed G3P[6] (48%, globally uncommon strain), G1P[8] (27%) and G2P[4] (7%) being the strains most commonly identified (Yassin *et al.,* 2012). Data from hospital based surveillance of rotavirus gastroenteritis among children less than five years from 2007-2011 in Addis Ababa, Ethiopia showed that rotavirus was prevalent in 20% of children enrolled from 1,749 diarrheal samples collected in the five year period (Tadesse, 2012). As the current study showed the prevalence of rotavirus 25% in children less than five years in northwest Ethiopia by Gelaw *et al.* (2018). Only one report by Abraham *et al.* (1992) indicated presence of 16.7% rotavirus in calves in central Ethiopia.

2.4. Virology of Rotavirus

2.4.1. Structure and its genome

Bovine rotavirus (BRVs) is a primary etiological agent of calf diarrhea. Rotaviruses are double stranded RNA (dsRNA) held in the inner core of the three-layered virus (Varani and Allain, 2002). Rotavirus is a non-enveloped virion possessing 11 dsRNA segments which a size range 16~21 kilo base pairs within the family Reoviridae and is very stable over a wide pH range with heat liability. There are seven serogroups (A-G) of rotaviruses based on antigenic and genetic similarities of the intermediate capsid protein of VP6. Group A rotaviruses are the major cause of rotavirus infection in domestic animals and initially known as neonatal calf diarrhea virus, was one of the first identified viral causes of diarrhea (Foster and Smith, 2009). Most BRVs (95%) belong to group A, although groups B and C rotaviruses have also been identified in field cases (Murphy *et al.,* 1999).

Genome segments code for structural proteins found in the virus particle and the non-structural proteins found in infected cells but not part of the mature particles. The genome consists of 18,555 nucleotides in total. Each segment is a gene, numbered 1 to 11 by decreasing size. The segmented genome can be separated by polyacrylamide gel electrophoresis (PAGE) to reveal an RNA migration pattern or electropherotype. The RNA pattern is both constant and characteristic for a particular strain and has been widely used in epidemiological studies for monitoring the transmission and spread of rotavirus (Ved, 2014).

2.4.2. Proteins

The nomenclature of the viral proteins designates the structural proteins as VP and nonstructural proteins as NSP followed by sequential numbering from 1 to 6 (Estes and Kapikian, 2007). Analysis of gene encoding segments shows that there are six structural proteins (VP1 to VP4, VP6 and VP7) and six non-structural proteins (NSP1 to NSP6). The structural proteins build up the viral particle (Figure 1) and the NSPs have function either in the viral replication cycle or interaction with host proteins to influence the pathogenesis or immune response. Each of the 11 segment of dsRNA encode a single viral protein except segment 11 which encodes two proteins (Anderson and Weber, 2004). Figure 1 is summarized the six structural (VP) and six non-structural protein (NSP). The functions of each protein are summarized in Table 3.

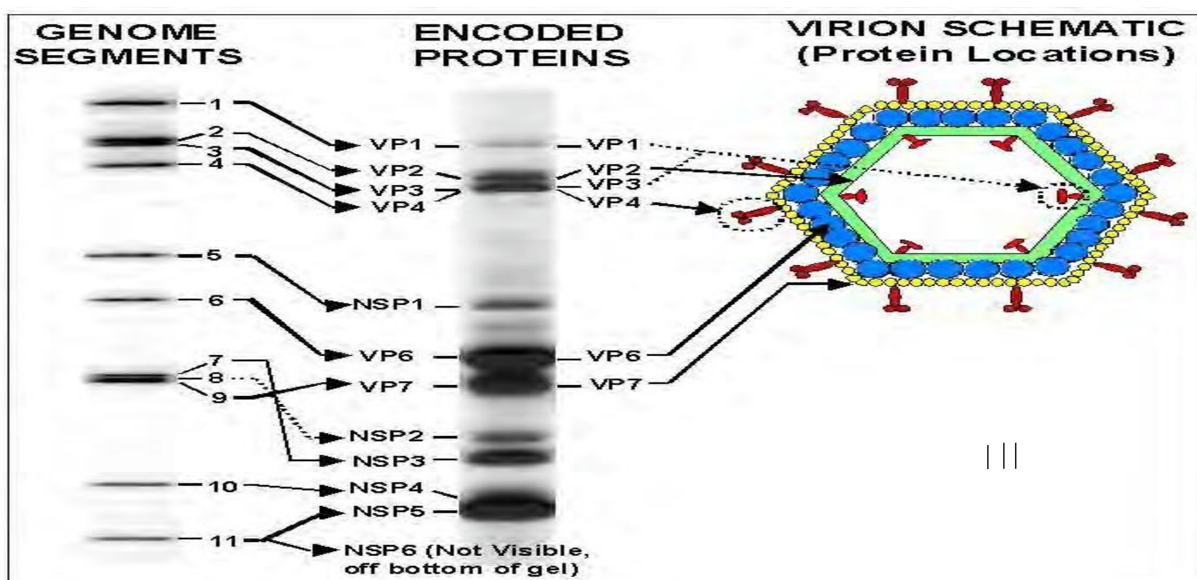

Figure 1: Diagrammatic representation of the rotavirus particle and its genome coding.

(I) The migration pattern of 11 dsRNA **genome segments** of rotavirus on a polyacrylamide gel. (II) Virus **proteins encoded** by specific genome segments in section I. The proteins were blotted onto a cellulose membrane and detected with rotavirus-specific antibodies. (III) Schematic diagram of rotavirus particle showing the cross-section arrangement of viral proteins through the three capsid layers namely: outer (VP4, red; VP7, yellow), inner (VP6, blue) and the inner core (VP2, green). **Source**: (Pak, 2011).

The proteins encoded by the rotavirus genes are well established. Except for segment 11, which encodes for two proteins NSP5 and NSP6, rest all segments encode a single protein. The six viral proteins (VP1, 2, 3, 4, 6 and 7) form the virus particle (virion). VP1 is the RNA-dependent, RNA polymerase for rotavirus, located in the core of the virus particle (Rodrigo *et al.,* 2010; Varani and Allain, 2002). VP2 is a replication intermediate, forms the core layer of the virion and binds the RNA genome while VP3 is an enzyme guanylyl transferase that catalyses the formation of the 5' cap in the post-transcriptional modification of mRNA. VP4 determines the rotavirus P serotype as well as host specificity, virulence and protective immunity, it also binds to molecules on the surface of cells called receptors and drives the entry of the virus into the cell (Maunula and von Bonsdorff, 2002). VP6 is highly antigenic and can be used to identify rotavirus species (Estes *et al.,* 2001) and it also determines the A-G groupings, and I, II sub-groupings of rotavirus. VP7 is a glycoprotein that determines the G serotype and that is involved in immunity to infection (Laird *et al.,* 2003).

Table 3. Rotavirus proteins, genome segments, and structural localization.

Protein	dsRNA segment No	Location In virus capsid	Function	Numbers of molecules/virion
VP1	1	Core	dsRNA synthesis (RNA dependent RNA polymerase)	12
VP2	2	Core	Inner shell protein	120
VP3	3	Core	Capping enzyme	12
VP4(Cleaved to VP5 and VP8)	4	Outer Capsid	Viral attachment, P-type neutralization antigen	120
VP6	6	Inner Capsid	Middle shell protein	780
VP7	9	Outer Capsid	Gtype neutralization antigen	780
NSP1	5		INF antagonist	----
NSP2	8		Viroplasm formation	----
NSP3	7		Enhance viral mRNA synthesis,	----
NSP4	10		Outer capsid assembly, Regulate calcium	----
NSP5	11		Viroplasm formation	----
NSP6	11		Viroplasm formation	----

Source:(Hagbom, 2015).

The six non-structural proteins (NSP1, 2, 3, 4, 5 and 6) are only produced in cells infected by rotavirus (Anderson and Weber, 2004; Graff *et al.,* 2002). NSP1 binds interferon regulatory factor 3 and may inhibit interferon response during rotavirus infection (Graff *et al.,* 2002). In conjunction with NSP5, NSP2 is involved in the synthesis and packaging of viral RNA, creation of viroplasms and is required for genome replication. NSP3 binds viral mRNA at the 3' end, promotes viral protein synthesis and is responsible for the shutdown of host cell protein synthesis. NSP4 is a viral enterotoxin and induces diarrhea during infection (Dong *et al.,* 1997). NSP6 is an RNA binding protein encoded by gene 11 from an out of phase open reading frame (Rainsford and McCrae, 2007).

In comparison to most cellular mRNAs, rotavirus mRNAs are unique in that they contain 5'-terminal caps but lack 3'-terminal poly (A) tails. During replication, the viral mRNAs serve two functions: (i) Direct synthesis and (ii) act as templates for the synthesis of minus-strand RNAs to produce dsRNAs (Chen *et al.,* 1994). The synthesis of dsRNAs is an event that follows or occurs simultaneously with the packaging of mRNA templates, as naked dsRNA cannot be detected in infected cells. Likewise, the absence of free dsRNA in the infected cell indicates that dsRNA remains particle associated once synthesized. Given that the 11 genomic dsRNAs are present in equimolar concentration in both infected cells and virions, the packaging and replication of the 11 species of viral mRNAs into dsRNAs must be a highly coordinated process (Patton and Gallegos, 1990).

Both outer capsid protein VP7 and VP4 (the spike protein) are targets for neutralizing antibodies. VP4, VP6, and VP7 play a major role in maintaining viral structure, virus attachment, and antigenicity. Although early studies implicated VP7 in the cell entry process, subsequent studies increasingly have indicated that VP4 is the major player in this process. VP4 is susceptible to proteolysis and viral infectivity is increases several folds when VP4 is proteolytic cleavaged and facilitates virus entry into cells. During proteolysis, VP4 is cleaved into VP8* (amino acids 1 to 247) and VP5* (amino acids 248 to 776), and the cleavage products remain associated with the virion (Arias *et al.,* 1996).

2.4.3. *Classification and serogroups*

Based on the group specific epitopes localized in an immunodominant site of VP6 between amino acid residue 48 and 75, rotaviruses have been divided into five serological species (A-E) and two additional tentative species (F and G) according to the International Committee on Taxonomy of Viruses (ICTV) (Matthijnssens *et al.,* 2011). These rotavirus species are commonly referred to as rotavirus groups. Rotaviruses belonging to group A, B, C and H (RVA, RVB, RVC and RVH, respectively) have been associated with acute gastroenteritis in humans and animals, whereas group D, E, F and G (RVD, RVE, RVF and RVG, respectively) rotaviruses are known to infect only animals, mostly birds (Estes and Greenberg, 2013). A novel tentative group I was recently described in Hungarian sheltered dogs (Mihalov-Kovács *et al.,* 2015). Table 4 is summarized the rotavirus group with respective host species.

Table 4: Rotavirus group detected so far in different mammalian and/or avian host species.

Rotavirus group/species	Host species
A	A wide variety of mammalian and avian species
B	Humans, cattle, goats, pigs, rat and sheep
C	Humans, cattle, dogs, goats, juvenile ferrets and pigs
D	Chicken and turkey
E	Pigs
F	Chicken
G	Chicken
H	Humans and pigs

Source: (Ghosh and Kobayashi, 2014).

Group A rotaviruses (RVA) can be further classified into P or G types based on genetic and antigenic similarities of VP4 and VP7. VP4 (P protein for 'protease-sensitive' due to its trypsin mediated cleavage required for virus adsorption into cells) determines the P serotypes. VP7 (G protein for 'glycoprotein' forming the matrix of the capsid) defines G serotypes (Laird *et al.,* 2003). For G types, serotypes (determined by neutralization assay) and genotypes (determined by RT-PCR) are largely identical, thereby allowing the use of the same numbering system. For P types, more genotypes than serotypes have been identified, owing to lack of mono-specific P antisera. As a result, P types are identified as serotypes by Arabic numbers and as genotypes by Arabic numbers in square brackets. Thus, the serotype of prototype human rotavirus strain Wa is described as G1P [8]. To date, at least 27 G types and 37 P types have been found in humans and animals (Matthijnssens *et al.,* 2011; Tonietti *et al.,* 2013). Unlike P types, correlation between G serotypes and genotypes is complete. Therefore, where available, P serotypes and genotypes are designated jointly with genotypes in square brackets, for instance, RVA/Human-tc/USA/DS-1/1976/G2P1B[4] (Matthijnssens *et al.,* 2011).

Although the dual typing system has been widely used in most epidemiological and molecular characterization studies, its use is primarily limited to classifying rotavirus strains. The dual typing system cannot determine factors that are involved in viral tropism and virulence of rotavirus strains. Furthermore, some evolutionary pathways like re-assortment and recombination followed by all the

11 genome segments of rotaviruses cannot be studied because the dual classification is restricted only to outer capsid encoding genome segments (Matthijnssens *et al.,* 2008).

In addition to the G and P genotyping of rotavirus, a whole genome-based genotyping system was recently proposed based on the assignment of genotypes to all the 11 gene segments (i.e., G/P and non-G/P genes) (Matthijnssens *et al.,* 2008). In the new genotyping system, the acronym Gx-P[x]-Ix-Rx-CxMx-Ax-Nx-Tx-Ex-Hx, where x is an integer, defines the genotype of the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 genes of a given rotavirus strain. Following the advent of hybridization techniques, researchers could investigate the occurrence of re-assortment events between human strains that belong to different genogroups or between human and animal strains which frequently lead to generation of novel rotavirus strains. Human rotaviruses were classified into two major (represented by the Wa and DS-1 reference strains) genogroups and one minor (represented by the AU-1 reference strain) genogroup (Nakagomi *et al.,* 2005).

The Wa-like strains are characterized by non-G/P genotypes (I1-R1-C1-M1-A1-N1-T1- E1-H1), and tend to have G/P genotypes G1P[8], G3P[8], G4P[8], or G9P[8] (Dennis *et al.,* 2014). In contrast, the DS-1-like strains are characterized by non-G/P genotypes (I2-R2-C2-M2- A2-N2-T2-E2-H2), and tend to have G/P genotype G2P[4]. The third minor AU-1-like strains are characterized by non-G/P genotypes (I3-R3-C3-M3-A3-N3-T3E3-H3) and tend to have G/P genotype G3P [9]. Whole genome-based analysis is a reliable method for obtaining conclusive data on the origin of an RVA strain and for tracing its evolutionary pattern (Ghosh and Kobayashi, 2011; Matthijnssens *et al.,* 2008). RVA of VP7 and VP4 genotypes with their respective host species are summarized in the Table 5.

Table 5: Common RVA G and P genotypes found in humans and animals.

Host species	**Typical RVA VP7 and VP4 genotypes**
Cattle	G6, G8, G10, P[1], P[5], P[11]
Pigs	G3-G5, G9, G11, P[6], P[7]
Horses	G3, G14, P[12]
Cats and dogs	G3, P[3], P[9]
Humans	G1-G4, G9, G12, P[4], P[6], P[8]

Source :(Ghosh and Kobayashi, 2014).

Rotavirus surveillance also generates valuable data on the circulating rotavirus strains (Table 6). These data are vital to improving vaccine development tracking emergent types, and helping to assess vaccine effectiveness and changes in strain diversity after vaccines are introduced. Globally, G1, G2, G3, G4, and G9 are the most prevalent VP7 serotypes; P[4], P[6], and P[8] are the most common VP4 genotypes, and G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] comprise 70–90% of circulating rotavirus strains (CDC, 2008a; Iturriza-Gomara *et al.,* 2009; Santos and Hoshino, 2005; Sharma *et al.,* 2009; Wu *et al.,* 2009). In Taiwan, G1 (40%), G3(27%), G9 (18%), and G2 (8%) are the most common VP7 serotypes (Chen *et al.,* 2007; Hwang *et al.,* 2011; Lai *et al.,* 2005; Lin *et al.,* 2008, 2006; Wu *et al.,* 2011). G6 and G10 type are reported to be the most prevalent in cattle (Martella *et al.,* 2007). The geographic distribution of rotavirus serotypes are summarized in Table 6.

Table 6: Geographic distribution of rotavirus serotypes.

Region	**Rotavirus serotypes**					
	G1P[8]	**G2P[4]**	**G3P[8]**	**G4P[8]**	**G9**	**Other**
North America	73%	11%	6%	1%	3%	5%
South America	34%	23%	2%	9%	16%	11%
Europe	72%	9%	2%	11%	4%	1.4%
Australia	82%	14%	1%	2%	0.5%	0.1%
Asia	34%	13%	1%	20%	12%	14%
Africa	23%	2%	21%	4%	7%	27%
Taiwan	40%	80%	27%	0%	18%	8%

Sources: (CDC, 2008a; Iturriza-Gomara *et al.,* 2009).

2.4.4. Re-assortment and antigenic variation

Re-assortment is one of the important mechanisms for generating genetic diversity of rotaviruses and eventually for viral evolution. Although host species barriers and host range restriction exist in rotavirus, re-assortment can result in interspecies transmission, which also contributes to the diversity and evolution of rotavirus (Martella *et al.,* 2010a; Zhou *et al.,* 2015). A crucial factor in the generation of re-assortant viruses is the frequency of co-infection. In developing countries, the rate of RV co-

infection can be as high as 20%, while in developed countries, the rate is typically less than 5% (WHO, 2011). It may be because of the high rate of co-infection that the genetic diversity of viruses in developing countries can be so much higher than in developed countries. Due to the high frequency of co-infection, large genetically distinct RV clades may not be detectable in some developing countries (Patton, 2012).

Sequence analysis has shown that the antigenic epitopes of VP7 and VP4 proteins assigned to the same G and P type, respectively, will frequently show amino acid variation (Jin *et al.,* 1996; McDonald *et al.,* 2009; Wu *et al.,* 2011; Zeller *et al.,* 2012). This has been seen for VP7 and VP4 proteins of viruses recovered from different countries in the same year or that belong to different co-circulating clades at one site. Such amino acid variation may ultimately have an impact on vaccine efficacy, particularly if protection is based chiefly on G and P type specific homotypic responses. In fact, Hoshino *et al.* (2005) have shown that the effective titer of a G type specific neutralizing antiserum is affected by the amino acid composition of VP7 antigenic epitopes, even if the VP7 proteins are of the same G type.

2.4.5. *Replication*

Viruses interact with the host at all stages of replication; cell entry, viral transcription, translation, genome synthesis and packaging, and cell exit. These interactions are not only important for producing new virus progeny, but also enable the host to recognize the presence of an infectious agent. As host species have evolved mechanisms to defend against pathogens, viruses have in turn evolved strategies to avoid the host immune response (Randall and Goodbourn, 2008).

Rotavirus replication takes place in the cytoplasm of infected cells, in viroplasms being electron dense structures near the nucleus and ER (Lamb and Kurg, 2001) . Newly made viruses budded out from viroplasms into ER, through binding to the tail of the ER transmembrane viral glycoprotein NSP4. Although the virus replication process includes synthesis and transport of glycoproteins, the Golgi apparatus is not involved in rotavirus replication. Instead rotavirus replication, morphogenesis and pathogenesis are regulated by intracellular calcium concentrations. The rotavirus toxin NSP4 has been shown to be released very early during an infection, first as a cleavage product including the toxic

region released from infected cells, starting at 4 hours post infection (Zhang *et al.,* 2000) and later during infection as fully glycosylated NSP4 (Bugarcic and Taylor, 2006).

Based on cell culture studies, the general steps of rotavirus replication are as follows (Lamb and Kurg, 2001) (Figure 2): Virus attachment to cell surface by VP4 or the cleavage product VP8. The conformational change is protease-dependent, where VP4 is cleaved into VP8 and VP5. Rotavirus has tropism for mature enterocytes but the exact receptor for viral binding in vivo has not yet been identified, although sialic acid, integrins, histo-blood group antigens (Diederichsen De Brito *et al.,* 2000; Svensson *et al.,* 2014) and toll-like receptors (TLR) have been suggested (Pott *et al.,* 2012). Cell entry, by receptor-mediated endocytosis occurs via VP5, thus indicating that cleavage of VP4 into VP5 and VP8 is required. Calcium dependent endocytosis has also been shown. Non-clathrin, non-caveolin-dependent endocytosis delivers the virion to the early endosome. It has also been suggested that rotavirus can enter the cell by direct entry or fusion. Uncoating of the TLP, reduced calcium concentrations in the endosome are thought to trigger the uncoating of VP7 and loss of the outer capsid (VP7, VP5 and VP8). Double-layered particles (DLP) (core proteins and inner capsid VP6) are released into the cytosol (Kaljot *et al.,* 1988).

Transcription and translation takes place in the cytoplasm of the cell. The internal polymerase complex (PC) (VP1 and VP3) starts to transcribe capped (+) RNAs from each of the eleven dsRNA segments. (+)RNA serves either as mRNA for direct translation, synthesis of viral proteins by cellular ribosomes or as a template for (-) RNA synthesis of viral genome replication, taking place in viroplasms (Kaljot *et al.,* 1988). Assembly is the NSP2 and NSP5 interact to form viroplasms, where replication and sub-viral particle assembly takes place. DLPs are formed within the viroplasms. The assembly process of the outer capsid is not fully understood but it is thought that the transmembrane protein NSP4 recruits DLPs and the outer capsid protein VP4 to the cytosolic side of the ER membrane. The NSP4/VP4/DLP -complex then buds into ER. The removal of the ER membrane and NSP4 takes place in the ER through interaction with ER-resident VP7 and the final TLP is formed. Virus release from the infected cell is through cell lysis or Golgi-independent non-classical vesicular transport. In the GIT the virion will be exposed to trypsin-like proteases, which will cleave the protease-sensitive VP4 into VP5 and VP8, thus resulting in a fully infectious virion (Lamb and Kurg, 2001).

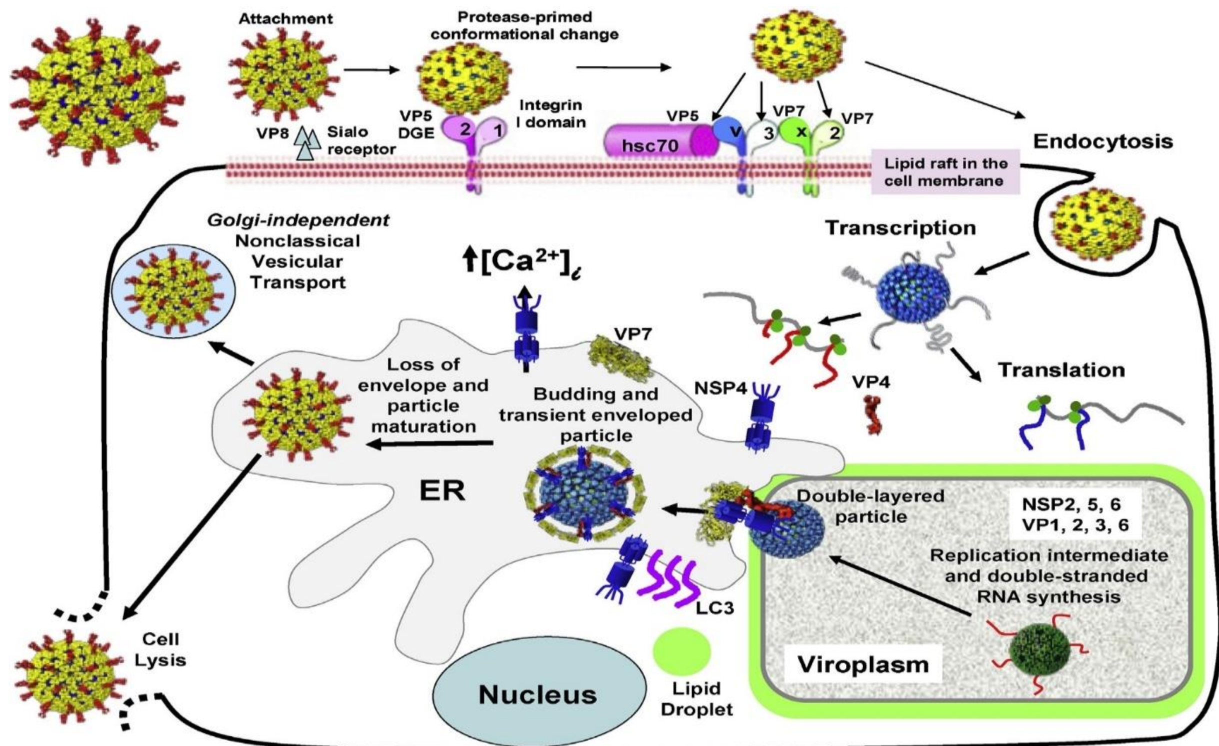

Figure 2: The rotavirus replication cycle.

The rotavirus triple layered particles (TLPs) first attach to sialo-glycans (or histo-blood group antigens) on the host cell surface, followed by interactions with other cellular receptors, including integrins. Virus is then internalized by receptor-mediated endocytosis. Removal of the outer layer, triggered by the low calcium of the endosome, results in the release of transcriptionally active double-layered particles (DLPs) into the cytoplasm. The DLPs start rounds of mRNA transcription, and these mRNAs are used to translate viral proteins. Once enough viral proteins are made, the RNA genome is replicated and packaged into newly made DLPs in specialized structures called viroplasms, which interact with lipid droplets. The newly made DLPs bind to NSP4, which serves as an endoplasmic reticulum (ER) receptor, and bud into the ER. NSP4 also acts as a viroporin to release Ca^{2+} from intracellular stores. Transiently enveloped particles are seen in the ER. The transient membranes are removed as the outer capsid proteins VP4 and VP7 assemble, resulting in the maturation of the TLPs. The progeny virions are released through cell lysis. In polarized epithelial cells, particles are released by a non-classical vesicular transport mechanism.

Source: (Estes and Greenberg, 2013).

2.5. General Pathophysiology

The severity and localization of rotavirus infection vary among animal species and between studies, but pathological changes are almost exclusively limited to the small intestine. Rotavirus infects the mature non-dividing enterocytes in the middle and top parts of the villi in the small intestine (Lundgren and Svensson, 2001). At the cellular level, the infection is characterized by vacuolization, blunting and shortening of the villi. Rotavirus also produces the enterotoxin NSP4, which is thought to play an important role in the pathophysiology and clinical symptom of rotavirus disease (Ball *et al.,* 1996; Ge *et al.,* 2013; Morris *et al.,* 1999). The incubation time is 24 to 48 hours and illness usually last from 3 to 5 days, longer in immune-compromised individuals (Fields *et al.,* 1996). There are few pathology studies of the duodenal mucosa of infants infected with rotavirus. Biopsies have displayed shortening and atrophy of villi, distended endoplasmic reticulum, mononuclear cell infiltration, mitochondrial swelling and loss of microvilli (Davidson and Barnes, 1979; Holmes *et al.,* 1975).

Systemic spread of rotavirus has been reported but is very rare and its clinical importance remains unclear (Bugarčić and Taylor, 2006). In a few cases rotavirus RNA has been detected in cerebrospinal fluid (CSF) (Medici *et al.,* 2011), possibly associated with meningitis (Wong *et al.,* 1984), encephalopathy (Nakagomi and Nakagomi, 2005) and encephalitis (Ushijima *et al.,* 1987). Several recent studies have demonstrated that antigenemia, viremia and limited systemic replication seems to occur frequently in different body sites, but there is little evidence that this systemic spread and replication is responsible for any specific pathologic findings in the host (Ramani *et al.,* 2010; Ushijima *et al.,* 1987). In severely immunocompromised infants it has been shown that rotavirus can replicate and cause abnormalities in the liver and other organs (Gilger *et al.,* 1992).

2.6. Pathogenesis of Rotavirus Infection

Bovine rotaviruses group A are entero-pathogenic agents more commonly associated with neonatal diarrhea in calves up to 30 days old (Alfieri *et al.,* 2006). The mechanism of rotavirus-induce diarrhea is not completely known. The major mechanism appears to be a decreased absorption of salt and water related to selective infection of the absorptive intestinal villous cells, resulting in net fluid secretion (Dhama *et al.,* 2009). The main place for rotavirus infection is brush border of villous epithelial cells

in the small intestine. The infected cells are rapidly replaced with undifferentiated crypt cells and results in reducing activity of lactase in villous (Carlton and McGavin, 1995).

The primary mode of transmission of rotavirus is fecal-oral, although some studies have reported low titers of virus in respiratory tract secretions and other body fluids, indicating the possibilities for air-borne and water-borne transmissions of rotavirus (Dennehy, 2000). After ingestion, the rotavirus particles exclusively infect the mature differentiated enterocytes in the mid and upper part of the villi of the small intestine leading to structural changes in the intestinal epithelium (Lundgren and Svensson, 2001). The virus replicates in the cytoplasm of epithelial cells of the mature absorptive and enzyme producing enterocytes of small intestinal villi. Destruction of mature entrecotes in the villi, leading to rupture and sloughing of the enterocytes with release of virus to infect adjacent cells (Martella *et al.,* 2010b). Unlike the parvovirus, rotavirus can infect neither the immature villous crypt cells nor the colonic enterocytes. Rotavirus attaches to its cellular receptors (sialoglyco-protein and integrins) via the VP4 protein. The virus is thought to invade target cells in two possible ways; by direct entry or fusion with enterocytes and through Ca^{2+}-dependent endocytosis (Pérez *et al.,* 1998).

Three mechanisms have been described by which rotavirus might cause diarrhoea. First, within 12-24 hours post-infection, enterocytes are intact but the levels of the brush-border dissacharidases (sucrase, maltase, and lactase) are greatly reduced. As a result, dissacharides in the diet cannot be hydrolysed to monosaccharides and thus cannot be absorbed, leading to osmotic diarrhoea (Anderson and Weber, 2004). Second, NSP4 has an effect in opening calcium channels in the enterocytes. This causes an efflux of sodium and water, producing secretory diarrhea (Dong *et al.,* 1997; Jayaram *et al.,* 2004). Finally, the raised intra-enterocyte calcium concentration causes enterocytes to die by oncosis. The rate of death of the mature villous tip enterocytes exceeds the rate of growth of immature enterocytes that are regenerated from the stem cells in the crypt, causing villous blunting and thus malabsorption (Leung *et al.,* 2005). Infection resolves both as the virus runs out of susceptible mature enterocytes and an immune response is generated (Lundgren and Svensson, 2001).

Recently, Hagbom *et al.* (2011) demonstrated that emesis, which is a hallmark of the rotavirus disease, is caused by serotonin (5-hydroxytryptamine, 5-HT). 5-HT is secreted by enterochromaffin cells (EC) that can be directly infected with and replicate rotaviruses in humans. The 5-HT activates vagal afferent nerves connected to the nucleus of the solitary tract and area postrema in the brainstem structures associated with nausea and vomiting.

2.7. Immune Response to Rotavirus

The mechanisms responsible for immunity to rotavirus infections are not completely understood. Animal models have been useful in elucidating the role of antibodies and in exploring the relative importance of systemic and local immunity (Desselberger and Huppertz, 2011). In humans, rotavirus infection has been shown to induce a good humoral immune response and protection increases with each new infection and reduces the severity of the diarrhea (Velázquez *et al.,* 1996).

Primary rotavirus infections induce production of rotavirus-specific memory B and T cells (Velázquez *et al.,* 2000). Since the immunity against severe diarrhea in humans resulting from series of childhood rotavirus infections often wanes with age, elderly persons become more susceptible to rotavirus re-infection (Glass *et al.,* 2006). The significance of the systemic presence of IgA, IgG and IgM antibodies towards protection against rotavirus infection in both humans and animals remain to be understood (Desselberger and Huppertz, 2011; Fischer *et al.,* 2005; Ramig, 2004). However, it is known that maternal IgG antibodies may play a role in protecting infants under the age of three months from developing severe diarrhoea caused by rotavirus infections as evidenced by the neutralising activity of antibodies detected from transitional milk and colostrum specimens (Chan *et al.*, 2011; Herrmann *et al.,* 1996; Yang *et al.,* 2001).

CD4+ helper T (TH) cells also play a vital role in the successful clearance of a rotaviral infection (Vancott *et al.,* 2001). Thus the correlates of immunity to rotavirus include both the presence of high amounts of cross reactive secretory IgA, and serotype specific serum IgA and IgG, which requires a rotavirus-specific TH cell response as well as a rotavirus-specific CTL response (Anderson and Weber, 2004). Protection of neonates against rotavirus infection appears to be conferred by both transplacentally acquired maternal antibodies and by antibodies and other factors in breast milk. Interestingly, rotavirus infection in neonates often results in asymptomatic infection unless novel serotypes emerge, and rotavirus can circulate silently in neonatal units (Patel *et al.,* 2009).

2.8. Factors Affecting Disease Severity

The factors that influence the severity of the disease as well as pathogenesis are reduced intake of colostrum, age and health status of the calves, immune status of the dam, degree of exposure and virulence of virus, and the presence of secondary pathogens (Steele *et al.,* 2004). If rotavirus infection occurs in combination with *E. coli* or corona virus, the mortality rate could be high. Several other factors like dehydration, unhygienic environment, temperature variations or chilling during winter and high population density in farms may also enhance disease severity. However the major stress factors that potentiate the infection have been found to be cold climate and marked fluctuations in the ambient temperature between day and night. An age related resistance has also been observed. As there is competition between the rate of replication of rotavirus and replacement of enterocytes in older animals; highly virulent strains can only cause diarrhea in adult calves (Dhama *et al*., 2009).

2.9. Clinical Features of Rotavirus Infection

2.9.1. Symptoms in animals

Rotavirus diarrhea in calves presents an acute disease having very short incubation period of 12–24 hours or at times ranging from 18–96 hours. Fortunately, most rotavirus infections are mild and self-limiting, although there is usually high morbidity. Variations in clinical disease observed in calves depend on a number of factors, including difference in virulence among rotavirus strains, age of the host, host immune status, dose of the inoculum, occurrence of mixed infections, environmental stress (weather conditions, housing, overcrowding) and nutrition. These factors, along with systemic consequences of electrolyte imbalances, fluid loss and metabolic acidemia, anorexia, profuse watery diarrhea and various degrees of systemic dehydration (Holland, 1990). In severe cases, death occurs as a result of electrolyte imbalances, dehydration and cardiac arrest (E Pérez *et al.,* 1998).

2.9.2. Clinical sign in human

Rotavirus is the major cause of acute gastroenteritis in young children, worldwide (Tate *et al.,* 2012). The outcome of rotavirus infection varies from asymptomatic through mild short-lived watery diarrhea, to an overwhelming gastroenteritis with dehydration leading to death. The onset of symptoms is abrupt after a short incubation period of 1-3 days. The disease is characterized by fever, frequent abdominal pain and vomiting for 2-3 days, followed by pale watery or loose non-bloody diarrhea for 3-8 days. Diarrhea can be profuse, with patients commonly having 10-20 bowel movements each day. Such severe diarrhea without fluid and electrolyte replacement may result in death. Patients continue to excrete virus for extended periods of time and may thus be a reservoir for infecting others. However, infections can also be asymptomatic, especially in neonates, older children and adults (Lundgren and Svensson, 2001). Cases of asymptomatic infections in older children and adults are probably due to active immunity. Usually all children have become infected several times during the 24 first months of life and by the time they reach 5 years of age most children have had repeated infections and developed a life-long lasting immunity to rotavirus disease (Lundgren and Svensson, 2001).

2.10. Transmission

Rotaviruses are highly contagious, ubiquitous in the environment and relatively resistant to disinfectants. The adult animals are the main source of infection in newborn animals, and serological surveys revealed that 50–100% of adult animals might show immune response against RVA. Young calves, especially 1-3 weeks aged are most vulnerable to the rotavirus infection and infection rates declines as age of calf increases (Soltan *et al.,* 2016). The infectious dose is low (as few as 10 particles) (Ward *et al.,* 1986), and the virus is shed in large quantities (as many as 10^{11} particles per gram of stool) both before the onset of symptoms and for several weeks afterward. The virus transmits through a fecal-oral route and calves are most often infected by contact with other calves, primarily or secondarily through objects, feed and water. It has been proposed that calves can also be infected by virus shed by the dam at birth. The infected calves shed virus through the feces from the second day of infection and the shedding may last for 7-8 days. The virus primarily affects neonatal individuals, and calves more than 3 months of age are usually not affected. Rotavirus that infects calves causes often severe and sometimes life threatening diarrhea (Dhama *et al.,* 2009).

Transmission to susceptible individuals occurs mainly by the fecal-oral route through direct contact with the rotavirus, including children and adults with asymptomatic illness and contact with contaminated fomites, food, water, and environmental surfaces (Barnes *et al.,* 2003; Ramani *et al.,* 2008). Rotavirus has been reported that improvements in hand hygiene in hospitals can decrease the incidence in healthcare-associated rotavirus infections (O'ryan *et al.,* 2009; Zerr *et al.,* 2005). It has also been suggested that aerosol transmission might be important. Evidence of the airborne spread of rotavirus gastroenteritis is primarily circumstantial, including the short incubation period (1-3 days) and the fact that the virus often presents in explosive outbreaks (Dennehy, 2000). Rotavirus has also been detected in the respiratory secretions from a small number of patients, and cases of pneumonia have been described. Rotavirus epidemics exhibit a seasonal pattern (Bernstein, 2009). In temperate climates, rotavirus infections peak in the winter months. Seasonality is less marked closer to the equator, but the disease is more common during drier and cooler months. Recent data suggest that the seasonality of rotavirus could have been changed by the introduction of rotavirus vaccines (CDC, 2008; Hull *et al.,* 2011).

2.11. Diagnosis of Rotavirus

Laboratory diagnosis of rotavirus is very important for management and control of outbreak of disease related with rotavirus infection in calves. Viral gastroenteritis is caused by different types of viral antigens like corona virus, noroviruses, astroviruses and adenoviruses. It is very difficult to diagnose specific causal agents by clinical examination, so laboratory diagnosis is vital for confirmatory diagnosis. This can be carried out by using various tests (Barua, 2019). Rapid and accurate detection of the etiological agent is important to further contain the spread of infection in animals. Rotavirus is shed in high concentration in the stool (~10^{12} viruses/gram) of children with gastroenteritis. Therefore measurement of rotavirus antigen in the stool has been used to identify rotavirus infected patients. Generally, the diagnosis of rotavirus is based on isolation and identification of the virus in intestinal contents or feces (Holland, 1990). Isolation of rotavirus has been performed in rotavirus specific cell line MA-104 (Simian origin), and direct detection has been facilitated by electromicroscopy. Immunofluorescence test (IFT), immunoperoxidase test (IPT) and viral RNA-based PAGE have also been employed to detect the infectious agent. Latex agglutination test (LAT) has also been used for the rapid detection of rotavirus antigens (Hammami *et al.,* 1990; Reidy *et al.,* 2006). ELISA, being a highly sensitive and specific test, has been developed by many workers and used for the identification of rotaviruses (Murphy *et al.,* 1999).

2.11.1. Antigen capturing enzyme-linked immunosorbent assay (Ag-ELISA)

Ag-ELISA is an assay for rapidly detecting a pathogen in a clinical specimen based on antibody (e.g., monoclonal antibody) recognition of the target antigen (Lequin, 2005). It has antibody attached to a solid surface which can be a glass, plastic material or membrane filter. This antibody captures the target antigen if present in the sample. Then there will be a cascade of colorimetric reactions to verify capturing of the antigen and visualize the antigen-antibody reaction. Antigen can be quantitatively estimated as optical density (OD) measured by a spectrometry positively correlates with the amount of antigen. In some situations, the commercial kit may be expensive, particularly for veterinary medicine (Barua, 2019).

2.11.2. Electron microscopy (EM)

Electron microscopy (EM) is used for virus detection and identification based on morphological characteristics. There are two types of EM methods: direct EM and immune-electron microscopy (IEM) (Brandt *et al.,* 1981). Two different staining techniques (positive and negative staining) are used to visualize the presence of target. In the direct EM, virus particles in a fluid sample matrix are applied directly to a solid support and then are visualized by EM after a contrast stain is applied. It is commonly referred to as "negative straining EM", whereas positive staining is generally used in a thin-section EM on fixed tissues. In comparison, IEM has a higher sensitivity and specificity than direct EM as a specimen is incubated with antibody specific for the target virus in order to agglutinate the virus before staining. The visualization of viruses, particularly non-cultivatable ones, is a major advantage of EM with rapid turnaround (Yong-il, 2012). Most of bovine enteric viruses, such as BRV, BToV and BCV, are difficult to isolate or propagate in cell culture, but these viruses can be differentiated by their morphology under an electron microscope. The cost of electron microscopes and requirement of skilled laboratory personnel is still a challenge for the EM test being used as routine diagnostic test (Duckmanton *et al.,* 1998).

2.11.3. Isolation of virus in cell culture

Virus isolation test is a confirmatory diagnostic test that still measured as 'gold standard' for detecting the presence of viral pathogens in specimens (Yong-il, 2012). Cell culture techniques are commonly used for virus isolation for diagnostic purpose, as well as virus propagation for vaccine production or further virus characterization such as antigenic variation or gene sequencing (Ribes *et al.,* 2002). The isolation of rotavirus in cell culture from fecal samples is the most conventional way of confirmatory diagnosis of rotavirus infection and gives the ultimate proof of virus association with the disease but it is less sensitive and is laborious process. Isolation of BRV is performed in rotavirus specific primary cell cultures (calf kidney cells) and cell lines (MA 104-Simian origin, MDBK, HT-29 and PK-15). Presence of virus is suspected by occurrence of cytopathic effect (CPE) including rounding and detachment of cells in cell culture system. Enhancement of CPE has been shown to be increased by incorporation of trypsin in the medium in minute quantities and by the pretreatment of fecal samples with trypsin (Steele *et al.,* 2004). The viability of target virus in a specimen is critical for the success

of virus isolation (Schielke *et al.,* 2011). Specimens should be kept at a low temperature and in a transport medium during shipping to a diagnostic laboratory and delivered to the lab as soon as possible after collection (Schielke *et al.,* 2011).

2.11.4. *Rotavirus dsRNA PAGE*

The rotavirus dsRNA can be detected in clinical specimens by extraction of viral RNA and analysis by electrophoresis on a polyacrylamide gel followed by silver staining. During electrophoresis the 11 segments of the rotavirus dsRNA, which are negatively charged molecules, separate according to size (WHO, 2009). The patterns of dsRNA can be visualized in the gel by staining with silver nitrate, because silver ions form a stable complex with nucleic acids. The gel can be stored after staining. The migration patterns of the segments of rotavirus dsRNA allow the classification of rotavirus strains into the "short" and "long" electropherotypes (Cho, 2012).

2.11.5. *Polymerase chain reaction (PCR)*

Polymerase chain reaction (PCR) is frequently used test method for detecting rotavirus. It is a thermocyclic enzymatic amplification of specific sequence of the target genes using a pair of oligonucleotide primers that hybridize on each cDNA strand of interest region in the genomic sequence. The detection of rotavirus dsRNA in fecal specimens consists of 4 steps: i) Viral dsRNA Extraction ii) Denaturation of the rotavirus dsRNA iii) Reverse transcription of dsRNA iv) Amplification of cDNA by PCR; PCR consists of: a) heating the DNA to be amplified to separate the template strands, b) annealing of two primers that are complimentary to the region to be amplified, c) extension of the primers by a heat stable DNA polymerase enzyme that uses each DNA strand as template, d) repeating the process 30-40 times with the newly synthesized cDNA heat denatured and the enzymes extending the primers attached to the separated single DNA strand. After completion of the reaction, the PCR products can be visualized on an agarose or acrylamide gel by electrophoresis technique and special staining with ethidium bromide. Amplification of the target sequence is determined based on molecular size and/or sequencing of the PCR product (Barua, 2019).

2.11.6. Real-time PCR

Real time PCR is a PCR method which amplifies the target sequence and also quantifies the amount of the target with higher sensitivity (Wong and Medrano, 2005). Real-time reverse transcription-PCR is a high throughput robust easy to perform, quantitative, sensitive and specific assay to detect viral nucleic acids (Espy *et al.,* 2006). Multiplex Real time PCR based on SYBR Green and TaqMan assay have been developed for detection of group A human rotavirus. Multiplex real-time PCR has also been described to detect rotavirus along with other enteric pathogens in bovine fecal samples (Cho *et al.,* 2010). Compared to conventional RT-PCR, real time RT-PCR has been shown to be more rapid and more sensitive for the detection and quantitation of rotavirus (Kang *et al.,* 2004; Pang *et al.,* 2004). For rapid diagnosis of rotavirus in faecal samples a SYBR Green based Real-Time PCR assay was developed targeting the NSP4 gene (Kang *et al.,* 2004).

2.11.7. Rotavirus genotyping using RT-PCR

Reverse transcription- polymerase chain reaction (RT-PCR) ever since the initial report by Kary Mullis and coworkers in 1986 about in vitro enzymatic amplification of specific DNA fragments from complex nucleic acid samples using PCR, a number of different applications of the technique have grown exponentially. Gouvea *et al.* (1990) was the first one to report a novel G-typing method based on RT-PCR amplification of the VP7 gene with type-specific primers. Subsequently, Nakagomi and Nakagomi, (1991) used RT-PCR for serotyping of rotavirus virus and reported that six VP7 serotypes or G-types (G1-G4, G8, and G9) Occur in group "A" human rotaviruses. In their study they could type about 89% of the samples. Gentsch *et al.* (1992) described the sequence information and developed a RT-PCR based typing method to detect four genetically distinct gene 4 types. Taniguchi *et al.* (1984) used PCR for identifying serotypes of human and bovine rotaviruses and PCR was shown to be more sensitive (93%) than ELISA (82%) in his study.

RT-PCR is more sensitive (100%) and specific (99%) in comparison to ELISA and PAGE (Hussain, 1996). As against RNA electrophoresis and ELISA, it provides for a more accurate detection of rotaviruses by 18.8% and 26.5%, respectively. In recent reports, it has been shown that increased detection and quantification of group "A" rotavirus can be done by real-time RT-PCR. For easy screening of the faecal samples for rotavirus A, a diagnostic RT-PCR assay was developed by targeting the group specific VP6 gene (Kang *et al.,* 2004).

Fukuda *et al.* (2012) developed a one-step multiplex RT-PCR method for the simultaneous detection of five viruses causing diarrhoea in adult cattle i.e. bovine group A rotavirus (rotavirus A), bovine group B rotavirus (rotavirus B), bovine group C rotavirus (rotavirus C/GCR), bovine coronavirus (BCV) and bovine torovirus (BToV). In his study, the one step multiplex RT-PCR was found to have higher sensitivity to detect rotavirus A than a single RT-PCR with conventional primers. The results indicate that the one-step multiplex RT-PCR developed can be used for the detection of rotavirus A, rotavirus B, rotavirus C, BCV and BToV and can be expected to be a useful tool for the rapid and cost-effective diagnosis and surveillance of viral diarrhea in adult cattle (CDC, 2008b).

2.11.8. *Restriction fragment length polymorphism (RFLP)*

Restriction Endonuclease (RE) analysis of field rotaviruses is a powerful tool to understand genomic diversity of rotaviruses circulating in environment. Apart from proving useful in monitoring the extent of genetic variation among rotavirus strains within a population, RFLP may also prove valuable in the examination of interspecies transmission and possible source of origin of rotavirus strain. Chang *et al.* (1996) used RFLF for P and G genotyping of bovine rotavirus A. Gouvea *et al.* (1990) analyzed 194 strains of rotavirus A representing all known G types digestion with three restriction enzymes (Sau96I, BstYI, HaeIII) by direct digestion of amplified cDNA copies or by deduction of the restriction patterns from known sequences. Digestion with Sau96I and HaeIII identified restriction sites commonly used for all, or mostly for all, strains of rotavirus studied, whereas BstYl was the most discriminating among rotavirus strains.

2.11.9. Reverse transcription loop-mediated isothermal amplification (RT-LAMP)

Nemoto *et al.* (2015) developed RT-LAMP for detection of equine rotavirus targeting P [12], the most predominant P genotype worldwide. The results indicated that the RT-LAMP assay was specific for equine rotavirus and was found more sensitive than semi-nested RT-PCR. Because RT-LAMP is easy to perform without the need for a thermal cycler or gel electrophoresis, so the RT-LAMP assay should be applicable to diagnosis of equine rotavirus infections in diagnostic laboratories.

2.11.10. Hybridization assays

The assessment of the genetic variability of rotavirus by hybridization assay, including blot techniques such as Northern and Southern blot and also liquid assays, has been an alternative approach to PCR assays. Most Northern blot and liquid hybridization assays have utilized cDNA or ssRNA probes synthesized from all segments in a single hybridization reaction and thus limit the amount of segment specific information available from the test (Nakagomi *et al.,* 1992). Non-radiolabeled cDNA probes have been used for G and P genotyping of bovine rotavirus A (Prasad *et al.,* 2005).

2.11.11. Latex agglutination test (LAT)

LAT is in principle similar to ELISA test (Polpanich *et al.,* 2007). Antigen or antibody is coated on the surface of latex particles, which captures antibody and the target antigen, respectively. The test has been applied for the detection of a wide range of targets, such as bacteria, virus, hormones, drugs and serum protein (Park *et al.,* 2004). Latex particles are made of synthetic rubber and emulsified as billions of micelles of the same size of a desired diameter. Usually the size of particles ranges between 0.05 to 2μm in diameter, and the presence of sulfate ions provides an inherent negative surface charge to the particles (Perez-Amodio *et al.,* 2001). This prepared latex particle can be further functionalized by special processing, such as amidation, amination, carboxyation, hydroxylation or magnetization, to increase their binding stability and analytic attachment depending upon the purpose of test (Perez-Amodio *et al.,* 2001). The latex agglutination test is frequently employed in diagnostic lab. because it can be a semi-quantified test and is relatively cheap with rapid turnaround (Gurjar *et al.,* 2008).

Caution should be taken in interpreting marginal results as false positive/negative results frequently occur due to non-specific binding or interference (Polpanich *et al.,* 2007).

2.12. Treatment

There is no specific treatment for rotaviral infections. Treatment is based in providing supportive care and managing clinical signs and potential complications. In livestock and companion animals, fluid administration is essential to replace losses from diarrhoea or vomiting, to correct acidosis and to restore electrolytes imbalance. Adequate sodium concentration and appropriate glucose to sodium ratios are the most important components of an efficient rehydration solution *(Lorenz et al.,* 2011). In young animals, administration of fluids can be performed by means of oesophageal catheter; in older animals, intravenous administration is preferable. In affected piglets, administration of a plasma protein mixture, consisting of immunoglobulins, growth factors and other biologically active peptides, has been advocated to enhance small intestine recovery (Corl *et al.,* 2008).

2.13. The Zoonotic Potential of Rotavirus

Rotaviruses have a wide host range, infecting many animal species as well as humans. As it was found that certain animal rotavirus strains had antigenic similarities to some human strains, speculation increased about whether animals play a role as a source of rotavirus infection in humans. There is however an alternative view that animal rotaviruses can indeed infect humans and cause disease whenever the chance exists. This is based on the identification of unusual rotavirus types, with properties of strains more commonly found in animals, which were isolated from various cases of human infection. These unusual human rotavirus types may have arisen either as whole virions or as genetic re-assortants between human and animal strains during co-infection of a single cell (Gorziglia *et al.,* 2006). The segmented nature of the genome suggests that, like other viruses with segmented genomes such as influenza virus, rotaviruses are able to form new strains by a mechanism of re-assortment. Re-assortment can occur when two rotaviruses of two different strains infect the same cell, and during replication and packaging they exchange genome segments (Ramig, 2002). The 11 genome segments of the parental virus strains can theoretically re-assort into 2048 (Flores *et al.,* 1983; Ramig, 2002) different possible genome constellations, if re-assortment is random.

Gouvea and Brantly (1995) hypothesized that rotaviruses exist as mixed populations of reassortants, and that re-assortment was the driving force behind diversity. A prerequisite of diversity is co-circulation of many different rotavirus types in a population; and more diversity, and more frequency of uncommon strains, is seen in years with the highest number of co-circulating strains (Iturriza-Gómara *et al.,* 2001; Jain *et al.,* 2001). Gouvea and Brandtly considered that mixed populations of rotaviruses are being continually propagated in human and animal hosts, resulting in new and diverse progeny populations of rotavirus. With regard to new rotavirus strains arising through re-assortment, a concept of zoonotic genes may be developed. These can be defined as genes originating in animal rotaviruses which can interact with genes of human rotaviruses, to form infectious rotavirus particles which are serially propagated in the human population (Cook *et al.,* 2004).

Until recently, specific rotavirus types have been associated with specific animal species. For example, human rotaviruses most commonly belong to G types 1– 4 and P types [4] and [8] (Gentsch *et al.,* 2011), whereas bovine rotaviruses most commonly belong to G types 6, 8 and 10 and P types [1], [5] or [11] (El-Attar *et al.,* 2002). The rotaviruses have been characterized, the host species specificity of P and G types has become less distinct. Human group A rotavirus strains that possess genes commonly found in animal rotaviruses have been isolated from infected children in both developed and developing countries. Strains such as G3 (found commonly in species such as cats, dogs, monkeys pigs, mice, rabbits and horses), G5 (pigs and horses), G6 and G8 (cattle), G9 (pigs and lambs), and G10 (cattle) have been isolated from the human population throughout the world (Desselberger *et al.,* 2003).

G and P type combinations which are found in man have also been found in animal species. For example, G10P[11] was found in American and Canadian cattle by Lucchelli *et al.* (1994). and in Indian cows and buffaloes by Gulati *et al.* (1999) G3P[6] and G4P[6] were found in pigs in Poland and the USA by Winiarczyk *et al.* (2002) and G1P[8] and G5P[8] were found in pigs in Brazil by Santos *et al.* (1999). The emerging G9 strains 26-28 may have arisen in humans through transfer from animals. They have been found in lambs and pigs (Koch-institut, 1995; Santos *et al.,* 1999).

In humans, they appear to cause more severe symptoms than the common rotavirus strains, (Cubitt *et al.,* 2000; Widdowson *et al.,* 2000), which might be due to less immunity to these emerging strains, or to greater virulence being conferred by their genetic makeup. Several studies have indicated symptomatic infection of humans by animal viruses. Nakagomi and Nakagomi, (1989) reported that almost all gene segments of a rotavirus G3 strain (AU228) isolated from a child with a pet cat were identical to those of a feline rotavirus strain (FRV-1). Strains very similar to this may have become established in humans (Nishikawa *et al.,* 1989). A three week-old baby in an Israeli household which had a young dog (< 6 months old) was infected with an animal rotavirus G3 strain (Nakagomi *et al.,* 1992). Das *et al.* (1993) reported that a G8 rotavirus which had widely circulated in newborn infants in India, causing asymptomatic infection, had VP7 and VP4 gene sequences which were identical to those of a bovine rotavirus strain.

Nakagomi and Nakagomi (1989) considered that available evidence suggested that whereas some feline and canine rotavirus strains have spread into human populations as whole virions, bovine rotaviruses were involved in re-assortment with human rotaviruses, leading to the emergence of unusual strains in various parts of the world. Apparent dual infection with human and animal rotaviruses has been observed Nakagomi and Nakagomi,(1989) recovered G1P[5] and G1P[8] strains from an infant with severe diarrhoea. The G1P [5] rotavirus was genotypically similar to bovine strains. It was not isolated from the infant in high titre, and possibly had little if any effect on the child's disease. Nonetheless it would have had the potential to reassort with the co-infecting strain.

2.14. Control and Prevention of Rotavirus Infections

Rotaviruses are very infectious and comparatively resistant to inactivation by chemical disinfectants and antiseptics. Control and prevention measures against rotavirus infection are not so easy for its mass distribution and tendency to stability in different climate situation and are shed in high concentrations in faeces of infected animals. The primary strategy to reduce the burden of rotavirus infections is vaccination. Vaccination protocol differs from the approaches implemented to protect infants and children against rotavirus disease (Martella *et al.,* 2010b).

In humans, the primary objective is the reduction of maternal antibody level by the age of 4-6 months, active immunity induced by vaccination is elicited to last during the first few years of children lives when the risk of severe infections is the greatest (Dóró *et al.,* 2015). In order to decrease the incidence of disease in the herd, a good producer should maximize colostrums transfer, increase environmental sanitation, reduce stressors such as overcrowding or poor nutrition and vaccinate bred cows for rotavirus at 60 and 30 days before calving (Izzo *et al.,* 2011).

First-milking colostrums are source of nutrients and of passively absorbed maternal antibodies, critical to protect the newborn calf against infectious disease in the first weeks and months of life. The calf is born without most antibodies, including those that fight the infectious agents which cause diarrhea. The calf will acquire these antibodies only from colostrums (Edwards *et al.,* 1982). Because of this, any effort to prevent diarrhea by vaccinating cows is wasted unless the calf actually receives colostrums, preferably before it is two to four hours old. As the calf grows older, it rapidly loses its ability to absorb colostral antibodies. Colostrums given to calves that are more than 24 to 36 hours old are practically useless; antibodies are seldom absorbed this late in life. The neonatal calf should ideally receive 2 to 3L (for beef calves) or 3 to 4L (in dairy calves) of colostrums within the first 6 hours after birth. The colostrums contains antibodies, immune cells (neutrophils, macrophages, T and B cells), complements, lactoferrin, insulin-like growth factor-1, transforming growth factor, interferon, and nutrients (Larson *et al.,* 2004).

To improve the passive immunization of calves against rotavirus and corona virus as well as against different strains of *E. coli* vaccination of the pregnant dam can be proposed. Usually cows are vaccinated twice (6 to 8 and 2 to 3 weeks) before parturition to stimulate the production of specific antibodies. The primary function of colostrums is to enhance the calf's immune system through the passive transfer of both antibody and cell-mediated immunity. Ideally, calves should receive colostrums from their dams although colostrums from several cows is often mixed and administration of colostrums feeding is the transmission of BVDV, bovine leukemia us, an John's disease that can be spread by infected or purchased colostrums (Berge *et al.,* 2006).

Specific IgG present in colostrums may protect against the more common Enteropathogens causing calf diarrhea, such as rotavirus, corona virus and *E. coli*. Although vaccination of the dam prior to calving may boost colostrums IgG concentrations (Heckert *et al.,* 2005; Lorenz *et al.,* 2011). Vaccinate the cows and pregnant heifers with any necessary calf diarrhea vaccines well prior to calving. Vaccines that contain rotavirus, corona virus, and the K99 *E. coli* antigens can be helpful in preventing calf diarrhea. These are best given to the cow prior to calving so it can make antibodies and secrete them into the colostrums. When the calf ingests this enriched colostrums, it will be protected against these major agents (Pithua *et al.,* 2009). In animals, the concept of passive immunization is based on maternal antibodies that are transferable through the placenta or are secreted in the colostrum providing transient protective immunity to offspring against clinically manifest RVA infection. Rotavirus vaccines have been developed to control the neonatal calf diarrhea associated with rotavirus infection. Most of the commercial vaccines are combined with more than one agent (Papp *et al.,* 2013b).

Commercial RVA vaccines are administered parenterally to cows and sows during the late stage of gestation, in order to elicit a strong maternal immunity that is readily conferred to newborn animals. Some studies have demonstrated vaccine failure or breakthroughs that have been related to a number of factors, including inadequate managing conditions of animals or antigenic differences between vaccine and field RVA strains, even if vaccine and field strains shared partially their surface antigen specificities (Saif and Fernandez, 1996). Moreover, optimum management and hygienic practices can minimize the incidence of rotaviral diarrhea in farm animals (Holland, 1990; Steele *et al.,* 2004). To control secondary bacterial infection antibiotics and fluid and electrolyte therapy to restore the fluid reserve, has to be given due importance so that the mortality rate in calves could be minimized (Steele *et al.,* 2004).

3. MATERIAL AND METHODS

3.1. Description of the Study Area

The current study was conducted in four selected areas of central part of Oromia, Ethiopia (Bishoftu, Sebata, Holeta and Addis Ababa) from November 2018 to May 2019 (Figure 3). Fecal samples were collected from four selected of dairy farms. There are many small, medium and large-scale dairy farms in four selected area that supply milk and milk products to consumers of the town and surrounding urban areas. These dairy farms contain either local or exotic breeds depending on the scale of production.

Bishoftu town is found in east Shewa Zone, Oromia Regional State, located about 45 km South-east of the capital city, Addis Ababa. The area is located at 9°N latitude and 40°E longitude at altitude of 1850 m.above sea level. According national meterology agence (NMA) (2016), annual rain fall of 866 mm of which 84% is in the long rainy season (June to September) with annual minimum and maximum temperature of 11and 29°C, respectively. The domestic animals reared in Bishoftu town are 30887cattle, 43138 poultry, 9322 equine, 9294 sheep and 4753 goats (Bishoftu City Administration Agricultural Desk, 2014).

Sebeta town is located in the Oromia Special Zone Surrounding Finfinne (Addis Ababa) of Oromia Region. The district is located 25 km south west of Addis Ababa at an altitude of 1800-3385 m above sea level and at latitude and longitude of 8°55-8.917°N and 38°37-38.617°E respectively. It receives an average annual rainfall of 1073 ml and has temperature that ranges from 11.3-28^{0}C. It has a total area of 102,758 km (SHFDO, 2018). According to the information obtained from Sebeta Hawas district Administration Office (2018), both livestock rearing and crop production are the main economic activities of the majority of communities. The major livestock reared in the district include cattle, sheep, goats and poultry.

Holeta Town is located in the central part of the country, 31 km west of Addis Ababa in Oromia Regional state, west Shewa Administrative Region. The area is bounded between latitude 8º 53' 75" to 9º 14' North and longitude 38º 21' 40" to 38º 36' 14" East. The Town has an area of 5550 hectares. Holeta Town found at an average 2449 m above sea level. The annual mean maximum and the minimum temperatures are 25.9 and 7.2^{0}C, respectively (HTRADO, 2009).

Addis Ababa, the capital city of Ethiopia, lies at an elevation of 2300 m above sea level and is featured by a grassland biome. It is geographically located at 9°1′48″N latitude and 38°44′24″E longitude. It has a typical highland climate with temperature ranging from 11°C-24 °C. Addis Ababa has a mean annual rainfall of 1300 mm with bimodal distribution (NMA, 2016).

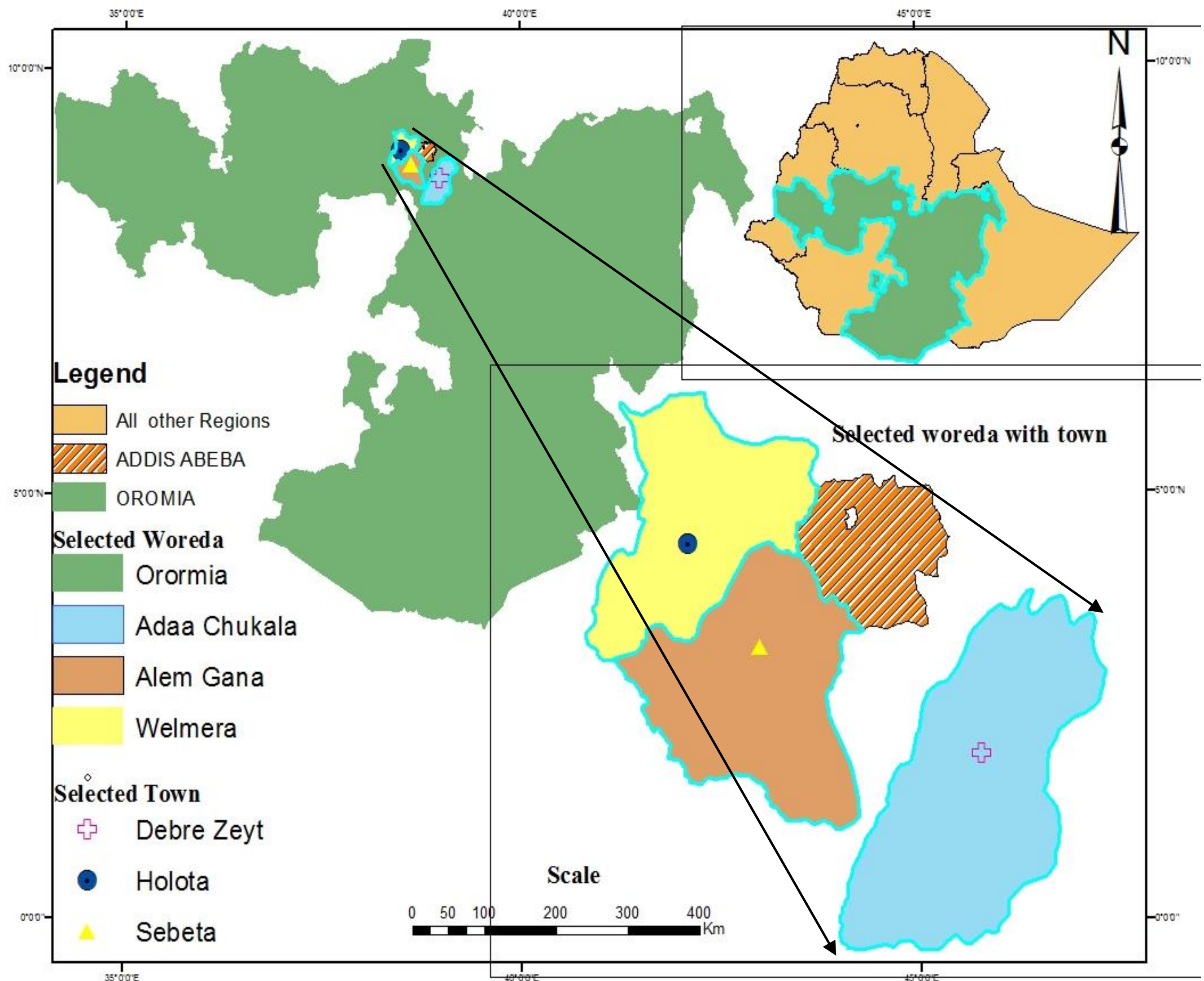

Figure 3: Map of study area and sampling sites.

3.2. Study Population

The study was conducted in both apparently healthy cow calves and calves having clinical sign of diarrhea namely profuse watery diarrhea, systemic dehydration and depressed during investigation. Cow calves up to 30 days of age groups, all breed and sex reared under intensive management conditions were included in the study. Diarrhea was considered if feces are semi-liquid to liquid, with or without other abnormal characteristics such as presence of blood or mucous. Any calf with feces without these characteristics was considered non-diarrheic or apparently healthy (Ammar *et al.,* 2014).

3.3. Study Design

A cross-sectional study was conducted in different dairy farms found in four selected site of (Bishuftu, Sebeta, Holeta and Addis Ababa) central part of Oromia, Ethiopia from November 2018 up to May 2019. Information about the calves was gathered by interviewing farm owners and animal health workers of selected study sites. The information collected were recorded on data collection sheet, and then calves were clinically examined for presence of diarrhea or not and fecal samples were collected for diagnostic testing as follows. At the time of sampling the name of the farm, date of sampling, consistency of feces, age, breed, and tag number was recorded for each calf on proper recording format (APPENDIX-I).

3.4. Sampling Technique and Sample Size Determination

Before the commencement of the actual study, preliminary data were sourced from the respective District Agricultural Office and dairy cooperatives to document the lists of dairy farm in to large scale, medium scale, and small scale dairy farm to estimate the size of study population. The Study areas were purposively selected and identified based on transport accessibility, geographical location and on the abundance of dairy farms to get more calves. Clinically diseased and non-diseased calves were sampled for isolation and characterization of rotavirus. The calves from the seven large scale dairy farms namely; Genesis farm, Asterwaqu dairy farm, Mama dairy Farm, Sisay dimma dairy farm, Haddish dairy Farm, Fantu dairy farm, Holeta agricultural research center dairy farm and a representative random sample of calves from 680 medium and small scale dairy farm were selected

for the study. The sampling units were both local and crossbred dairy calves aged between birth and 1 month. Farms were categorized in to small, medium and large based on the herd size of (5-20), (21-50), and greater than 50 heads of cattle, respectively. In larger farms, a minimum of 10% of the all calves in the farm were sampled.

Considering individual members of dairy cooperatives in each study location as a cluster, cluster sampling method was used to select calves from medium and small scale dairy farm. In this study, sampling frame for study herds was taken from the dairy cooperatives located in Bishuftu, Sebeta, Addis Ababa and Holeta. A total of 680 medium and small scale dairy producers were registered in the dairy cooperatives of study areas. Accordingly, 170 dairy producer were sampled by using systematic random sampling technique (every 4nd dairy producer) from the documented sampling frame. When a selected dairy farmer did not have calf or no pregnant cows with due calving date in the six month cohort period, it was then replaced by another dairy farmer mostly from the nearby area. Sample size for cluster sampling was determined by adjusting the sample size calculated for simple random sampling. The adjustment is the function of average cluster size and intraculster correlation, and mathematically expressed as follows;

$n' = n[(1+ ((m -1)*\rho)]$

Where; **n'** = sample size for cluster sampling

n = sample size calculated for simple random sampling

m = average cluster size

ρ = intracluster correlation

However, in the present study the average herd (cluster) size (calves per each dairy farm) was 1.6. As clustering was found small, the effect of intracluster correlation would be small and **n'** would approximate **n**. So the sample size calculated for random sampling was taken directly to be the sample size for this study. To estimate the prevalence of bovine rotavirus in calves, sample size was determined by using simple random sampling method (Martin *et al.,* 1987; Thrusfield and Christley, 2018).

$$n = \frac{1.96^2 (p)(1-p)}{d^2}$$

p= Expected prevalence

d= Desired level of precision (5%)

n= Sample size

Using expected rotavirus prevalence 16.7% in central Ethiopia (Abraham *et al*., 1992), confidence level of 95% and required absolute precision of 5%; a total of 214 sample size was determined for medium and small scale dairy farm of selected study area. However, a total of 245 calves were enrolled during the study period to enhance precision and to compare prevalence across different herd sizes. Of which, 214 calves were from 680 medium and small scale dairy farm and 31 calves from seven large scale dairy farms.

3.4.1. *Collection of fecal samples*

Fecal samples were collected in sterile tube after cleaning of the anal area with a paper towel and beats by rectal stimulation with the index finger using disposable sterile plastic gloves (Ammar *et al.,* 2014). Approximately 30 grams of fecal material was collected directly from the rectum of calves using disposable latex glove. Collected samples were placed into universal ice box containing ice packs and transported to the virology laboratory at National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebeta and were stored at -80 °C until processing.

3.5. Laboratory Techniques

3.5.1. *Detection of bovine rotavirus antigen by ELISA*

Multiscreen Ag ELISA Calf digestive (BIO K 314/1, Belgium) is the type a sandwich ELISA capturing mixture of monoclonal antibodies (MAbs) against bovine rotavirus (BRV) was used to detect BRV Ag in the fecal suspensions. These antibodies capture the corresponding Ag in the fecal samples. The sandwich ELISA procedure was performed according to the manufacturer instruction (Kit reference BIO K 314/1, Belgium).

The ELISA was performed to detect rotavirus Ag in the fecal samples. The 96 well plates provided in the kit contained two different capture antibodies. Rows C, E, D, F, H and G were coated with rotavirus specific capture antibodies and rows A and B coated with non-specific antibodies, which acted as controls (positive and negative control). These control rows allow the differentiation between specific immunological reaction and non-specific bindings so as to eliminate false positives.

Feces were diluted in the dilution buffer provided in the kit. A volume of 100µl of diluted sample was added to corresponding wells of specific and non-specific antibody coated rows, respectively. Similarly both the positive and negative controls were added to their respective well per plate. The plate was incubated at 25°C for 0.5 hour and washed 3 times with washing solution (diluted in the ratio 1:20 with distilled water) provided in the kit. Ready to use conjugate of rotavirus specific monoclonal antibody labelled peroxidase was used as such and poured in 100µl quantities per well. The plates were covered with a lid and incubated at 25°C for 0.5 hour in a dark room and washed three times with the provided washing buffer. Then 100 µl of the chromogen (tetramehtyl benzidine) solution added to each well on the plate. The plates were then incubated for 10 minutes at 25°C without covering and away from direct light.

Finally the reaction was stopped by adding stop solution (1M phosphoric acid) provided in the kit. The optical density was measured at 450nm after stopping the reaction with 50µl per well of stop solution. The optical density was measured at 450 nm using an ELISA plate reader at 450nm immediately after stopping the reaction with the stop solution. The test was validated using the positive control and data sheet provided by the kit. The net optical density of each sample was calculated by subtracting the reading for each sample well from corresponding negative control. Net optical density (O.D.) = (O.D. of specific binding -O.D. of non-specific binding. The ELISA reader was used to transfer optical density values to excel spread sheet of computer connected to the reader. Even positive and negative reaction results in ELISA was decided based on color changes after adding stop solution as well as calculating the optical density value. Blue color changed to yellow after adding stop solution was recognized as positive and optical density value was > 0.15 Elisa units (EU) for positive and < 0.15 Elisa units (EU) for negative to bovine rotavirus.

3.5.2. Extraction of rotavirus RNA

Rotavirus RNA was extracted from the fecal suspension using QIAamp viral RNA mini kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer's instructions. About 1g of fecal sample was added to 1ml of phosphate buffer saline (PBS). The mixture was vortexed vigorously for 40 seconds followed by centrifugation at 10,000 rpm for 5 minutes. All the supernatant (about 500µl) was transferred to new tubes.

Briefly, 140 µl of original fecal supernatant was added in to 560 µl buffer AVL-carrier RNA in the microcentrifuge tube, vortexed for 15 seconds to ensured efficient lysis and homogeneous solution, then the incubated at room temperature (15-25°C) for 10 minutes to lysis viral particle. The solution was centrifuged to remove drops from the inside of the lid then 560 µl ethanol (96%) was added in to the sample, mixed by vortexing for 15 seconds and again centrifuged the tube to remove drops from inside the lid. Then 630 µl from the solution was taken and pipetted in to the QIAamp Mini column and centrifuged at 8000 rpm for 1 minutes and the filtered was discarded. This action was repeated twice.

Then 500 µl buffer AW1 was added to QIAamp Mini column and centrifuged at 8000 rpm for 1 min. The filtrate was discarded and the column was placed in a fresh 2ml collection tube .Then 500µl of buffer AW2 were added to the column then centrifuged at 14,000 rpm for 3 min and the filtrate was discarded. Carefully the QIAamp mini column was opened and added 60 µl Buffer AVE equilibrated to room temperature. Then 65µl of Buffer AVE was added to the column, equilibrated at room temperature for 1 minute then centrifuged at 8000 rpm for 1 minute. Finally, the nucleic acid of the virus was obtained. A double elution using 2 x 40 µl Buffer AVE was performed to increased yield. Final extracted viral RNA was stored in -80^{0}C for further processing.

3.5.3. Viral isolation

All ELISA positive fecal samples were taken forward for virus isolation. Approximately 1 gram fecal sample was mixed with 9 ml sterile PBS containing antibiotic. The fecal suspension was then centrifuged at 800 rpm for 15 minutes. The supernatant fluids contain rotavirus positive were filtered through 0.45 µm membrane syringe filter and filtrates were mixed with an equal volume of Dulbecco's Modified Eagle Medium (DMEM) containing 5% fetal calf serum (FCS) and 10 µg/ml crystalline trypsin and incubated at 37°C for 60 minutes. After incubation, one ml of the mixture was inoculated into the culture flasks with confluent monolayer of Madin Darby bovine kidney (MDBK) cell lines and kept for 1hour incubation to adsorption virus. After the adsorption at 37°C for 1 hour, the cells were washed three time with plain of DMEM maintenance media and incubated at 37°C in a humidified incubator having 5% CO_2. Monolayers were observed daily for development CPE for five days and viruses were sub-cultured blindly every two days after being subjected to 3 cycle of freezing and thawing. CPE was observed after 48 hours in positive case and it was characterized by a destruction of the monolayer cell, cell rounding and infected cells were disrupted and detached from the flask. Cells showing characteristic CPE were harvested by freezing and thawing thrice and centrifuged at 16,000 rpm for 20 minutes at 4°C for the removal of cell debris. The supernatant containing the virus was collected and stored at -80°C for further passages. If no CPE was observed, the sample was considered as 'no virus detected' (NVD) and the culture was frozen at -80°C, then thawed and centrifuged at 3,000 rpm for 10 minutes to collect supernatant for second blind passage (P2). This was repeated for third passage (P3); and if no CPE was observed on the third passage after 48 hours inoculation, then the sample was considered negative for rotavirus.

3.5.4. Reverse transcription polymerase chain reaction (RT- PCR)

The RT-PCR reaction was performed by using a RT-PCR Kit (QIAGEN) for the confirmation of bovine rotavirus A. Primers for the amplification of gene segments VP4 were synthesized (Table 7) based on the previous study (Park *et al.,* 2006). For the desired amplification of VP4 gene (880 base pairs) of bovine rotavirus, optimization of PCR conditions was done by using the varying concentrations of dsRNA, primers and Taq polymerase in RT-PCR reaction mixture with total volume of 25 µl. Optimized reaction mixture for RT-PCR was dsRNA 2.5 µl, PCR buffer 2.5 µl, dNTPs 2.5

μl, MgC_2 2.5 μl, Forward Primer F (10 pmol) 3.0 μl, Reverse Primer 10μM (6μl), DNase/RNase free water 6 μl. Conditions for the amplification of VP4 gene of bovine rotavirus includes, before applying RT-PCR reaction, 2.5μl of viral dsRNA were denatured at 95°C for 5 minutes and chilled immediately for 5 minutes. Then, reaction was carried out under the following conditions. RT-PCR was carried out with an initial reverse transcription step of 60 minutes at 42^0C, followed by PCR for activation at 94^0C for 15 minutes, 40 cycles of amplification (30 seconds at 94^0C, 45 seconds at 55^0C (annealing), and 45 seconds at 72^0C (extension)), with a final extension of 7 minutes at 72^0C. To analyze the PCR product, agarose gel electrophoresis was performed. For this, 1.5% gel was prepared and 1 μl of 100 base pair (bp) ladder along with the PCR product was run at 110 volts for 45 minutes. The size of the PCR product for VP4 gene (880 bp) was illuminated in a gel documentation system and a photograph was taken.

Table 7: Primer details.

Primer	Primer sequences	Size of amplicons	Melting Temp	Primer Length
VP4-F	TGGCTTCGCTCATTTATAGACA	880 bp	54.2	22
VP4-R	ATTTCGGACCATTTATAACC	880 bp	47.4	20

3.6. Data Management

The collected data were entered in Microsoft Excel. The contingency table was used at 5% significance to assess the differences among the proportions of fecal samples positive to rotavirus variables such as age group and sex of the animals studied by using Chi Square. Quantitative data was coded and entered in a computer spread sheets and the Stata 13 software was used for the data analysis.

3.7. Ethical Considerations

All procedures for this study was approved by the Animal research ethics and review committee of College of Veterinary Medicine and Agriculture, Addis Ababa University.

4. RESULTS

In total, 245 diarrheic and non-diarrheic fecal samples of calves and their relevant herd and farm level information were collected and analyzed to determine the prevalence of rotavirus infection in dairy calves, in central part of Oromia, Ethiopia. Among 245 calves, 83 (33.88%) had diarrhea and 162 (66.12%) had no diarrhea at the time of sampling. The overall prevalence of rotavirus infection in calves less than four weeks of age was estimated as 2.45% (6/245) by antigen capture sandwich ELISA (Figure 4) in selected dairy farm.

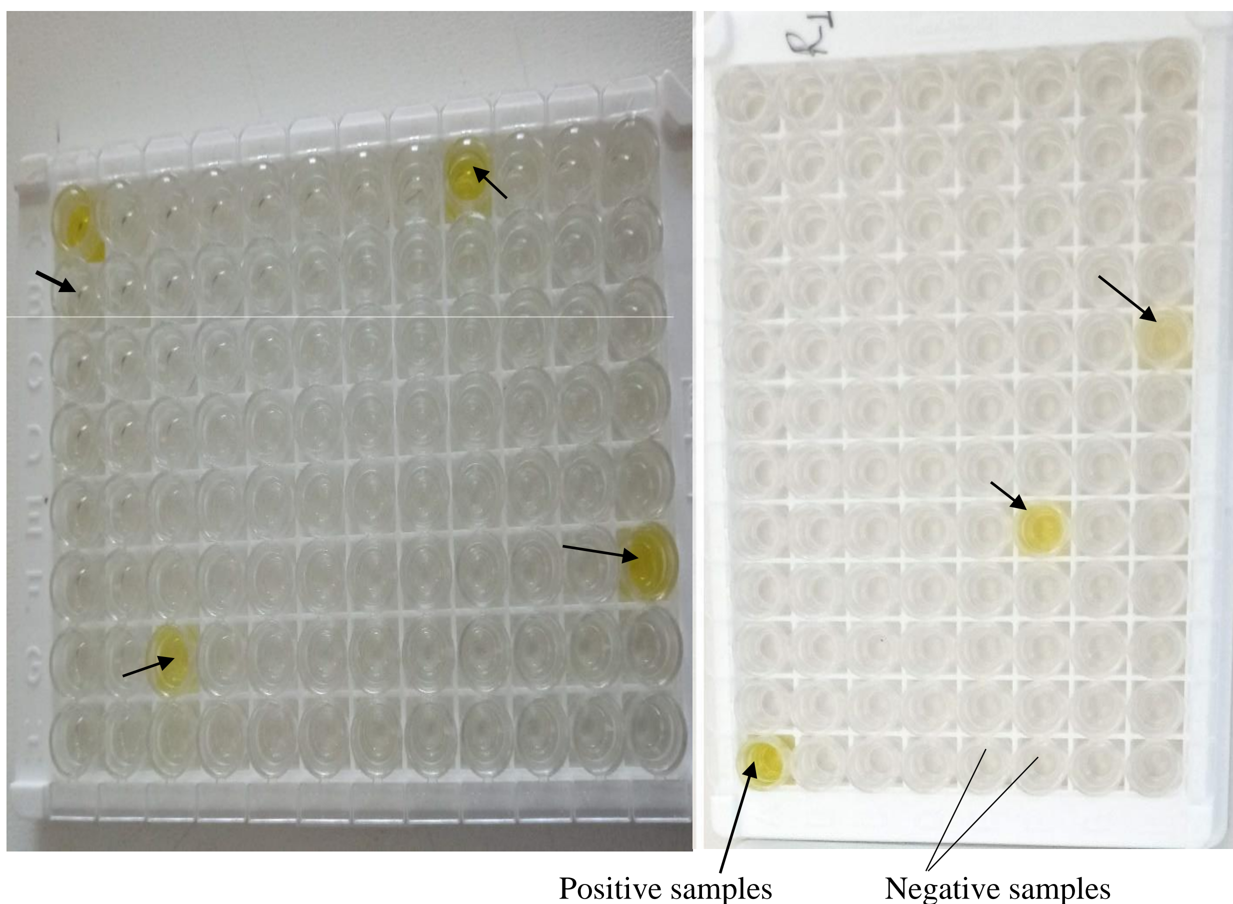

Figure 4: Results of ELISA in a Plate.

All the arrow with yellow color indicated the positive sample and non-arrow with yellow color positive control while arrow with white color was negative control.

When the results was calculated separately for the two groups of calves (i.e. diarrheic and none diarrheic calves), a prevalence of 7.23% (6/83) of rotavirus was observed in diarrheic calves and all none diarrheic samples were negative (0/162, Table 8). Distribution of antigen positive samples corresponding to ages, breed and sex of calves were shown in Table 8. The results indicate a prevalence for calves in the first week of ages was 4%. This shows that new born calves of 1-2 weeks of age were more susceptible to rotavirus infection. But the observed rotavirus prevalence in different calve ages were not significant ($P > 0.05$).

Current research showed that the males were more susceptible to rotavirus infection as compared to female calves. A higher prevalence of 6.2% (5/81) was associated with male calves, while a prevalence of 0.6% (1/164) was recorded in female calves. The prevalence were significant for rotavirus between male and female calves (Chi-square = 7.0239; p = 0.008). A higher prevalence (4.9%) of rotavirus Ag was observed among calves fed colostrum's from 30 minutes to 2 hours compared to calves given colostrum's within 30 minutes of birth (0 .8%). New born calves of cross breed cows were more susceptible to rotavirus infection than local breed (1.4%). The result showed a prevalence of cross breed was 2.9%. The rotavirus prevalence was not significant ($P > 0.05$) for breed and between times of colostrums feeding. The current study indicated that the prevalence of rotavirus infection was higher in Sebeta (4%) as compared with other selected site. The rotavirus prevalence was not significant ($P > 0.05$) between the selected locations.

Calves floor area was concrete, more susceptible to rotavirus infection than the calves floor area was brick or muddy. The result showed a prevalence of 2.8%. The rotavirus prevalence was not significant ($P > 0.05$) between the calves floor area. The result indicated that 6.9% calves separated immediately after birth from dam were found positive, whereas rotavirus was detected in 2.6% samples of calves separated greater than 24 hours after birth from dam (Table 8). The prevalence's were not significant ($P > 0.05$) for rotavirus between the times of separations of calves from dam. See Table 8 for stratified prevalence of other variables.

Table 8: Distribution of bovine rotavirus in screened both diarrheic and none-diarrheic samples.

Distributions of calf parameters	**Level**	**Number of samples screened**	**Number of samples positive**	**Prevalence (%)**	**Chi square**	**P-value**
Calves Health	Diarrheic	83	6	7.23	12.0048	0.001*
	Non-diarrheic	162	0	0		
Sex	Male	81	5	6.2	7.0239	0.008*
	Female	164	1	0.6		
Age	1st week	100	4	4	2.1263	0.547
	2nd week	106	2	1.8		
	3rd week	18	0	0		
	4th week	21	0	0		
Location	Bishoftu	60	2	3.3	1.3771	0.711
	Addis Ababa	123	3	2.4		
	Sebeta	25	1	4		
	Holeta	37	0	0		
Breed	Cross	174	5	2.9	0.0461	0.830
	Local	71	1	1.4		
Floor of the calves area	Concrete	179	5	2.8	0.4364	0.804
	Brick	60	1	1.7		
	Muddy	6	0	0		
First colostrum feeding after birth	Within 30 minutes	132	1	0.8	4.3263	0.115
	Within 2 hours	103	5	4.9		
	Within 2-6 hours	10	0	0		
Separation of calves from dam	Immediately after birth	29	2	6.9	4.2418	0.120
	<24 hours	71	0	0		
	>24 hours	145	4	2.6		

NOTE: P value - Level of significance. Significant when P-value ≤0.05.

In this study, MDBK cell line was used to isolate the virus from all samples of Ag-ELISA test positive samples. Out of 6 samples cultured on MDBK cell line, CPE was observed in 4 (66.7%) samples, while CPE was not observed on the remaining 2 samples (33.33%) even on third blind passage. In the first passage, infected cells did not show any CPE. But from second passage onwards the infected cells started showing characteristic CPE. At 24 hours post infection (p.i,) the infected cells became round and clumped. At 48 hours p.i, the cells were thin and round shaped. At 72 hours p.i, the cells became small and majority of monolayer detached (Figure 5).

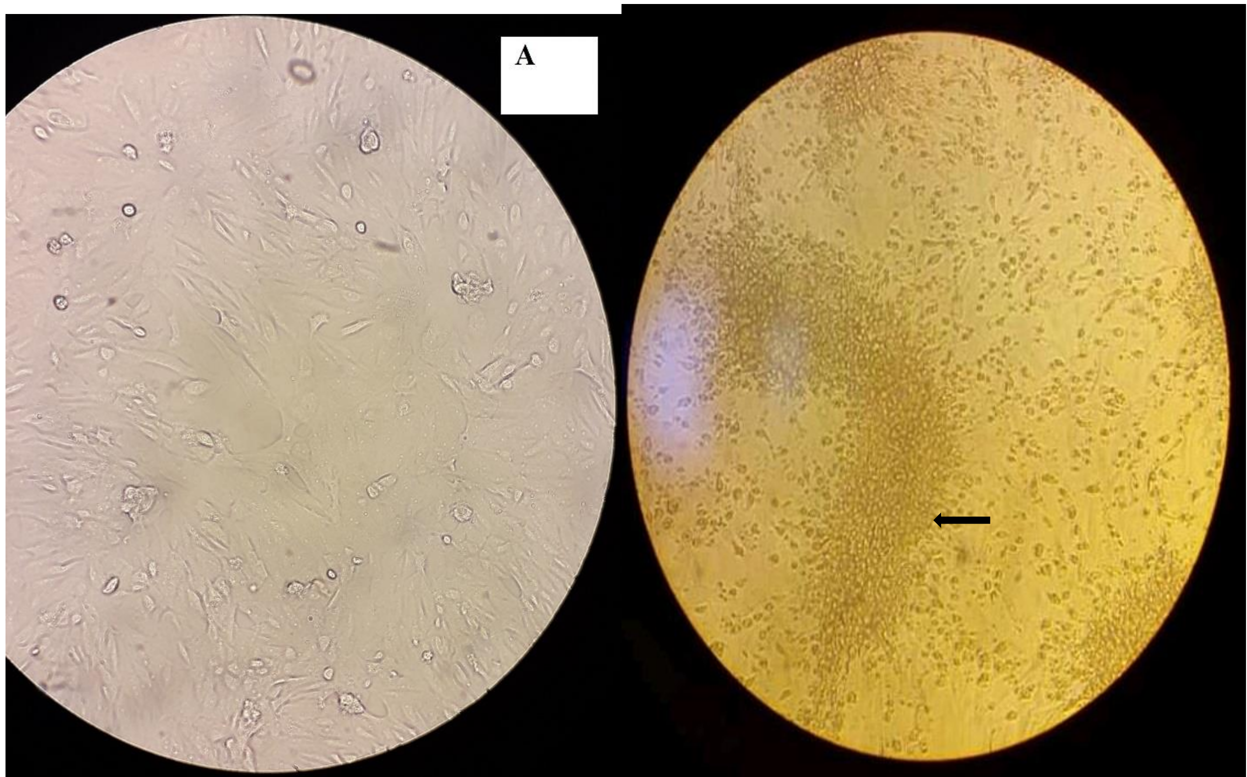

Figure 5: Characteristic CPE of rotavirus inoculated on Madin Darby bovine kidney cell line.

Shows rounding and cell detachment from the flask and lysis of cells (indicated by arrow) against respective controls (A).

Out of 6 positive samples 4 samples were screened by RT-PCR for molecular characterization due to the non-availability of sufficient quantity of fecal sample in the remaining samples. RT-PCR amplification of the 880 base pair fragment of the VP4 gene in the four samples that developed a CPE allowed to confirm the presence of rotaviruses (Figure 6). The Ag-ELISA negative calves sample and negative on cell culture did not amplify by PCR. After RNA extraction and RT-PCR was done for amplification of VP4 gene. Out of the 4 fecal samples examined by RT-PCR technique, all samples were identified as positive (100%) and as expected, length of VP4 gene (880 bp) was generated for rotaviruses (Fig.6).

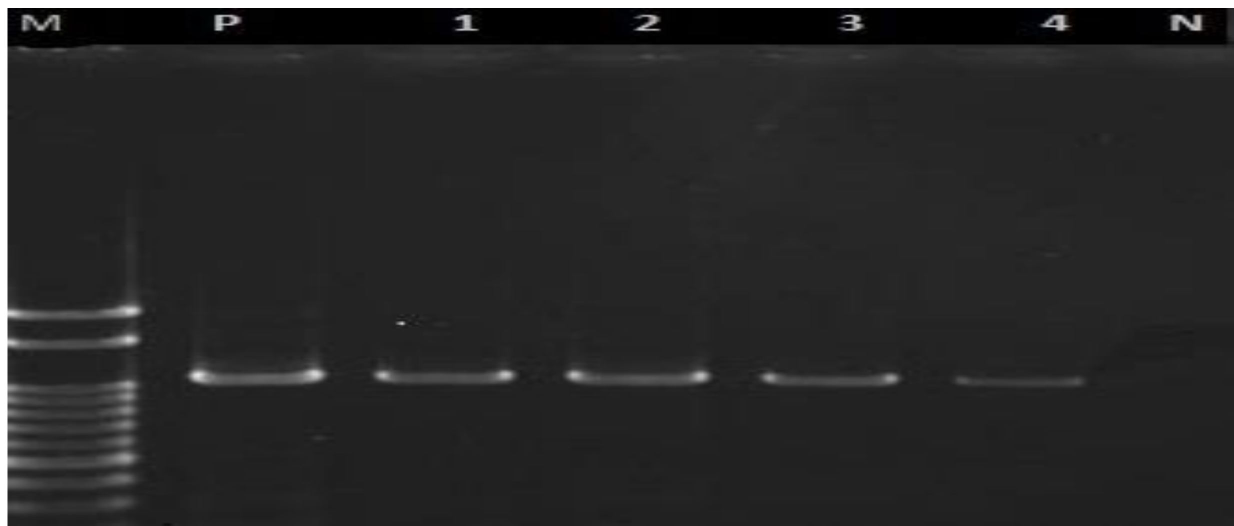

Figure 6: cDNA band corresponding to rotavirus VP4 genes detected on agarose gel.

From left to right: Lane M, Molecular weight markers; Lane P, is positive control; Lane N, is negative control; Lane 1,2,3,4, is positive sample.

5. DISCUSSIONS

Newly born calves represent worldwide an important source in livestock production for meat or breeding i.e. replacement stock (Radostits *et al.,* 2007). These industry faces many series disease problems like calf diarrhea, which usually affect it dramatically. Neonatal calf diarrhea is a prime disease affecting newborn calves leading to morbidity and mortality in newborn calves, causing economic losses due to the costs of treatment, diagnostics, weight loss or death in infected animals and poor growth performance. A crucial period for these calves is the first few days following birth. In developing countries like Ethiopia, domestic animals are the major income source for poor families. These families suffered badly due to the neonatal calf mortality curse. Among numerous viral, bacterial and parasitic causative agents, bovine rotavirus is the foremost cause of neonatal calf diarrhea in domestic animals. The cause of neonatal calf mortality is specifically related to group A bovine rotaviruses (Estes and Kapikian, 2007). Fecal contamination plays an important role in transmission of rotavirus infection and the infection is widespread globally in cattle populations. For the effective control measures, prompt diagnosis of the disease is important (Dhama *et al.,* 2009).

In the present study, 6 of 245 fecal samples screened using Ag-ELISA was positive for rotaviral infection and all the positive animals were diarrheic calves while all the non-diarrheic calves were negative. The prevalence was found to be 7.23% in diarrheic calves. This result is in agreement with those reported by Prez *et al.* (1998) in Costa Rica (7%), Duman and Aycan (2010) in Turkey (8.5%), Yilmaz (2016) in Turkey (8.92%), and Rajendran and Kang (2014) in India (5.5%). Higher prevalence rate of rotavirus have been reported from many countries including Abraham *et al*. (1992) in Ethiopia (16.7%), Ammar *et al.* (2014) in Algeria (14.63%), Kyle (2007) in Vietnam (15%) , Pisanelli *et al.* (2005) in Southern Italy (16.8%), Jindal *et al.* (2016) in India (27.02%) and Uhde *et al.* (2008) in Switzerland (58.7%). However, the result of this study is higher when compared to that reported by Fiedler *et al.* (1982) in Oldenburg (1.96%). The discrepancy of results could be attributed to the age and the sample size difference. Prevalence of rotaviral infection varies depending on the country and region under study (Basera *et al.,* 2010; Radostitis *et al.,* 2007). All the 6 rotavirus positive samples were from diarrheic calves under the age of 4 weeks. Similar results were recorded by Sharma (2004) in bovine calves.

The current result could suggest that male calves (6.2%) were higher susceptible for rotavirus infection than female calves (0.6%). Other studies like Dash *et al.* (2011) and Sharma (2004) also reported higher susceptibility of male bovine calves (20.37%) and (42.85%) in comparison to female calves (12.76%) and (28.2%), respectively. The possible justification for this could be due to immune system in that Odde (1988) reported higher anti-rotavirus IgG concentrations for female calves compared to male calves. It could be due to the managemental practices as in most of the dairy farms female calves are better looked after than male calves. Previously, Ammar *et al.* (2014) and Dash *et al.* (2011) also reported higher susceptibility of male bovine calves in comparison to female calves against rotavirus infection. In line with this, Clement *et al*. (1995) noticed that males calves were more susceptible to diarrhea as compared to female calves.

Age wise, the susceptibility of newborn calves of 1st week up to 2nd weeks of age to rotavirus infection were more than older calves. The occurrence of rotavirus in the fecal samples of diarrheic calves was found to decrease with increase in the age of the calves. The finding of the present study is in agreement with the earlier workers reported by Abraham *et al.* (1992), Jindal *et al.* (2000) and Singh *et al.* (2009), higher occurrence of rotavirus infection in diarrheic calves were mainly restricted to the first 2 weeks of life. Maximum prevalence of rotavirus diarrhea was observed in 5-21 days old calves (Radostits *et al.,* 2007). The 2 weeks old calves were the most susceptible to rotavirus infections, which may be due to decreasing of passive immunity and the absence of the natural resistance against infection. The 3-weeks-old calves are characterized by absence of rotavirus, this may be highlighted by an increased natural resistance against infection (Ammar *et al.,* 2014).

The results showed that the prevalence was slightly higher in the Sebeta (4%) than in Bishoftu (3.3%) and Addis Ababa (2.4%) towns. This could be attributed to sample size of the areas and presence of higher number of factories in the near farm that can be a source of contamination for animals. In the present study higher prevalence was recorded in crossbred calves (4.2%) than local calves (2.8%) that similar to previous report of Sharma (2013).

In the present study, viral growth in cell culture was assessed by examining inoculated cells for CPE. Out of 6 samples, Only 4 of the 6 Ag-ELISA positive samples established infection in MDBK cells as determined by production of characteristic CPE on the second passage and it continued up to third passages. The CPE observed were characterized by rounding, detachment as well as destruction of mono-layer cell. The CPE produced in this study were in agreement with previous reports *(McNulty et al.,* 1977; Nagesha *et al.,* 1985; Saravanan *et al.,* 2006).

The RT-PCR technique confirmed the presence of rotaviruses in fecal samples that were previously diagnosed by ELISA and growth in cell culture. The RT-PCR-based genotyping method used was further confirmed to be a useful epidemiological tool and to determine the presence of rotavirus nucleic acid by using specific generic primers for VP4 genetic regions in feces samples (Desselberger *et al.,* 2003). This study is comparable with previous reports, (Hammami *et al.,* 1990; Reidy *et al.,* 2006).

6. CONCLUSION AND RECOMMENDATIONS

The present investigation was undertaken to investigate the prevalence, isolation and characterization of rotavirus in calves lessen than one month of age at different selected farms in central Oromia. The effect of age, sex, breed and house floor of calves on prevalence of diarrhea was also studied. Using Ag-ELISA, 6 samples were identified as positive, and all of the isolates were obtained from diarrheic calves in the 1st and 2nd weeks of age. The result indicated that there is an association between rotavirus detection and sex of calves in that the prevalence of rotavirus is higher in male calves than female calves. In addition, the prevalence is higher in calves kept in concrete floor, these fed colostrum later (within 2 hours) as well as in local bred calves and in calves separated from their dams immediately after birth. When the 6 Ag-ELISA positive samples were cultured on MDBK cell line, only 4 samples showed cytopathic effect (CPE). Observational study and questionnaire survey also indicated that only awareness of the advantage of colostrum feeding is not enough, but also times of colostrum administration to neonate calves are crucial for the ultimate development of immune status against pathogens including rotavirus infection. Calving areas should have well-drained grass lots or pastures visible from the barn area and calving areas should be selected or landscaped to allow for adequate drainage. Enteric disease like rotavirus infection is a vital health problem in calves that interrupts production benefits with reduced weight gain and increased mortality, and the virus potential for its zoonotic spread, it is imperative to determine the disease burden and responsible risk factors. This is very useful to execute effective preventive measures such as practicing early colostrum feeding in newborn calves, vaccination in dams and improving livestock management.

Based on the above conclusion the following recommendations were forwarded:

- Awareness creation for researcher and government regarding the effect of rotavirus infection in calves health and growth performance and national economy is very important.
- Further study of rotavirus infection in calves covering larger areas of the country need to be conducted so that representative information of the circulating strains cloud be generated and understood. Availability of such data are critical for control of the disease.
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7. REFERENCES

Abraham, G., Roeder, P., Zewdu, R.. (1992): Agents associated with neonatal diarrhoea in Ethiopian dairy calves. *Trop. Anim. Health Prod*. **24**: 74–80.

Acha, S., Kühn, I., Jonsson, P., Mbazima, G., Katouli, M., Möllby, R. (2004): Studies on calf diarrhoea in Mozambique: prevalence of bacterial pathogens. *Acta Vet. Scand*. **45**: 27.

Agutu, M., Ongus, J., Kombich, J., Kamenwa, R., Nyangao, J., Kagira, J., Ogutu, A., Bitek, A. (2017): Prevalence and genetic diversity of rotavirus infection in children with acute gastroenteritis in a hospital setting, nairobi Kenya in post vaccination era: A cross-sectional study. *Pan Afr. Med. J*. **26**: 1–12.

Al-Robaiee, I., Al-Farwachi, M. (2013): Prevalence of rotaviral infection in diarrheic neonatal calves in Mosul city, Iraq. *Vet. World*. **6**: 538.

Alfieri, A., Parazzi, M., Takiuchi, E., Médici, K., Alfieri, A. (2006): Frequency of group A rotavirus in diarrhoeic calves in Brazilian cattle herds, 1998–2002. *Trop. Anim. Health Prod*. **38**: 521.

Ammar, S., Mokhtaria, K., Tahar, B., Amar, A., Redha, B., Yuva, B., Mohamed, H., Abdellatif, N., Laid, B. (2014): Prevalence of rotavirus (GARV) and coronavirus (BCoV) associated with neonatal diarrhea in calves in western Algeria. Asian Pac. *J. Trop. Biomed*. **4**: S318–S322.

Anderson, E., Weber, S. (2004): Rotavirus infection in adults. *Lancet Infect. Dis*. **4**: 91–99.

Arias, C., Romero, P., Alvarez, V., Lopez, S. (1996): Trypsin activation pathway of rotavirus infectivity. *J. Virol*. **70**: 5832–5839.

Athanassious, R., Marsolais, G., Assaf, R., Dea, S., DescOteaux, J., Dulude, S., Montpetit, C. (1994): Detection of bovine coronavirus and type A rotavirus in neonatal calf diarrhea and winter dysentery of cattle in Quebec: evaluation of three diagnostic methods. *Can. Vet. J*. **35**: 163.

Ball, J., Tian, P., Zeng, C., Morris, A., Estes, M. (1996): Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein. *Science*. **272**: 101–104.

Barnes, G., Callaghan, S., Kirkwood, C., Bogdanovic-Sakran, N., Johnston, L., Bishop, R. (2003): Excretion of serotype G1 rotavirus strains by asymptomatic staff: A possible source of nosocomial infection. *J. Pediatr*. **142**: 722–725.

Barrington, G., Gay, J., Evermann, J. (2002): Biosecurity for neonatal gastrointestinal diseases. *Vet. Clin. North Am. Food Anim. Pract*. **18:** 7–34.

Bartels, C., Holzhauer, M., Jorritsma, R., Swart, W., Lam, T. (2010): Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. *Prev. Vet. Med*. **93:** 162–169.

Barua, S. (2019): Clinico-pathology and Molecular characterization of bovine rotavirus infection in calves in south-eastern part of Bangladesh.

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

Bendali, F., Bichet, H., Schelcher, F., Sanaa, M. (1999): Pattern of diarrhoea in newborn beef calves in south-west France. *Vet. Res*. **30**: 61–74.

Berge, A., Moore, D., Sischo, W. (2006): Field trial evaluating the influence of prophylactic and therapeutic antimicrobial administration on antimicrobial resistance of fecal Escherichia coli in dairy calves. *Appl. Environ. Microbiol*. **72**: 3872–3878.

Bernstein, D. (2009): Rotavirus overview. *Pediatr. Infect. Dis. J.* **28**: S50–S53.

Bhat, S., Juyal, P., Singla, L. Das, (2013): Bovine cryptosporidiosis: brief review of its distribution in india. *Trends Parasitol. Res* **2**: 5–13.

Bhat, S., Juyal, P., Singla, L. (2012): Prevalence of cryptosporidiosis in neonatal buffalo calves in Ludhiana district of Punjab, India. *Asian J Anim Vet Adv.* **7**: 512–520.

Bishop, R., Davidson, G., Holmes, I., Ruck, B. (1974): Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis. *Lancet.* **303**: 149–151.

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

Bizuneh, T., S/Mariam, Z., Abebe, A., Lema, E. (2004): Rotavirus infection in under-five children in Jimma Hospital, Southwest Ethiopia. *Ethiop. J. Heal. Dev*. **18:** 19–24.

Björkman, C., Svensson, C., Christensson, B., De Verdier, K. (2003): Cryptosporidium parvum and Giardia intestinalis in calf diarrhoea in Sweden. *Acta Vet. Scand*. **44**: 145.

Blanchard, P. (2012): Diagnostics of dairy and beef cattle diarrhea. *Vet. Clin. Food Anim. Pract*. **28:** 443–464.

Brandt, C., Kim, H, Rodriguez, W., Thomas, L., Yolken, R., Arrobio, J., Kapikian, A., Parrott, R. and, Chanock, R. (1981): Comparison of direct electron microscopy, immune electron microscopy, and rotavirus enzyme-linked immunosorbent assay for detection of gastroenteritis viruses in children. *J. Clin. Microbiol*. **13**: 976–981.

Bugarc̀ić, A., Taylor, J. (2006): Rotavirus nonstructural glycoprotein NSP4 is secreted from the apical surfaces of polarized epithelial cells. *J. Virol*. **80**: 12343–12349.

Bwogi, J., Malamba, S., Kigozi, B., Namuwulya, P., Tushabe, P., Kiguli, S., Byarugaba, D., Desselberger, U., Iturriza-Gomara, M., Karamagi, C. (2016): The epidemiology of rotavirus disease in under-five-year-old children hospitalized with acute diarrhea in central Uganda, 2012-2013. Arch. Virol. **161**: 999–1003.

Carlton, W., McGavin, M. (1995): Thomson's special veterinary pathology, Mosby-Year Book. St. Louis 152–153.

Carvalho-Costa, F., de Assis, R., Fialho, A, Araújo, I., Silva, M., Gómez, M., Andrade, J., Rose, T., Fumian, T., Volotão, E., Miagostovich, M., Leite, J. (2019): The evolving epidemiology of rotavirus A infection in Brazil a decade after the introduction of universal vaccination with Rotarix®. *BMC Pediatr*. **19**: 42.

CDC, (2008a): Rotavirus surveillance--worldwide, 2001-2008. *MMWR. Morb. Mortal. Wkly. Rep*. **57**: 1255.

CDC, (2008b): Delayed onset and diminished magnitude of rotavirus activity--United States, November 2007-May 2008. *MMWR. Morb. Mortal. Wkly. Rep*. **57**: 697.

Chan, J., Nirwati, H., Triasih, R., Bogdanovic-Sakran, N., Soenarto, Y., Hakimi, M., Duke, T., Buttery, J., Bines, J., Bishop, R. (2011): Maternal antibodies to rotavirus: could they interfere with live rotavirus vaccines in developing countries? Vaccine **29**: 1242–1247.

Chang, K., Parwani, A., Saif, L. (1996): The characterization of VP7 (G type) and VP4 (P type) genes of bovine group A rotaviruses from field samples using RT-PCR and RFLP analysis. *Arch. Virol*. **141**: 1727–1739.

Chen, D., Zeng, C., Wentz, M., Gorziglia, M., Estes, M., Ramig, R. (1994): Template-dependent, in vitro replication of rotavirus RNA. *J. Virol.* **68**: 7030–7039.

Chen, S., Chang, Y., Lee, Y., Chao, H., Tsao, K., Lin, T., Ko, T., Tsai, C., Chiu, C. (2007): Molecular epidemiology and clinical manifestations of viral gastroenteritis in hospitalized pediatric patients in Northern Taiwan. *J. Clin. Microbiol*. **45**: 2054–2057.

Cho, Y., Kim, W., Liu, S., Kinyon, J., Yoon, K. (2010): Development of a panel of multiplex real-time polymerase chain reaction assays for simultaneous detection of major agents causing calf diarrhea in feces. *J. Vet. diagnostic Investig*. **22**: 509–517.

Cho, Y. (2012): Ecology of calf diarrhea in cow-calf operations.

Cho, Y., Yoon, K. (2014): An overview of calf diarrhea-infectious etiology, diagnosis, and intervention. *J. Vet. Sci.* **15**: 1–17.

Clement, J., King, M., Salman, M., Wittum, T., Casper, H., Odde, K. (1995): Use of epidemiologic principles to identify risk factors associated with the development of diarrhea in calves in five beef herds. *J. Am. Vet. Med. Assoc*. **207**: 1334–1338.

Collins, P., Mulherin, E., Cashman, O., Lennon, G., Gunn, L., O'Shea, H., Fanning, S. (2014): Detection and characterisation of bovine rotavirus in Ireland from 2006–2008. *Ir. Vet. J*. **67**: 13.

Cook, N., Bridger, J., Kendall, K., Gomara, M., El-Attar, L., Gray, J. (2004): The zoonotic potential of rotavirus. *J. Infect.* **48**: 289–302.

Corl, B., Mathews Oliver, S., Lin, X., Oliver, W., Ma, Y., Harrell, R., Odle, J. (2008): Conjugated linoleic acid reduces body fat accretion and lipogenic gene expression in neonatal pigs fed low- or high-fat formulas. *J. Nutr*. **138**: 449–454.

CSA, Centeral Statics Agences of the F.D.R. of Ethiopia,(2015): Report on Livestock and Livestock Characteristics (Private Peasant Holdings). *Statistical Bulletin*. **578**: 1–181.

Cubitt, W., Steele, A., Iturriza, M. (2000): Characterisation of rotaviruses from children treated at a London hospital during 1996: emergence of strainsG9P2A [6] and G3P2A [6]. *J. Med. Virol*. **61**: 150–154.

DACA, S.V.T.G.L. (2006): for Veterinary Practice.

Das, M., Dunn, S., Woode, G., Greenberg, H., Rao, C. (1993): Both surface proteins (VP4 and VP7) of an asymptomatic neonatal rotavirus strain (1321) have high levels of sequence identity with the homologous proteins of a serotype 10 bovine rotavirus. *Virology*. **194**: 374–379.

Dash, S., Tewari, A., Kumar, K., Goel, A., Bhatia, A. (2011): Detection of Rotavirus from diarrhoeic cow calves in Mathura, India. *Vet. World*. **4**: 554.

Davidson, G., Barnes, G. (1979): Structural and functional abnormalities of the small intestine in infants and young children with rotavirus enteritis. *Acta Pædiatrica* **68**: 181–186.

de Graaf, D., Vanopdenbosch, E., Ortega-Mora, L., Abbassi, H., Peeters, J. (1999): A review of the importance of cryptosporidiosis in farm animals. *Int. J. Parasitol*. **29**: 1269–1287.

De la Fuente, R., Garcıa, A., Ruiz-Santa-Quiteria, J., Luzon, M., Cid, D., García, S., Orden, J., Gomez-Bautista, M. (1998): Proportional morbidity rates of enteropathogens among diarrheic dairy calves in central Spain. *Prev. Vet. Med*. **36**: 145–152.

De Leeuw, P., Ellens, D., Straver, P., Van Balken, J., Mofrman, A., Baanvinger, T. (1980): Rotavirus infections in calves in dairy herds. *Res. Vet. Sci*. **29**: 135–141.

De Rycke, J., Bernard, S., Laporte, J., Naciri, M., Popoff, M., Rodolakis, A. (1986): Prevalence of various enteropathogens in the feces of diarrheic and healthy calves, in: *Annales de Recherches Vétérinaires*. pp. 159–168.

De Verd Er, K. (2006): Infektionspanoramat vid diarréer hos svenska kalvar. *Sven. Vet*. **58**: 29.

Dennehy, P. (2000): Transmission of rotavirus and other enteric pathogens in the home. *Pediatr. Infect. Dis. J*. **19**: S103–S105.

Dennis, A., McDonald, S., Payne, D., Mijatovic-Rustempasic, S., Esona, M., Edwards, K., Chappell, J., Patton, J. (2014): Molecular Epidemiology of Contemporary G2P[4] Human Rotaviruses Cocirculating in a Single U.S. Community: Footprints of a Globally Transitioning Genotype. *J. Virol*. **88**: 3789 LP-3801.

Desselberger, U., Huppertz, H. (2011): Immune responses to rotavirus infection and vaccination and

associated correlates of protection. *J. Infect. Dis*. **203**: 188–195.

Desselberger, U., Iturriza-Gómara, M., Gray, J. (2003): Rotavirus Epidemiology and Surveillance 125–152.

Dhama, K., Chauhan, R., Mahendran, M., Malik, S. (2009): Rotavirus diarrhea in bovines and other domestic animals. *Vet. Res. Commun*. **33**: 1–23.

Diederichsen De Brito, W., Munford, V., Villaç, A., Ramos Caruzo, T., Rácz, M. (2000): Characterization of mixed infections with different strains of bovine rotavirus in an outbreak of diarrhea in dairy herds in Goiás, Brazil. *Brazilian J. Microbiol*. **31**: 140–145.

Dong, Y., Zeng, C., Ball, J., Estes, M., Morris, A. (1997): The rotavirus enterotoxin NSP4 mobilizes intracellular calcium in human intestinal cells by stimulating phospholipase C-mediated inositol 1, 4, 5-trisphosphate production. *Proc. Natl. Acad. Sci.* **94**: 3960–3965.

Dóró, R., Farkas, S., Martella, V., Bányai, K. (2015): Zoonotic transmission of rotavirus: surveillance and control. *Expert Rev. Anti. Infect. Ther*. **13**: 1337–1350.

Duckmanton, L., Carman, S., Nagy, É., Petric, M. (1998): Detection of bovine torovirus in fecal specimens of calves with diarrhea from Ontario farms. *J. Clin. Microbiol.* **36**: 1266–1270.

Duman, R., Aycan, A. (2010): Prevalence of rotavirus infections in calves with diarrhea hi Konya region. *J. Anim. Vet. Adv*.

Edwards, S., Broom, D., Collis, S. (1982): Factors affecting levels of passive immunity in dairy calves. *Br. Vet. J*. **138**: 233–240.

El-Attar, L., Dhaliwal, W., Iturriza-Gómara, M., Bridger, J. (2002): Identification and molecular characterization of a bovine G3 rotavirus which causes age-independent diarrhea in cattle. *J. Clin. Microbiol*. **40**: 937–942.

Espy, M., Uhl, J., Sloan, L., Buckwalter, S., Jones, M., Vetter, E., Yao, J., Wengenack, N., Rosenblatt, J., Cockerill, F. (2006): Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin. Microbiol. Rev*. **19**: 165–256.

Estes, M., Greenberg, H. (2013): Rotaviruses, p 1347–1401. Fields Virol. 6th ed. Lippincott Williams Wilkins, Philadelphia, PA.

Estes, M. Kang, G., Zeng, C., Crawford, S., Ciarlet, M. (2001): Pathogenesis of rotavirus gastroenteritis, in: Novartis Foundation Symposium. Chichester; *New York; John Wiley*; **1999**: pp. 82–100.

Estes, M., Kapikian, A. (2007): Rotaviruses. Fields Virology. Eds) Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE, Philadelphia Kluwer Heal. Williams Wilkins 1917–1974.

FAO (Food and Agricultural Organization) of the U.N. (2011): World Livestock 2011 Livestock in

food security World Livestock 2011 Livestock in food security, World.

Fiedler, H., Bahr, K., Hirchert, R. (1982): REPORT OF CRYPTOSPORIDIUM INVASION IN CALVES. Tierarztl. *Umsch*. **37**: 497–500.

Fields, B., Knipe, D., Howley, P., Everiss, K., Kung, H. (1996): Fundamental virology. Lippincott-Raven Philadelphia ePA PA.

Fischer, T., Ashley, D., Kerin, T., Reynolds-Hedmann, E., Gentsch, J., Widdowson, M., Westerman, L., Puhr, N., Turcios, R., Glass, R. (2005): Rotavirus antigenemia in patients with acute gastroenteritis. *J. Infect. Dis*. **192**: 913–919.

Flores, J., Sereno, M., Lai, C., Boeggeman, E., Perez, I., Purcell, R., Kalica, A., Greenberg, H., Wyatt, R., Hansen, J. (1983): Use of single-stranded rotavirus RNA transcripts for the diagnosis of rotavirus infection, the study of genetic diversity among rotaviruses, and the molecular cloning of rotavirus genes. Double-stranded RNA viruses. *Elsevier, Amsterdam* 1115–1127.

Foster, D., Smith, G. (2009): Pathophysiology of diarrhea in calves. *Vet. Clin. North Am. Food Anim. Pract*. **25**: 13–36.

Fukuda, M., Kuga, K., Miyazaki, A., Suzuki, T., Tasei, K., Aita, T., Mase, M., Sugiyama, M., Tsunemitsu, H. (2012):. Development and application of one-step multiplex reverse transcription PCR for simultaneous detection of five diarrheal viruses in adult cattle. *Arch. Virol*. **157**: 1063–1069.

Garaicoechea, L., Bok, K., Jones, L., Combessies, G., Odeon, A., Fernandez, F., Parreno, V. (2006): Molecular characterization of bovine rotavirus circulating in beef and dairy herds in Argentina during a 10-year period (1994–2003). *Vet. Microbiol.* **118**: 1–11.

Ge, Y., Mansell, A., Ussher, J.E., Brooks, A., Manning, K., Wang, C., Taylor, J. (2013): Rotavirus NSP4 triggers secretion of proinflammatory cytokines from macrophages via Toll-like receptor 2. *J. Virol*. **87**: 11160–11167.

Gelaw, A., Pietsch, C., Liebert, U.G., 2018. Molecular epidemiology of rotaviruses in Northwest Ethiopia after national vaccine introduction. Infect. Genet. Evol. 65, 300–307.

Gentsch, J., Glass, R., Woods, P., Gouvea, V., Gorziglia, M., Flores, J., Das, B., Bhan, M. (1992): Identification of group A rotavirus gene 4 types by polymerase chain reaction. *J. Clin. Microbiol.* **30**: 1365–1373.

Gentsch, J., Woods, P., Ramachandran, M., Das, B., Leite, J., Alfieri, A., Kumar, R., Bhan, M., Glass, R. (2011): Review Of G And P Typing Results From A Global Collection Of Rotavirus Strains: Implications For Vaccine Development. *J. Infect. Dis*. **174**: S30–S36.

Ghosh, S., Kobayashi, N. (2014): Exotic rotaviruses in animals and rotaviruses in exotic animals. VirusDisease. **25**: 158–172.

Ghosh, S., Kobayashi, N.(2011): Whole-genomic analysis of rotavirus strains: current status and

future prospects. *Future Microbiol*. **6**: 1049–1065.

Gilger, M., Matson, D., Conner, M., Rosenblatt, H., Finegold, M., Estes, M. (1992): Extraintestinal rotavirus infections in children with immunodeficiency. *J. Pediatr*. **120**: 912–917.

Giri, S., Nair, N, Mathew, A., Manohar, B., Simon, A., Singh, T., Suresh Kumar, S., Mathew, M.A., Babji (2019): Rotavirus gastroenteritis in Indian children < 5 years hospitalized for diarrhoea, 2012 to 2016. *BMC Public Health.* **19**: 69.

Glass, R., Bresee, J., Jiang, B., Parashar, U., Yee, E., Gentsch, J. (2006): Rotavirus and rotavirus vaccines, in: Hot Topics in Infection and Immunity in Children III. *Springer*, pp. 45–54.

Gorziglia, M., Larralde, G., Kapikian, A., Chanock, R. (2006): Antigenic relationships among human rotaviruses as determined by outer capsid protein VP4. *Proc. Natl. Acad. Sci*. **87**: 7155–7159.

Gouvea, V., Brantly, M. (1995):Is rotavirus a population of reassortant. *Trends Microbiol*. **3**:159-162.

Gouvea, V., Glass, R., Woods, P., Taniguchi, K., Clark, H., Forrester, B., Fang, Z. (1990): Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. *J. Clin. Microbiol*. **28**: 276–282.

Graff, J., Mitzel, D., Weisend, C., Flenniken, M., Hardy, M. (2002): Interferon regulatory factor 3 is a cellular partner of rotavirus NSP1. *J. Virol*. **76**: 9545–9550.

Gulati, B.R., Nakagomi, O., Koshimura, Y., Nakagomi, T., Pandey, R. (1999): Relative frequencies of G and P types among rotaviruses from Indian diarrheic cow and buffalo calves. *J. Clin. Microbiol*. **37**: 2074–2076.

Gulliksen, S., Lie, K., Løken, T., Østerås, O. (2009): Calf mortality in Norwegian dairy herds. *J. Dairy Sci*. **92**: 2782–2795.

Gurjar, A., Hegde, N., Love, B., Jayarao, B. (2008): Real-time multiplex PCR assay for rapid detection and toxintyping of Clostridium perfringens toxin producing strains in feces of dairy cattle. *Mol. Cell. Probes*. **22**: 90–95.

Hagbom, M. (2015): Rotavirus Disease Mechanisms Diarrhea, Vomiting and Inflammation : How and Why. Rotavirus Dis. Mech. Diarrhea, Vomiting Inflamm. How Why.

Hagbom, M., Istrate, C., Engblom, D., Karlsson, T., Rodriguez-Diaz, J., Buesa, J., Taylor, J., Loitto, V., Magnusson, K., Ahlman, H. (2011): Rotavirus stimulates release of serotonin (5-HT) from human enterochromaffin cells and activates brain structures involved in nausea and vomiting. *PLoS Pathog*. **7**: e1002115.

Hammami, S., Castro, A., Osbum, B. (1990): Comparison of polyacrylamide gel electrophoresis, an enzyme-linked-immunosorbent assay, and an agglutination test for the direct identification of bovine rotavirus from feces and coelectrophoresis of viral RNA's. *J. Vet. Diagnostic Investig*. **2**: 184–190.

Heckert, H., Bardella, I., Brunner, B., Brunner, R. (2005): Effects of maternal immunization of cows-a field study. *Prakt. Tierarzt*. **86**: 500–+.

Herrmann, J., Chen, S., Fynan, E., Santoro, J., Greenberg, H., Wang, S., Robinson, H. (1996): Protection against rotavirus infections by DNA vaccination. *J. Infect. Dis*. **174**: S93–S97.

Holland, R. (1990): Some infectious causes of diarrhea in young farm animals. *Clin. Microbiol. Rev*. **3**: 345–375.

Holmes, I., Ruck, B., Bishop, R., Davidson, G. (1975): Infantile enteritis viruses: morphogenesis and morphology. *J. Virol.* **16**: 937–943.

Hoshino, Y., Honma, S., Jones, R., Ross, J., Santos, N., Gentsch, J., Kapikian, A., Hesse, R. (2005): A porcine G9 rotavirus strain shares neutralization and VP7 phylogenetic sequence lineage 3 characteristics with contemporary human G9 rotavirus strains. *Virology*. **332**: 177–188.

Hoshino, Y., Jones, R., Ross, J., Honma, S., Santos, N., Gentsch, J., Kapikian, A. (2004): Rotavirus serotype G9 strains belonging to VP7 gene phylogenetic sequence lineage 1 may be more suitable for serotype G9 vaccine candidates than those belonging to lineage 2 / 3. *J. Virol*. **78**: 7795-7802.

Hoshino, Y., Kapikian, A. (2000): Rotavirus serotypes: classification and importance in epidemiology, immunity, and vaccine development. *J. Heal. Popul. Nutr*. 5–14.

HTRADO, (2009). Holeta Town Rural Agricultural Development Office. Annual summary of work report. Unpublished 24.

Hull, J., Teel, E., Kerin, T., Freeman, M., Esona, M., Gentsch, J. (2011): United States rotavirus strain surveillance from 2005 to 2008: genotype prevalence before and after vaccine introduction. *Pediatr Infect Dis J*. **30**.

Hur, T., Jung, Y., Choe, C., Cho, Y., Kang, S., Lee, H., Ki, K., Baek, K., Suh, G. (2013): The dairy calf mortality: the causes of calf death during ten years at a large dairy farm in Korea. *Korean J. Vet. Res*. **53**: 103–108.

Huyen, D., Hong, D., Trung, N., Hoa, T., Oanh, N., Thang, H., Thao, N., Hung, D., Iijima, M., Fox, K., Grabovac, V., Heffelfinger, J., Batmunkh, N., Anh, D. 2018. Epidemiology of acute diarrhea caused by rotavirus in sentinel surveillance sites of Vietnam, 2012–2015. *Vaccine* **36**: 7894–7900.

Hwang, K., Huang, Y., Bányai, K., Wu, H., Chang, F., Yang, D., Hsiung, C., Lin, J., Jiang, B., Gentsch, J. (2011): Severe gastroenteritis associated with G3P [9] rotavirus in Taiwan. *Infection*. **39**: 271–275.

Iturriza-Gomara, M., Dallman, T., Bányai, K., Böttiger, B., Buesa, J., Diedrich, S., Fiore, L., Johansen, K., Korsun, N., Kroneman, A. (2009): Rotavirus surveillance in Europe, 2005-2008: web-enabled reporting and real-time analysis of genotyping and epidemiological data. *J. Infect. Dis*. **200**: S215–S221.

Iturriza-Gómara, M., Isherwood, B., Desselberger, U., Gray, J. (2001): Reassortment in vivo: driving force for diversity of human rotavirus strains isolated in the United Kingdom between 1995 and 1999. *J. Virol*. **75**: 3696–3705.

Izzo, M., Kirkland, P., Mohler, V., Perkins, N., Gunn, A., House, J. (2011): Prevalence of major enteric pathogens in Australian dairy calves with diarrhoea. *Aust. Vet. J*. **89**: 167–173.

Jain, V., Das, B., Bhan, M., Glass, R., Gentsch, J., Laboratories, I. (2001): Great diversity of group A rotavirus strains and high prevalence of mixed rotavirus infections in India. *J. Clin. Microbiol*. **39**: 3524–3529.

Jayaram, H., Estes, M., Prasad, B. (2004): Emerging themes in rotavirus cell entry, genome organization, transcription and replication. *Virus Res*. **101**: 67–81.

Jin, Q., Ward, R., Knowlton, D., Gabbay, Y., Linhares, A., Rappaport, R., Woods, P., Glass, R., Gentsch, J. (1996): Divergence of VP7 genes of G1 rotaviruses isolated from infants vaccinated with reassortant rhesus rotaviruses. *Arch. Virol*. **141**: 2057–2076.

Jindal, S., Maiti, N., Oberoi, M. (2000): Genomic diversity and prevalence of Rotavirus in cow and buffalo calves in northern India. *Rev. Sci. Tech. Int. des Epizoot*. **19**: 871–876.

Jindal, S., MAITI, N., OBEROI, M. (2016): Genomic diversity and prevalence of Rotavirus in cow and buffalo calves in Northern India. *Rev. Sci. Tech. l'OIE*. **19**: 871–876.

Jonesteller, C., Burnett, E., Yen, C., Tate, J., Parashar, U. (2017): Effectiveness of rotavirus vaccination: a systematic review of the first decade of global postlicensure data, 2006–2016. *Clin. Infect. Dis*. **65**: 840–850.

Kaljot, K., Shaw, R., Rubin, D., Greenberg, H. (1988): Infectious rotavirus enters cells by direct cell membrane penetration, not by endocytosis. *J. Virol*. **62**: 1136–1144.

Kam, A., Khelef, D., Kaidi, R., Rahal, K., Tali-Maamar, H., Yabrir, B., Laoun, A., Mostfaoui, A., Boutaiba, S., Cozma, V. (2011): The Frequency of the Shedding of Cryptosporidium parvum, F5 Escherichia coli, Rotavirus, Coronavirus and Salmonella spp. in Young Dairy Calves in Mitidja Area (Algeria). *Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca. Vet. Med.* **68**.

Kaneene, J., Hurd, H. (1990): The national animal health monitoring system in Michigan. III. Cost estimates of selected dairy cattle diseases. *Prev. Vet. Med*. **8**: 127–140.

Kang, G., Iturriza-Gomara, M., Wheeler, J., Crystal, P., Monica, B., Ramani, S., Primrose, B., Moses, P., Gallimore, C., Brown, D. (2004): Quantitation of group A rotavirus by real-time reverse-transcription-polymerase chain reaction: Correlation with clinical severity in children in South India. *J. Med. Virol*. **73**: 118–122.

Khagayi, S., Burton, D., Onkoba, R., Ochieng, B., Ismail, A., Mutonga, D., Muthoni, J., Feikin, D., Breiman, R., Mwenda, J., Odhiambo, F., Laserson, K. (2014): High burden of rotavirus gastroenteritis in young children in rural western Kenya, 2010-2011. *Pediatr. Infect. Dis. J. 33 Suppl.* **1**: S34-40.

Kirkwood, C., Bogdanovic-Sakran, N., Palombo, E., Masendycz, P., Bugg, H., Barnes, G., Bishop, R. (2003): Genetic and antigenic characterization of rotavirus serotype G9 strains isolated in Australia between 1997 and 2001. *J. Clin. Microbiol*. **41**: 3649–3654.

Koch-institut, R., 1995. Vi rology ©. *Arch. Virol*. **4**: 737–743.

Kyle, H. (2007): Infection of rotavirus in dairy calves in south Vietnam.

Lai, H., Lin, S., Lin, H.., Ku, C., Wang, L., Yang, C. (2005): Phylogenetic analyses of human rotavirus in central Taiwan in 1996, 2001 and 2002. *J. Clin. Virol.* **32**: 199–217.

Laird, A., Gentsch, J., Nakagomi, T., Nakagomi, O., Glass, R. (2003): Characterization of serotype G9 rotavirus strains isolated in the United States and India from 1993 to 2001. *J. Clin. Microbiol*. **41**: 3100–3111.

Lamb, R., Kurg, R. (2001): Orthomyxoviridae: the viruses and their replication. *Fields Virol*. 1487–1531.

Langoni, H., Linhares, A., Avila, F., de, Da Silva, A., Elias, A. (2004): Contribution to the study of diarrhea etiology in neonate dairy calves in São Paulo state, Brazil. *Brazilian J. Vet. Res. Anim. Sci.* **41**: 313–319.

Larson, R., Tyler, J., Schultz, L., Tessman, R., Hostetler, D. (2004): Management strategies to decrease calf death losses in beef herds. *J. Am. Vet. Med. Assoc*. **224**: 42–48.

Lequin, R. (2005): Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clin. Chem*. **51**: 2415–2418.

Leung, A., Kellner, J., Davies, H. (2005): Rotavirus gastroenteritis. *Adv. Ther*. **22**: 476–487.

Lin, Y., Chang, S., Kao, C., Huang, L., Chung, M., Yang, J., Chen, H., Taniguchi, K., Tsai, K., Lee, C. (2006): Molecular epidemiology of G9 rotaviruses in Taiwan between 2000 and 2002. *J. Clin. Microbiol*. **44**: 3686–3694.

Lin, Y., Kao, C., Chang, S., Taniguchi, K., Hung, P., Lin, H., Huang, L., Huang, H., Yang, J., Lee, C. (2008): Determination of human rotavirus VP6 genogroups I and II by reverse transcription-PCR. J. Clin. Microbiol. 46, 3330–3337.

Lorenz, I., Fagan, J., More, S. (2011): Calf health from birth to weaning. II. Management of diarrhoea in pre-weaned calves. *Ir. Vet. J*. **64**: 9.

Lucchelli, A., Kang, S., Jayasekera, M., Parwani, A., Zeman, D., Saif, L. (1994): A survey of G6 and G10 serotypes of group A bovine rotaviruses from diarrheic beef and dairy calves using monoclonal antibodies in ELISA. *J. Vet. Diagn. Invest*. **6**: 175–181.

Lundgren, O., Svensson, L. (2001): Pathogenesis of rotavirus diarrhea. *Microbes Infect*. **3**:1145-1156.

Malik, S., Verma, A., Kumar, A., Gupta, M., Sharma, S. (2012): Incidence of calf diarrhea in cattle and buffalo calves in Uttar Pradesh, India. *Asian J. Anim. Vet. Adv*. **7**: 1049–1054.

Malik, Y., Kumar, N., Sharma, K., Sharma, R., Kumar, H., Anupamlal, K., Kumari, S., Shukla, S., Chandrashekar, K. (2013): Epidemiology and genetic diversity of rotavirus strains associated with acute gastroenteritis in bovine, porcine, poultry and human population of Madhya Pradesh, Central India, 2004–2008. *Virus*. **2013**: 5–9.

Martella, V., Bányai, K., Matthijnssens, J., Buonavoglia, C., Ciarlet, M. (2010a): Zoonotic aspects of rotaviruses. *Vet Microbiol*.**140**: 246–255.

Martella, V., Bányai, K., Matthijnssens, J., Buonavoglia, C., Ciarlet, M. (2010b): Zoonotic aspects of rotaviruses. *Vet. Microbiol*. **140**: 246–255.

Martella, V., Ciarlet, M., Banyai, K., Lorusso, E., Arista, S., Lavazza, A., Pezzotti, G., Decaro, N., Cavalli, A., Lucente, M. (2007): Identification of group A porcine rotavirus strains bearing a novel VP4 (P) genotype in Italian swine herds. *J. Clin. Microbiol*. **45**: 577–580.

Martin, S., Meek, A., Willeberg, P. (1987): Veterinary epidemiology: principles and methods. Iowa State University Press, Ames IA.

Matthijnssens, J., Ciarlet, M., Heiman, E., Arijs, I., Delbeke, T., McDonald, S., Palombo, E., Iturriza-Gómara, M., Maes, P., Patton, J., Rahman, M., Van Ranst, M. (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. *J. Virol*. **82**: 3204–3219.

Matthijnssens, J., Ciarlet, M., McDonald, S., Attoui, H., Bányai, K., Brister, J., Buesa, J., Esona, M., Estes, M., Gentsch, J. (2011): Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). *Arch. Virol*. **156**: 1397–1413.

Maunula, L., von Bonsdorff, C., (2002): Frequent reassortments may explain the genetic heterogeneity of rotaviruses: analysis of Finnish rotavirus strains. *J. Virol.* **76**: 11793–11800.

Mayameei, A., Mohammadi, G., Yavari, S., Afshari, E., Omidi, A. (2010): Evaluation of relationship between Rotavirus and Coronavirus infections with calf diarrhea by capture ELISA. *Comp. Clin. Path*. **19**: 553–557.

McDonald, S., Matthijnssens, J., McAllen, J., Hine, E., Overton, L., Wang, S., Lemey, P., Zeller, M., Van Ranst, M., Spiro, D., Patton, J. (2009): Evolutionary dynamics of human rotaviruses: balancing reassortment with preferred genome constellations. PLoS Pathog. **5**: e1000634.

Mcnulty, M. (1983):Etiology, Pathology and Epidemiology of Viral Gastroenteritis. *Ann Rech Vet*.

McNulty, M., Allan, G., McFerran, J. (1977): Cell culture studies with a cytopathic bovine rotavirus. *Arch. Virol*. **54**: 201–209.

Medici, M., Abelli, L., Guerra, P., Dodi, I., Dettori, G., Chezzi, C. (2011): Case report: detection of rotavirus RNA in the cerebrospinal fluid of a child with rotavirus gastroenteritis and meningism. *J. Med. Virol*. **83**: 1637–1640.

Meganck, V., Hoflack, G., Piepers, S., Opsomer, G. (2015): Evaluation of a protocol to reduce the

incidence of neonatal calf diarrhoea on dairy herds. *Prev. Vet. Med*. **118**: 64–70.

Megersa, B., Yacob, A., Regassa, A., Abuna, F., Asmare, K., Amenu, K. (2009): Prevalence and incidence rates of calf morbidity and mortality and associated risk factors in smallholder dairy farms in Hawassa, Southern Ethiopia. *Ethiop. Vet. J*. **13**: 59–68.

Mihalov-Kovács, E., Gellért, Á., Marton, S., Farkas, S., Fehér, E., Oldal, M., Jakab, F., Martella, V., Bányai, K. (2015): Candidate new Rotavirus species in sheltered Dogs, Hungary. *Emerg. Infect. Dis*. **21**: 660–663.

Morris, A., Scott, J., Ball, J., Zeng, C., O'Neal, W., Estes, M. (1999): NSP4 elicits age-dependent diarrhea and Ca2+ mediated I− influx into intestinal crypts of CF mice. *Am. J. Physiol. Liver Physiol*. **277**: G431–G444.

Murphy, F., Gibbs, E., Horzinek, M., Studdert, M. (1999): Veterinary virology. *Elsevier*.

Mwenda, J., Mpabalwani, E., Pazvakavambwa, I., Seheri, L., Page, N., Widdowson, M., Duncan Steele, A., Ntoto, K., Abebe, A., Enweronu-Laryea, C., Armah, G., Amina, I., Kiulia, N., Mchomvu, J., Kisakye, A. (2010): Burden and Epidemiology of Rotavirus Diarrhea in Selected African Countries: Preliminary Results from the African Rotavirus Surveillance Network. *J. Infect. Dis*. **202**: S5–S11.

Nagesha, H., Raghavan, R., Lal, S. (1985): Madin-Darby bovine kidney cell line for isolation of bovine rotaviruses from clinical cases of neonatal calf diarrhoea. *Indian J Virol.* **1**: 224–235.

Nakagomi, O., Mochizuki, M., Aboudy, Y., Shif, I., Silberstein, I., Nakagomi, T. (1992): Hemagglutination by a human rotavirus isolate as evidence for transmission of animal rotaviruses to humans. *J. Clin. Microbiol*. **30**: 1011–1013.

Nakagomi, O., Nakagomi, T. (1991): Molecular evidence for naturally occurring single VP7 gene substitution reassortant between human rotaviruses belonging to two different genogroups. *Arch. Virol*. **119**: 67–81.

Nakagomi, O., Nakagomi, T., Takahashi, Y., Enoki, M., Suzuki, T., Kilgore, P. (2005): Incidence and Burden of Rotavirus Gastroenteritis in Japan, as Estimated from a Prospective Sentinel Hospital Study. *J. Infect. Dis*. **192**: S106–S110.

Nakagomi, T., Nakagomi, O. (2005): Rotavirus antigenemia in children with encephalopathy accompanied by rotavirus gastroenteritis. *Arch. Virol*. 150, 1927–1931.

Nakagomi, T., Nakagomi, O. (1989): RNA-RNA hybridization identifies a human rotavirus that is genetically related to feline rotavirus. *J. Virol*. 63, 1431–1434.

Nataraju, S., Chattopadhyay, U., Krishnan, T. (2009): A study on the possibility of zoonotic infection in rotaviral diarrhoea among calves and buffalo calves in and around Kolkata, *India. Eur. Rev. Med. Pharmacol. Sci.* **13**: 7–11.

Nemoto, M., Morita, Y., Niwa, H., Bannai, H., Tsujimura, K., Yamanaka, T., Kondo, T. (2015): Rapid

detection of equine coronavirus by reverse transcription loop-mediated isothermal amplification. *J. Virol. Methods*. 215–216, 13–16.

Nishikawa, K., Hoshino, Y., Taniguchi, K., Green, K., Greenberg, H., Kapikian, A., Chanock, R., Gorziglia, M. (1989): Rotavirus VP7 neutralization epitopes of serotype 3 strains. *Virology* **171**: 503–515.

National Meteorological Service agency (NMA), (2016): Addis Ababa, Ethiop.

Nyangao, J., Page, N., Esona, M., Peenze, I., Gatheru, Z., Tukei, P., Duncan Steele, A. (2010): Characterization of human rotavirus strains from children with diarrhea in Nairobi and Kisumu, Kenya, between 2000 and 2002. *J. Infect. Dis.* **202**: S187–S192.

O'ryan, M., Lucero, Y., Prado, V., Santolaya, M., Rabello, M., Solis, Y., Berríos, D., O'Ryan-Soriano, M., Cortés, H. (2009): Symptomatic and asymptomatic rotavirus and norovirus infections during infancy in a Chilean birth cohort. Pediatr. *Infect. Dis. J*. **28**: 879-884.

Odde, K. (1988): Survival of the neonatal calf. *Vet. Clin. North Am. Food Anim. Pract*.**4**: 501-508.

Page, N., Esona, M., Armah, G., Nyangao, J., Mwenda, J., Sebunya, T., Basu, G., Pyndiah, N., Potgieter, N., Geyer, A. (2010): Emergence and characterization of serotype G9 rotavirus strains from Africa. *J. Infect. Dis*. **202**: S55–S63.

Pang, X., Lee, B., Boroumand, N., Leblanc, B., Preiksaitis, J., Yu Ip, C. (2004): Increased detection of rotavirus using a real time reverse transcription-polymerase chain reaction (RT-PCR) assay in stool specimens from children with diarrhea. *J. Med. Virol*. **72**: 496–501.

Papp, H., László, B., Jakab, F., Ganesh, B., De Grazia, S., Matthijnssens, J., Ciarlet, M., Martella, V., Bányai, K. (2013): Review of group A rotavirus strains reported in swine and cattle. *Vet. Microbiol*. **165**: 190–199.

Parashar, U., Gibson, C., Bresee, J., Glass, R. (2006): Rotavirus and severe childhood diarrhea. *Emerg. Infect. Dis*. **12**: 304–306.

Paredes, A., Brown, D., Rothnagel, R., Chiu, W., Schoepp, R., Johnston, R., Prasad, B. (1993): Three-dimensional structure of a membrane-containing virus. *Proc. Natl. Acad. Sci*. **90**: 9095–9099.

Park, J., Kurosawa, S., Watanabe, J., Ishihara, K. (2004): Evaluation of 2-methacryloyloxyethyl phosphorylcholine polymeric nanoparticle for immunoassay of C-reactive protein detection. *Anal. Chem*. **76**: 2649–2655.

Park, S., Saif, L., Jeong, C., Lim, G., Park, S., Ki, H., Park, S., Kim, Y., Jeong, J., Kang, M., Cho, K. (2006): Molecular characterization of novel G5 bovine rotavirus strains. J. Clin. Microbiol. 44, 4101–4112.

Patel, M., Shane, A., Parashar, U., Jiang, B., Gentsch, J., Glass, R. (2009): Oral Rotavirus Vaccines : How Well Will They Work Where They Are Needed Most ? **2220**: 39–48.

Patton, J. (2012); Rotavirus diversity and evolution in the post-vaccine world.*Discov. Med*. **13**:85-97.

Patton, J., Gallegos, C. (1990): Rotavirus RNA replication: single-stranded RNA extends from the replicase particle. *J. Gen. Virol*. **71**: 1087–1094.

Payne, D., Sulemana, I., Parashar, U. (2016): Evaluation of effectiveness of mixed rotavirus vaccine course for rotavirus gastroenteritis. *JAMA Pediatr*. **170**: 708–710.

Perez-Amodio, S., Holownia, P., Davey, C., Price, C. (2001): Effects of the ionic environment, charge, and particle surface chemistry for enhancing a latex homogeneous immunoassay of C-reactive protein. *Anal. Chem*. **73**: 3417–3425.

Pérez, E., Kummeling, A., Janssen, M., Jiménez, C., Alvarado, R., Caballero, M., Donado, P., Dwinger, R. (1998): Infectious agents associated with diarrhoea of calves in the canton of Tilarán, Costa Rica. *Prev. Vet. Med*. **33**: 195–205.

Phiri, B. (2008): Epidemiology of morbidity and mortality on smallholder dairy farms in Eastern and Southern Africa: a dissertation presented in partial fulfillment of the requirements for the degree of Master of Veterinary Studies in Epidemiology at Massey University.

Pisanelli, G., Martella, V., Pagnini, U., De Martino, L., Lorusso, E., Iovane, G., Buonavoglia, C. (2005): Distribution of G (VP7) and P (VP4) genotypes in buffalo group A rotaviruses isolated in Southern Italy. *Vet. Microbiol*. **110**: 1–6.

Pithua, P., Wells, S., Godden, S., Raizman, E. (2009): Clinical trial on type of calving pen and the risk of disease in Holstein calves during the first 90 d of life. *Prev. Vet. Med*. **89**: 8–15.

Polpanich, D., Tangboriboonrat, P., Elaissari, A., Udomsangpetch, R. (2007): Detection of malaria infection via latex agglutination assay. *Anal. Chem*. **79**: 4690–4695.

Pott, J., Stockinger, S., Torow, N., Smoczek, A., Lindner, C., McInerney, G., Bäckhed, F., Baumann, U., Pabst, O., Bleich, A. (2012): Age-dependent TLR3 expression of the intestinal epithelium contributes to rotavirus susceptibility. *PLoS Pathog*. **8**: e1002670.

Prasad, G., Malik, Y., Pandey, R. (2005): G and P genotyping of bovine group A rotaviruses in faecal samples of diarrhoeic calves by DIG-labelled probes.

Radostitis, O., Gay, C., Blood, D., Hinchcliff, K. (2000): Veterinary medicine: A textbook of the diseases of cattle, sheep, pigs, goats and horses. WB Saunders company. Philadelphia. USA. Pp 572–577.

Radostits, O., Gay, C., Hinchcliff, K., Constable, P. (2007): A textbook of the diseases of cattle, horses, sheep, pigs and goats. *Vet. Med*. **10**: 2045–2050.

Rai, R., Hansha, A., Rai, S., Singh, B., Kumar, H., Singh, A., Damodaran, T., Dhama, K. (2011): Prevalence of rota and coronavirus infections in calves of Barabanki and Raebareli districts of Uttar Pradesh. *Indian J Vet Pathol.* **35**: 73–74.

Rainsford, E., McCrae, M. (2007): Characterization of the NSP6 protein product of rotavirus gene 11. *Virus Res*. **130**: 193–201.

Rajendran, P., Kang, G. (2014): Molecular epidemiology of rotavirus in children and animals and characterization of an unusual G10P[15] strain associated with bovine diarrhea in south India. *Vaccine 32 Suppl*.**1**: A89-94.

Ramani, S., Arumugam, R., Gopalarathinam, N., Mohanty, I., Mathew, S., Gladstone, B., Jana, A., Kuruvilla, K., Kang, G. (2008): Investigation of the environment and of mothers in transmission of rotavirus infections in the neonatal nursery. *J. Med. Virol*. **8**: 1099–1105.

Ramani, S., Paul, A., Saravanabavan, A., Menon, V., Arumugam, R., Sowmyanarayanan, T., Samuel, P., Gagandeep, K. (2010): Rotavirus antigenemia in Indian children with rotavirus gastroenteritis and asymptomatic infections. *Clin. Infect. Dis*. **51**: 1284–1289.

Ramig, R. (2004): Pathogenesis of intestinal and systemic rotavirus infection. *J. Virol*. **78**: 10213-10220.

Ramig, R. (2002): Genetics of the Rotaviruses. *Annu. Rev. Microbiol*. **51**: 225–255.

Randall, R., Goodbourn, S. (2008): Interferons and viruses: An interplay between induction, signalling, antiviral responses and virus countermeasures. *J. Gen. Virol*. **89**: 1–47.

Reidy, N., Lennon, G., Fanning, S., Power, E., O'Shea, H. (2006): Molecular characterisation and analysis of bovine rotavirus strains circulating in Ireland 2002–2004. *Vet. Microbiol*. **117**: 242-247.

Reynolds, D., Morgan, J., Chanter, N., Jones, P., Bridger, J., Debney, T., Bunch, K. (1986): Microbiology of calf diarrhoea in southern Britain. *Vet. Rec*. **119**: 34–39.

Ribes, J., Seabolt, J., Overman, S. (2002): Performance characteristics of VIDAS and Directigen respiratory syncytial virus (RSV) antigen detection assays and culture for the identification of RSV in respiratory specimens. *J. Clin. Microbiol*. **40**: 1818–1820.

Rodrigo, C., Salman, N., Tatochenko, V., Mészner, Z., Giaquinto, C. (2010): Recommendations for rotavirus vaccination: A worldwide perspective. *Vaccine.* **28**: 5100–5108.

Saif, L., Fernandez, F.(1996): Group A rotavirus veterinary vaccines. *J. Infect. Dis*. **174**:S98-S106.

Samad, M. (2008): Animal husbandry and veterinary science. Volume I; Volume II. *Anim. Husb. Vet. Sci*. Vol. I; Vol. II.

Santos, N., Hoshino, Y. (2005): Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. *Rev Med Virol.* **15**.

Santos, N., Lima, R., Nozawa, C., Linhares, R., Gouvea, V. (1999): Detection of porcine rotavirus type G9 and of a mixture of types G1 and G5 associated with Wa-like VP4 specificity: Evidence

for natural human- porcine genetic reassortment. *J. Clin. Microbiol*. **37**: 2734–2736.

Saravanan, M., Parthiban, M., Wilson, A. (2006): Isolation of bovine rotavirus in cell culture from Neonatal calves with diarrhea. MV Sc.

Schielke, A., Filter, M., Appel, B., Johne, R. (2011): Thermal stability of hepatitis E virus assessed by a molecular biological approach. *Virol. J*. **8**: 487.

Sharma, R. (2004): Isolation and molecular characterization of rotavirus associated with diarrhea in bovine calves. MV Sc AH Thesis, JNKVV, Jabalpur 380.

Sharma, S., Paul, V., Bhan, M., Ray, P. (2009): Genomic characterization of nontypeable rotaviruses and detection of a rare G8 strain in Delhi, India. *J. Clin. Microbiol*. **47**:3998-4005.

Sharma, S. (2013): Epidemiological, Clinical and Haemato-biochemical Characterization of Calf Diarrhoea and Evaluation of Therapeutic Regimens.

SHFDO (Sebeta Hawas Finance and Economic Development Office), 2018. Annual Socio Economic profile and Statistical Abstract Report.

Singh, D., Kumar, M., Choudhary, P., Singh, H. (2009): Neonatal calf mortality–an overview. *Intas Polivet.* **10**: 165–169.

Singla, L., Gupta, M., Singh, H., Singh, S., Kaur, P., Juyal, P. (2013):Antigen based diagnosis of Cryptosporidium parvum infection in faeces of cattle and buffalo calves. *Indian J. Anim. Sci.***83**:

Snodgrass, D., Herring, J. (1977): The action of disinfectants on lamb rotavirus. *Vet. Rec*. **101**: 81.

Snodgrass, D., Terzolo, H., Sherwood, D., Campbell, I., Menzies, J., Synge, B. (1986): Aetiology of diarrhoea in young calves. *Vet. Rec*. **119**: 31–34.

Snodgrass, D., Wells, P. (1978): The immunoprophylaxis of of rotavirus infections in lambs. *Vet. Rec*. **102**: 146–148.

Soltan, M., Tsai, Y., Lee, P., Tsai, C., Chang, H., Wang, H., Wilkes, R. (2016): Comparison of electron microscopy, ELISA, real time RT-PCR and insulated isothermal RT-PCR for the detection of Rotavirus group A (RVA) in feces of different animal species. *J. Virol. Methods.* **235**: 99–104.

Steele, A., Geyer, A., Gerdes, G. (2004): Rotavirus infections. *Infect. Dis. Livest*. 1256–1264.

Steiner, L., Busato, A., Burnens, A., Gaillard, C. (1997): Frequency and etiology of calf losses and calf diseases before weaning in cow-calf farms. II. Microbiological and parasitological diagnoses in diarrheic calves. *Dtsch. Tierarztl. Wochenschr*. **104**: 169–173.

Straw, B., D'Allaire, S., Mengeling, W., Taylor, D. (2006): Diseases of swine. *Ames.*

Svensson, C., Linder, A., Olsson, S. (2006): Mortality in Swedish dairy calves and replacement heifers. *J. Dairy Sci*. **89**: 4769–4777.

Svensson, L., Sharma, S., Nordgren, J., Bucardo, F., Larson, G., Nasir, W., Günaydın, G., Hammarström, L., Ouermi, D., Simpore, J., Nitiema, L.W., Becker-Dreps, S. (2014): Both Lewis and Secretor Status Mediate Susceptibility to Rotavirus Infections in a Rotavirus Genotype–Dependent Manner. *Clin. Infect. Dis*. **59**: 1567–1573.

Tadesse, A. (2012): Hospital-based surveillance of rotavirus gastroenteritis in children less than five years-old: 2007-2011, Addis Ababa, Ethiopia. *Int. J. Infect. Dis*. **16**: e146.

Taniguchi, K., Urasawa, T., Urasawa, S., Yasuhara, T. (1984): Production of subgroup-specific monoclonal antibodies against human rotaviruses and their application to an enzyme-linked immunosorbent assay for subgroup determination. *J. Med. Virol*. **14**: 115–125.

Tate, J., Burton, A., Boschi-Pinto, C., Parashar, U., Agocs, M., Serhan, F., de Oliveira, L., Mwenda, J, Mihigo, R. (2016): Global, regional, and national estimates of rotavirus mortality in children< 5 years of age, 2000–2013. *Clin. Infect. Dis*. **62**: S96–S105.

Tate, J., Burton, A., Boschi-Pinto, C., Steele, A., Duque, J., Parashar, U. (2012): 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. *Lancet Infect. Dis*. **12**: 136–141.

Thrusfield, M., Christley, R. (2018): Veterinary epidemiology. John Wiley & Sons.

Tonietti, P., Hora, A., Silva, F., Ruiz, V., Gregori, F. (2013): Phylogenetic analyses of the VP4 and VP7 genes of porcine group a rotaviruses in São Paulo State, Brazil: First identification of G5P[23] in piglets. *J. Clin. Microbiol*. **51**: 2750–2753.

Troeger, C., Khalil, I., Rao, P., Cao, S., Blacker, B., Ahmed, T., Armah, G., Bines, J., Brewer, T., Colombara, D. (2018): Rotavirus vaccination and the global burden of rotavirus diarrhea among children younger than 5 years. *JAMA Pediatr*. **172**: 958–965.

Uhde, F., Kaufmann, T., Sager, H., Albini, S., Zanoni, R., Schelling, E., Meylan, M. (2008): Prevalence of four enteropathogens in the faeces of young diarrhoeic dairy calves in Switzerland. *Vet. Rec*. **163**: 362.

Ushijima, H., Araki, K., Abe, T., Shinozaki, T. (1987): Anti-rotavirus antibody in cerebrospinal fluid. *Arch. Dis. Child*. **62**: 298–299.

Vancott, J., Mcneal, M., Flint, J., Bailey, S., Choi, A., Ward, R. (2001): Role for T cell-independent B cell activity in the resolution of primary rotavirus infection in mice 3380–3387.

Varani, G., Allain, F. (2002): How a rotavirus hijacks the human protein synthesis machinery. *Nat. Struct. Mol. Biol*. **9**: 158.

Ved, N. (2014): Modecular Characterisation and Genotyping of Human and Animal Rotaviruses Isolates from Northern India.

Velázquez, F., Matson, D., Calva, J., Guerrero, M., Morrow, A., Carter-Campbell, S., Glass, R., Estes,

M., Pickering, L., Ruiz-Palacios, G. (1996): Rotavirus infection in infants as protection against subsequent infections. *N. Engl. J. Med*. **335**: 1022–1028.

Velázquez, F., Matson, D., Guerrero, M., Shults, J., Calva, J., Morrow, A., Glass, R., Pickering, L., Ruiz-Palacios, G. (2000): Serum antibody as a marker of protection against natural rotavirus infection and disease. *J. Infect. Dis*. **182**: 1602–1609.

Ward, R., Bernstein, D., Young, E., Sherwood, J., Knowlton, D., Schiff, G. (1986): Human rotavirus studies in volunteers: determination of infectious dose and serological response to infection. *J. Infect. Dis*. **154**: 871–880.

WHO (World Health Organization), (2013): Global rotavirus information and surveillance bulletin. Geneva WHO.

WHO, (2011): Global Rotavirus Information and Surveillance Bulletin Table of Contents. WHO **4**: 1–10.

WHO, (2009): Manual of rotavirus detection and characterization methods. World Health Organization, Geneva.

Widdowson, M., van Doornum, G., van der Poel, W., de Boer, A., Mahdi, U., Koopmans, M. (2000): Emerging group-A rotavirus and a nosocomial outbreak of diarrhoea. *Lancet*. **356**: 1161–1162.

Winiarczyk, S., Paul, P., Mummidi, S., Panek, R., Gradzki, Z. (2002): Survey of porcine rotavirus G and P genotype in Poland and the United States using RT-PCR. *J. Vet. Med. Ser. B*. **49**: 373–378.

Wong, C., Price, Z., Bruckner, D. (1984): Aseptic meningitis in an infant with rotavirus gastroenteritis. *Pediatr. Infect. Dis. J*. **3**: 244–245.

Wong, M., Medrano, J. (2005): Real-time PCR for mRNA quantitation. Biotechniques **39**: 75–85.

Woode, G. (1978): Epizootiology of bovine rotavirus infection. *Vet. Rec*. **103**: 44–46.

Wu, F., Banyai, K., Huang, J., Wu, H., Chang, F., Yang, J., Hsiung, C., Huang, Y., Lin, J., Hwang, K., Jiang, B., Gentsch, J. (2011): Diverse origin of P[19] rotaviruses in children with acute diarrhea in Taiwan: Detection of novel lineages of the G3, G5, and G9 VP7 genes. *J. Med. Virol*. **83**: 1279–1287.

Wu, F., Bányai, K., Huang, J., Wu, H., Chang, F., Hsiung, C., Huang, Y., Lin, J., Hwang, K., Jiang, B. (2011): Human infection with novel G3P [25] rotavirus strain in Taiwan. *Clin. Microbiol. Infect*. **17**: 1570–1573.

Wu, F., Liang, S., Tsao, K., Huang, C., Lin, C., Lin, J., Su, C., Eng, H., Yang, J., Chen, P. (2009): Hospital-based surveillance and molecular epidemiology of rotavirus infection in Taiwan, 2005–2007. *Vaccine*. **27**: F50–F54.

Wudu, T., Kelay, B., Mekonnen, H., Tesfu, K. (2008): Calf morbidity and mortality in smallholder dairy farms, Ada'a Liben district of Oromia, Ethiopia.*Trop. Anim. Health Prod*. **40**:369-376.

Yang, K., Wang, S., Chang, K., Lu, S., Saif, L., Greenberg, H., Brinker, J., Herrmann, J. (2001): Immune responses and protection obtained with rotavirus VP6 DNA vaccines given by intramuscular injection. *Vaccine*. **19**: 3285–3291.

Yassin, M., Kirby, A., Mengistu, A., Arbide, I., Dove, W., Beyer, M., Cunliffe, N., Cuevas, L., (2012): Unusual norovirus and rotavirus genotypes in Ethiopia. *Paediatr. Int. Child Health*. **32**: 51–55.

Yilmaz, V. (2016): Investigation of Rotavirus Infection in Calves with Diarrhea in Northeast Turkey. *Anim Vet Sci.* **4**: 1–4.

Yong-il, C. (2012): Ecology of calf diarrhea in cow-calf operations.

Yu, J., Lai, S., Geng, Q., Ye, C., Zhang, Z., Zheng, Y., Wang, L., Duan, Z., Zhang, J., Wu, S., Parashar, U., Yang, W., Liao, Q., Li, Z. (2019): Prevalence of rotavirus and rapid changes in circulating rotavirus strains among children with acute diarrhea in China, 2009-2015. *J. Infect.* **78**: 66–74.

Zeller, M., Patton, J., Heylen, E., De Coster, S., Ciarlet, M., Van Ranst, M., Matthijnssens, J. (2012): Genetic analyses reveal differences in the VP7 and VP4 antigenic epitopes between human rotaviruses circulating in Belgium and rotaviruses in Rotarix and RotaTeq. *J. Clin. Microbiol*. **50**: 966–976.

Zerr, D., Allpress, A., Heath, J., Bornemann, R., Bennett, E. (2005): Decreasing hospital-associated rotavirus infection: a multidisciplinary hand hygiene campaign in a children's hospital. *Pediatr. Infect. Dis. J*. **24**: 397–403.

Zhang, M., Zeng, C., Morris, A., Estes, M. (2000): A functional NSP4 enterotoxin peptide secreted from rotavirus-infected cells. *J. Virol*. **74**: 11663–11670.

Zhou, X., Wang, Y., Ghosh, S., Tang, W., Pang, B., Liu, M., Peng. J.(2015): Genomic characterization of G3P [6], G4P [6] and G4P [8] human rotaviruses from Wuhan, China: Evidence for interspecies transmission and reassortment events. *Infect. Genet. Evol*.**33**:55-71.

Zimmerman, J. (2006): Porcine Reproductive and Respiratory Syndrome Viruses (Porcine Arterivirus). *Dis. swine.* 387–417.

Zrelli, M., Messadi, L., Ben Miled, L., Jemli, M., Haddad, N. (1990): Infectious agents associated with calf diarrhea in Tunisia [neonatal enteritis]. *Rev. Med. Vet*.

8. ANNEXS

ANNEX- I Record Format for Calf Diarrhea

Date--------------------

Sampling format for calf diarrhea

I. Owners name--------------------------------------

II. Location

III. Farm name---

IV. Sample ID---

V. Address-district/PA---------------------------------

VI. General information

Calf ID--------------------Sex-------------Age-------------Breed-------------

VII. Sample type: Feces

VIII. Floor of the calf area……………………

IX. First colostrum feeding after birth……………………….

X. Separation of calf from dam

XI. Apparent health condition of the calf---

XII. Remark--

ANNEX - II Principle of the ELISA Test

In this test, the entire microtitration plate is sensitized with a mixture of antibodies that are specific for the pathogen (see the diagram on the last page). These antibodies capture the corresponding pathogens in the faecal samples. The faecal material is diluted in dilution buffer and incubated on the microplate for 1/2 hour at 21°C +/ - 3°C. Positive and negative controls are also deposited on the plate. The plate is incubated and washed and then ready-to-use conjugates are added to the wells. The choice of conjugates is left up to the user. The diagram on the next page gives an example of the arrangement of samples and conjugates on the plate. Following a second incubation for 1/2 hour at 21°C +/- 3°C, the plate is washed again and the chromogen tetramethylbenzidine (TMB), is added. This chromogen has the two advantages of being more sensitive than the other peroxidase chromogens and not being carcinogenic. If the pathogens being sought is present in the faeces, the corresponding conjugate or conjugates remain bound to the corresponding micro wells and the enzyme catalyses the transformation of the colourless chromogen into a blue compound. The intensity of the resulting colour is proportionate to the titre of the pathogen in the sample.

ANNEX- III Composition of the Kit

- ✓ Microplate: 96-well microtitration plate. The entire plate is sensitised by antibodies specific for the 2 pathogens for which the test is designed.
- ✓ Washing solution: One 100 ml bottle of 20 X concentrated washing solution. The solution crystallises spontaneously when cold. If only part of the solution is to be used, bring the bottle to 21°C +/- 3°C until all crystals have disappeared. Mix the solution well and take up the necessary volume. Dilute the buffer 1:20 with distilled or demineralised water.
- ✓ Dilution buffer: One 50 ml bottle of 5x colored and concentrated buffer for diluting samples. Dilute this concentrated dilution buffer 1:5 with distilled or demineralised water. If a deposit forms at the bottom of the container filter the solution on Whatman filter paper.
- ✓ Conjugate: 2 X 12 ml vials of coloured conjugate.The specificity of each conjugate is indicated on the bottle. The reagents are ready to use.
- ✓ Positive control: 1 vial containing 3 ml of the positive control. The reagent is ready to use.
- ✓ Negative control: 1 vial containing 3 ml of the negative control. The reagent is ready to use.

- ✓ Single component TMB: One bottle of the chromogen tetramethylbenzidine (TMB). Store between +2°C and +8°C.protected from light. This solution is ready to use.
- ✓ Stop solution: One bottle of 1 M phosphoric acid stop solution. This reagent is ready to use.

ANNEX- IV Procedure of ELISA Test

1- Bring all the reagents at 21°C +/- 3°C before use.

2- Dilute faecal samples volume per volume into dilution buffer. This is a qualitative dilution only, which must allow the pipetting of faecal suspensions. Discard any gruds by natural decantation for about 10 minutes. Do not centrifuge the suspensions.

3- Remove the microplate from its wrapper.

4- Pipette the diluted samples into the wells at the rate of 100 µl. Take care to change pipettes between two different samples. The arrangement of samples on the plate must be set by the user according to the number of faecal samples to test and the valences selected for each sample. Distribute the positive and negative controls over the plate as well (one well per valence tested). The control solutions are ready to use. If the distribution scheme for the samples and conjugates is complicated, fill out the layout forms.

5- Cover with a lid and incubate the plate at 21°± 3°C for 1/2 hour.

6- Rinse the plate with the washing solution prepared as instructed in the section "Composition of the Kit". To do this, dispose of the microplate's contents by flipping it sharply over a container filled with an inactivating agent. Let the microplate drain upside-down on a sheet of clean absorbent paper so as to eliminate all liquid. Add 300 µl of the washing solution, and then empty the plate once again by flipping it over above the containment vessel. Repeat the entire operation two more times, taking care to avoid the formation of bubbles in the microwells. After the plate has been washed three times proceed to the next step.

7- Add the ready to use conjugates into the wells at the rate of 100 µl per well.

8- Cover with a lid and incubate the plate at 21°± 3°C for 1/2 hour.

9- Wash the plate as instructed in Step 6.

10- Add 100 µl of the chromogen solution to each well on the plate. The chromogen solution must be absolutely colourless when it is pipetted into the wells. If a blue colour is visible, this means that the solution in the pipette has been contaminated.

11- Incubate 10 minutes at 21°C +/- 3°C without covering and away from light

12- Interpret the results visually by checking for a blue colour, unless you want to record the signals using a plate reader. In the latter case, skip to Step 13 and stop the reaction with the stop solution (read in the yellow range).

13- Add 50 µl of the stop solution to each well directly from the bottle. The blue colour will change into a yellow colour.

14- Record the optical densities using a plate reader and a 450 nm filter. The readings must be made as soon as possible after applying the stop solution, for in the event of a strong signal the chromogen can crystallise and lead to incorrect measurements.

ANNEX-V Interpreting the Results

If spectrophotometer readings are made, calculate the net optical density of each sample by subtracting from the reading for each sample well the optical density of the corresponding negative control. Proceed in the same way for the positive control antigens. The test is validated only if the positive control antigens yield difference in the optical densities at 10 minutes that are greater than the values:

Rotavirus	> 1000

Divide the signal read for each sample well by the corresponding positive control signal and multiply this result by 100 to express it as a percentage.

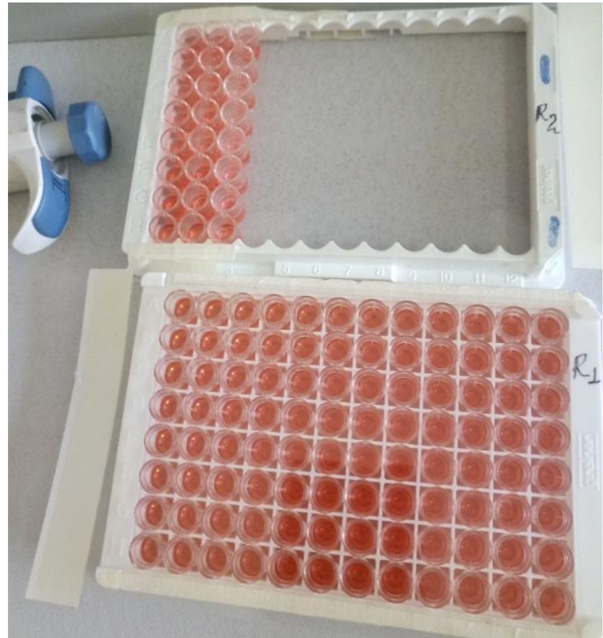
$$\text{Val(ue)} = \frac{\text{Delta OD Sample} * 100}{\text{Delta OD positive}}$$

Using the following table, determine each sample's status (positive, negative).

Rotavirus	> = 600 %

Any sample that yields a difference in optical density that is greater or equal than the percentages above is considered positive for the valence in question. Conversely, any sample that yields a difference in the optical density that is less than the percentages above is considered negative for the valence in question. If the results are interpreted visually (reading of the blue colour), the samples that produce a more intense blue colour than the colour in the corresponding negative control wells are considered to be positive.

ANNEX-VI: Miscellaneous photo During Study Period and Sample Collection.



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