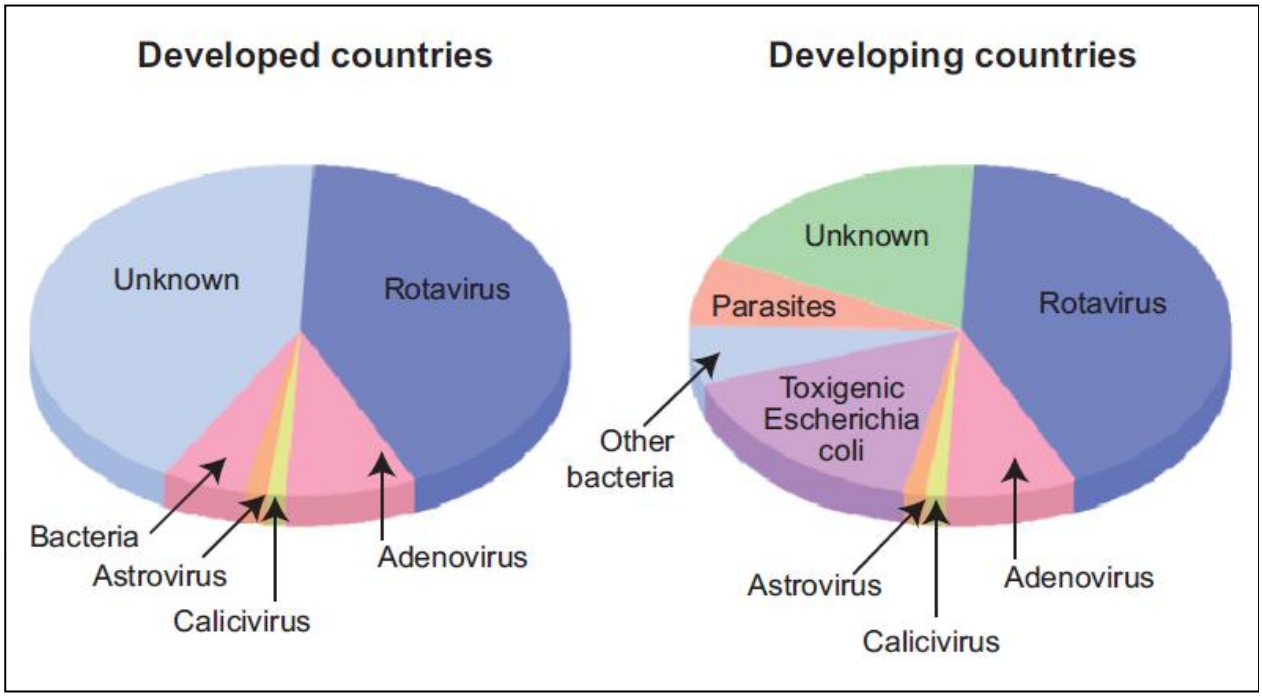
CHAPTER 1

1. Introduction

1.1 Background

Diarrhoeal diseases are a significant cause of morbidity and mortality in children worldwide. They are responsible for 4 to 6 million deaths per year according to the WHO, diarrhoea is especially dangerous for infants and young children. Globally, it is estimated that 1.4 billion episodes of diarrhoea occur in children less than 5 years of age annually (Parashar *et al*, 2003). There are many different diarrhoeal agents including bacteria, parasites and viruses. Figure 1 shows the causative agents of severe diarrhoea cases which necessitate hospitalization of children under the age of 5 years. The bacterial agents that cause severe diarrhoea are; *Vibrio cholerae*, *Shigella dysenteriae*, *Salmonella typh*i, Campylobacter, *Clostridium difficile*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enteritidis* (Knoop *et al*, 1993). Protozoan parasites such as *Cryptosporidium parvum* and *Giardia lamblia* are the most common agents of severe protozoan diarrhoea (http://www.brown.edu/courses/Bio_160/Projects2004/rotavirus/index.html. 30 October 2010). Viral gastroenteritis is caused by a variety of agents: for example, noroviruses, adenoviruses, astroviruses, rotavirus and many others can be responsible for diarrhoea. This study focused on rotavirus which is a primary cause of diarrhoea in infants worldwide. (http://www.brown.edu/courses/Bio_160/Projects2004/rotavirus/index.html. 30 October 2010). People of all ages are susceptible to rotavirus infection, but children 6 months to 2 years of age, premature infants, the elderly and immunocompromised individuals are particularly prone to more severe symptoms (http://vm.cfsan.fda.gov/~mow/chap33.html. 30 October 2010).

Figure 1: Causes of severe diarrhoea requiring hospitalization of infants and young children



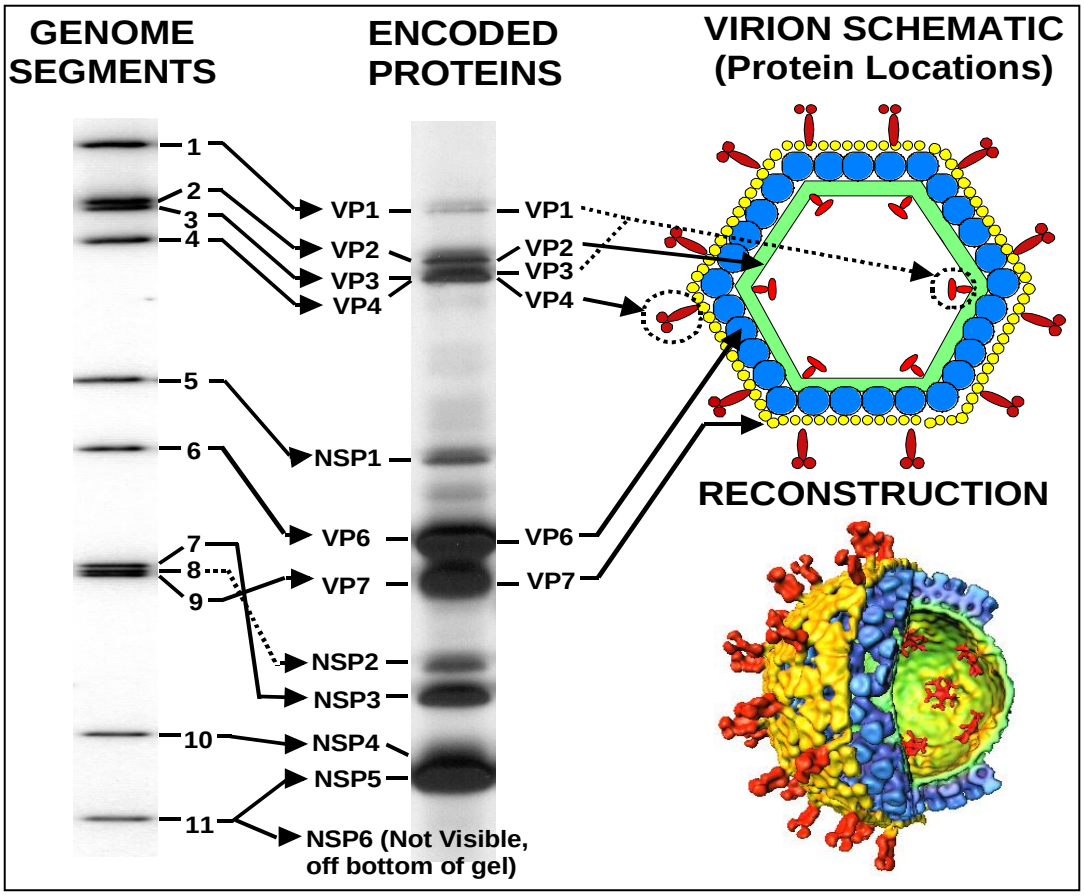
Source: Introduction of rotavirus vaccines into national immunization programmes – Management manual, World Health Organization, 2009

1.2 The rotaviruses

Rotaviruses are non-enveloped double stranded ribonucleic acid (dsRNA) viruses, with a wheel-like morphology when viewed by electron microscopy. These viruses belong to the genus *Rotaviruses* under the *Reoviridae* family. Rotaviruses are divided into groups A, B, C, D, E, F and G. This division is based on antigens present on the VP6 gene which is the major determinant of group and subgroup antigen reactivity. Groups D, E, F and G only infect animals, groups A, B, and C rotaviruses are responsible for most human infections, with group A being implicated in most severe diarrheal cases in both animals and humans (Donelli and Superti, 1994). The group A rotavirus genome is composed of eleven segments which

encode structural viral proteins (VP) and non-structural proteins (NSP), as illustrated in Figure 2. The viral structural proteins are VP1, VP2, VP3, VP4, VP6 and VP7 and the non-structural proteins include NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6 (Estes and Kapikian, 2007), (Ramani *et al*., 2007). The virion is composed of an inner core layer which is made up of VP1, VP2, and VP3, an intermediate layer consisting of VP6, and an outer shell composed of VP7 and VP4. The spikes of VP4, whose proteolytic cleavage activity gives rise to VP5 and VP8 (Glass *et al*, 2006) protrude from the outer shell. VP4 is designated as P antigenic protein because it is cleaved by the protease enzyme at intestinal level and VP7 is known as the G antigenic protein because it is glycosylated. The VP4 and VP7 proteins are important for the development of group A rotavirus vaccine because they are targets for neutralizing antibodies that give genotype specific protection (O'Ryan *et al*, 2009). There are 23 G genotypes and 32 P-genotypes of group A rotaviruses (Matthijnssens *et al*, 2007). The G-types: G1, G2, G3, G4 and G9 together with P-types P4, P6 and P8 are the most common human rotavirus types reported in studies worldwide.

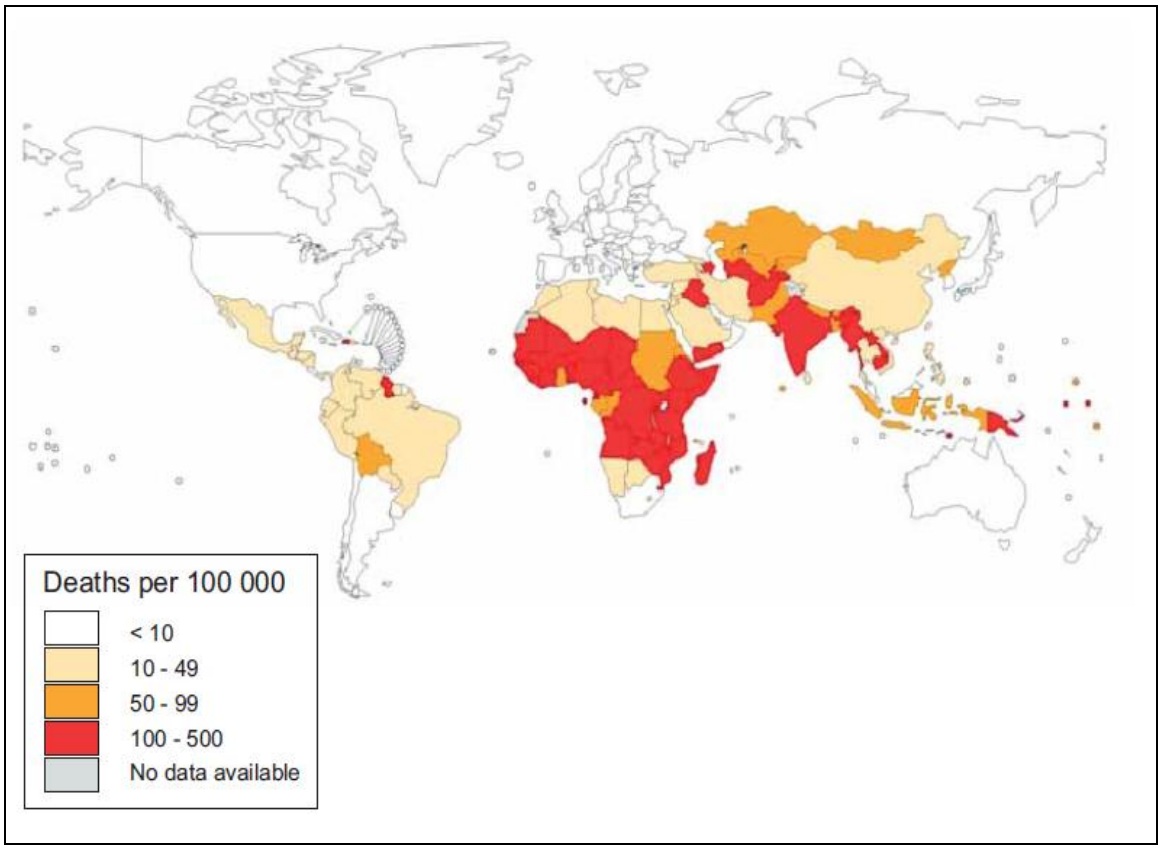
Figure 2: Coding assignments and virion locations of rotavirus proteins and 3D structure of the rotavirus particle.



Source: The 10th Rotavirus surveillance workshop laboratory manual, University of Limpopo-Medunsa Compus, 2009.

1.2.1 Epidemiology and Pathogenesis of rotavirus

Figure 3: Rotavirus mortality rate per 100 000 children <5 years of age, by country, in 2004



Source: Introduction of rotavirus vaccines into national immunization programmes – Management manual, World Health Organization, 2009

Rotavirus is a common cause of severe viral diarrhea in children less than 5 years old worldwide. The detection of Group A rotaviruses worldwide, particularly in non-bacterial and non-parasitic induced diarrhoeal cases has led to the fact that rotavirus is the leading cause of acute severe diarrhoea in infants and children under the age of five. The incidence of rotavirus infection is uniform in both the developed and developing countries (Bourdett-Stanziola *et al*, 2008). Gastrointestinal illness resulting from rotavirus infection among young children under the age of five years contribute greatly to morbidity and mortality rates in

many countries (Bourdett-Stanziola *et al*, 2008). According to Parashar *et al* (2006), 40 % of all diarrhoeal cases and 600 000 deaths worldwide are caused by rotavirus every year. More deaths, about 80 % of 600 000 deaths, occur in developing countries, particularly the poorest countries of Africa, Asia and Latin America, as shown in Figure 3. Transmission of rotaviruses is by ingestion of 10 to 100 infectious particles (Hoshino *et al*, 2003). Pathogenesis involves destruction of the villi, reticular cell enlargement, stunted microvilli and mitochondrial swelling. This results in poor absorption of salts and water causing diarrhoea and death (Donelli and Superti, 1994). The symptoms of rotavirus infection are fever and vomiting for several days followed by non-bloody diarrhoea. The diarrhoea can be very severe leading to life threatening dehydration. Rotavirus caused dehydration is the significant cause of death. Rotavirus infections are an important cause of hospitalization causing significant economic impact on poor countries (Bourdet-Stanziola *et al*, 2008).

The enterocytes lining the small intestine are divided into two types; the enterocytes and the crypt cells (Ramig, 2004). Villus enterocytes are mature, nonproliferating cells covering the villi that are differentiated into digestive and absorptive functions (Ramig, 2004). The absorptive enterocytes synthesize a number of disaccharidases, peptidases, and other enzymes that are expressed on the apical surface, where they carry out their digestive functions (Ramig, 2004). Absorption across the enterocyte barrier occurs both by passive diffusion of solutes along electrochemical or osmotic gradients and by active transport (Ramig, 2004). While the majority of water transport is passive along osmotic gradients, transporters such as the sodium-glucose cotransporter 1 (SGLT1) transport water along with solute (Loo *et a*l, 2002). The crypt epithelium lines the crypts and is the progenitor of the

villus enterocytes (Ramig, 2004). Crypt cells lack the well-defined microvilli and absorptive functions of the enterocyte and actively secrete Cl^- ions into the intestinal lumen. In the non-infected human, the combined activity of the enterocytes and crypt cells results in a constant bidirectional flux of electrolytes and water across the epithelium. On the villi, the balance is toward absorption, and in the crypts, the balance favours secretion (Ramig, 2004).

Rotaviruses replicate in the nondividing mature enterocytes near the tips of the villi, suggesting that differentiated enterocytes express factors required for effective infection and replication (Corner and Ramig, 1997). The pathological changes due to rotavirus infection are exclusively limited to the small intestine (Ramig, 2004). Rotavirus infection is associated with slight lesions, such as enterocyte vacuolization and loss; or larger changes, such as villus blunting and crypt hyperplasia (Ramig, 2004). Inflammation is generally mild compared to that caused by other intestinal pathogens (Ramig, 2004).

Rotavirus infection alters the function of the small intestinal epithelium, resulting in diarrhoea (Ramig, 2004). The diarrhoea was generally considered to be malabsorptive, secondary to enterocyte destruction (Kapikian *et al*, 2001). In addition to enterocyte destruction, absorption of Na^+ ions, water, and mucosal disaccharidases are decreased while mucosal cyclic AMP appears not to be altered (Matthijnssens *et al*, 2007). Malabsorption results in the transit of undigested monosaccharides and disaccharides, carbohydrates, fats, and proteins into the colon. The undigested bolus is osmotically active, and the colon is unable to absorb sufficient water, leading to an osmotic diarrhea (Graham and Estes, 1988). The gut lesions correlate with the presence of diarrhoea. The other mechanism of diarrhoea induction involves the viral nonstructural protein NSP4. A secreted fragment of NSP4, or

certain NSP4 peptides have toxin-like activity and can induce diarrhoea when inoculated into mice (Zhang *et al*, 2000). The NSP4 enterotoxin activity provides a way to mediate diarrhoeagenic changes in the absence of significant damage or to mediate changes at uninfected sites.

Some rotavirus infections are asymptomatic which suggests that both viral and host factors can affect disease severity (Matthijnssens *et al*, 2007), (Corner and Ramig, 1997). Among the viral factors are the following. (i) Some alleles of VP4 may be associated with asymptomatic disease (Flores *et al*, 1986). (ii)Some virus strains have restricted ability to replicate and cause disease in the host (Hall *et al*, 1993). (iii) Virus strains should be adapted for growth in a particular host species (Broome *et al*, 1993). The host factors which affect severity of rotavirus disease include the following. (i) Malnutrition is documented to increase the severity of rotavirus diarrhoea where it delays small intestinal recovery and modifies intestinal inflammatory responses (Steel and Torres-Medina, 1984), (Zijlstra *et al*, 1997), (Zijlstra *et al*, 1999).(ii) Rotavirus symptomatic infections are generally age restricted (Corner and Ramig, 1997). The age dependence appears to be unrelated to receptor expression, as both the viral and the NSP4 receptors are expressed in adult hosts. However, signaling downstream of the NSP4 receptor does appear to be age dependent but age restriction may be related to immunity, as neutralizing antibodies increase with age and virus exposure (Morris *et al*, 1999). (iii) Rotavirus disease may be related to age-dependent protease expression, as viral infectivity requires protease cleavage of VP4 and newborns have low levels of protease in the gut (Greenberg *et al*, 1994). (iv) The expression of intestinal mucins and the rate of epithelial cell replacement and fluid absorption are both age

dependent and have been shown to affect rotavirus infection and disease in the host (Moon, 1994).

1.2.2 **Laboratory diagnosis of rotavirus**

Laboratory diagnosis of rotavirus infection plays a crucial role in patient management, and it makes management and control of outbreaks possible. Viral gastroenteritis is caused by many agents such as noroviruses astroviruses and adenoviruses, differential diagnosis of gastroenteritis caused by these agents and rotavirus cannot be achieved by clinical examination but through laboratory diagnosis, (http://www.brown.edu/courses/Bio_160/Projects2004/rotavirus/index.html. 30 October 2010). Laboratory diagnosis of rotavirus can be achieved by direct detection of the virus or viral antigens in faecal specimens (Pyadian *et al*, 1988).

This can be carried out using electron microscopy to detect the virus, enzyme immunoassay for viral antigen, or polyacrylamide gel electrophoresis to detect viral RNA of the rotavirus genome (Herry *et al*, 1982). These techniques are very specific but they are technically demanding and require specialized equipment which limits their application in routine work (WHO Rotavirus detection manual, 2009). The EIA, latex agglutination tests, and immunochromatographic tests using specific monoclonal or polyclonal antibodies are specific and sensitive techniques which can be used for rapid detection of rotavirus in faecal specimens (Herrmann *et al*, 1985). Detected rotavirus strains are characterized by RT-PCR (Herry *et al*, 1982).

1.2.2.1 **Rotavirus EIA**

The most suitable detection format for rotavirus surveillance studies is the EIA which uses the rotavirus specific antibodies to capture antigen onto wells of plastic plates (WHO Rotavirus detection manual, 2009). The antigen is then detected in a colorimetric reaction using an enzyme conjugated rotavirus specific antibody. The optical density results can be easily read with standard plate reader, and the analysis of the results can be performed by a standard computer programme.

1.2.2.2 **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 Rotavirus detection manual, 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 (MRC DPRU Laboratory manual, 2009). The migration patterns of the segments of rotavirus dsRNA allow the classification of rotavirus strains into the 'short' and 'long' electropherotypes.

1.2.2.3 **Rotavirus detection by RT-PCR**

Rotavirus in clinical specimens can be detected and the G and P types determined by extraction of the viral RNA from faecal specimens and analysis by semi-nested RT-PCR with primers specific for regions of genes encoding the VP7 (G-type) or VP4 (P-type) (WHO rotavirus characterization manual, 2009). The obtained genotype-specific PCR products are

analyzed by performing agarose gel electrophoresis. The detection of rotavirus dsRNA in faecal 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 polymerase chain reaction (PCR), PCR consists of :

- heating the DNA to be amplified to separate the template strands
- annealing of two primers that are complimentary to the region to be amplified
- extension of the primers by a heat stable DNA polymerase enzyme that uses each DNA strand as template
- 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

1.2.2.4 **Rotavirus genotyping using RT-PCR**

The rotavirus genotyping methods are based on semi-nested RT–PCR, in which viral RNA extracted from faecal specimens is reverse-transcribed and then amplified by PCR in the presence of consensus primers for the rotavirus genes specifying G (gene 9) or P (gene 4) genotype (Das *et al*). The primers are homologous to strains from different genotypes so that one primer pair can be used to amplify most human rotavirus strains (Gouvea *et al*, 1990). The DNA products from the first amplification cycle are used as template in the second PCR

cycle in the presence of one of the original consensus primers and a mixture of genotypic-specific primers of the opposing polarity from the consensus primer, each designed to yield a product of different size (Das, *et al*). The genotypes are determined based on the size of the PCR product after analysis by agarose gel electrophoresis (WHO Manual of rotavirus detection and characterization, 2009). Since RT-PCR genotyping can determine both G and P types, confirm results, and characterize non-typeable strains with nucleotide sequencing, it is the method of choice for most rotavirus laboratories.

1.2.3 Treatment and prevention

The administration of intravenous or oral rehydration fluids for treatment of rotavirus-caused dehydration and vaccination of children at risk can prevent or reduce the severity of infection (Armah *et al*, 2010). Significant reduction of deaths, morbidity and hospitalization caused by rotavirus infection can be achieved by inclusion of rotavirus vaccines, RotaTeq or Rotarix, in childhood immunization programmes (Armah *et al*, 2010). The 2 available rotavirus vaccines are live attenuated types which can be administrated orally. The Rotarix vaccine is a monovalent vaccine developed by attenuation of a human G1P[8] rotavirus strain, in cell culture (WHO Introduction of rotavirus vaccines, 2009). The significant efficacy of the Rotarix vaccine against infection by rotavirus genotype G1P[8] infection was reported. The pentavalent rotavirus vaccine, RotaTeq, has been derived from reassortment of human rotavirus strains with bovine strains that replicate poorly in the human body (WHO Introduction of rotavirus vaccines, 2009). The RotaTeq vaccine offers effective protection against infection by the following human rotavirus G-types: G1, G2, G3, G4 and G9 (Armah *et al*, 2010). These strains are the most common cause of severe diarrhoea in children less

than 5 years old. The WHO recommends that countries considering introducing rotavirus vaccine should carry out strain surveillance first in order to determine the predominant and circulating strains.

1.3 Literature review

1.3.1 Highlights of the Worldwide Rotavirus Surveillance findings

In a review of rotavirus surveillance worldwide over a 15 years period, Santos and Hoshino (2005) reported that the 5 most common rotavirus genotypes were detected in approximately 95 % of rotavirus infections worldwide. For example, G1P[8] caused 70 % of infections in North America, Australia and Europe, and 30 % of infections in South America, Asia and Africa. G2P[4] was common in South America (23 %) and Asia (13 %) whereas; G3P[8] was common in Africa (21 %) (Santos, and Hoshino, 2005). The unusual genotypes fluctuate in many parts of the world, but because trends could change, they should be continuously monitored (O'Ryan, 2009). The G and P combinations which are unusual are indicators of naturally occurring reassortments of various human rotavirus genotypes, reassortments between animal and human rotavirus strains or direct transmission from animal to human (Santos and Hoshino, 2005) Detection rate of the unusual rotavirus genotypes in some continents is as follows: Africa 27 %, Asia 14 %, South America 11 %, Europe 1.4 %, Australia 0.1 % and North America 5 % (O'Ryan, 2009).

For example, the G8P[4] and G8P[6] have been identified in Africa since 1990s responsible for about 13 % of gastroenteritis caused by rotavirus. The G5P[8] strain has been reported in South America and the G12P[8] and G12[P6] strains are being increasingly detected in cases of acute gastroenteritis worldwide (O'Ryan, 2009). High frequency of unusual genotypes in certain

geographical regions suggests that the genotypes may have genetic stability and the potential to spread in a population depending on the compatibility with the human intestinal receptors. Some studies have reported nontypable rotavirus strains which are probably occur as a result of point mutation, genetic reassortment, genomic rearrangement, or intragenic recombination (O'Ryan, 2009). The detection rate of non-typeable rotavirus strains is influenced by type of the technique used: enzyme immunoassay is generally associated with higher rate of non-typeable strains compared to reverse transcription polymerase chain reaction (RT-PCR) (Santos and Hoshino, 2005).

It is important to determine the predominantly circulating rotavirus electropherotype (e-type) in order to detect replacement of circulating phenotype (Zuridah *et al*, 2004). The combination of electropherotyping and genotyping techniques avoids the problems associated by reports of rotavirus without group antigen (Zuridah *et al*, 2004). In a study carried out in Malaysia by Zuridah *et al* (2004) it was documented that rotavirus e-types could coexist or any e-types could replace each other. The e-type replacement cycle can take 2 years, 3 years, 6 years, 10 years and 12 years. In Malaysia and other parts of the world the rotavirus with 'long' e-type was associated with outbreaks, the 'short' e-type was detected with low incidence (Zuridah *et al*, 2004). Electrophoresis of RNA is invaluable for group A rotavirus identification, both its 'long' e-type and 'short' e-type can be detected without ambiguity (Zuridah *et al*, 2004). The association between the e-types and genotypes of rotavirus is conflicting, authors of some studies associated the 'short' e-type with G2 and the 'long' e-type with G1, G3 and G4.

1.3.2 Circulating rotavirus genotypes in America

Rotavirus infection shows a distinct seasonality pattern particularly in temperate climates (O'Ryan, 2009). In the United States, gastroenteritis caused by rotavirus reaches its peak in the Southwest states in the November to December period and April to May in the Northeastern states (Centers for Disease Control and Prevention, 2006) The epidemiological surveillance of rotavirus strains has led to the identification of five common group A rotavirus strains worldwide. The common strains are; G1P[8]; G2P[4]; G3P[8]; G4P[8] and G9P[8]. Due to substantial rotavirus diversity, over 100 G and P genotype combinations have been identified in various studies (Matthijnssens *et al*, 2009). Circulating rotavirus G and P genotype combinations can vary in different regions or within the same country. The incidence of genotypes can vary from year to year within the same region or country. The study by Clark *et al* (2004) on prevalence of circulating rotavirus strains in Philadelphia between 1994 and 1999 has shown that the predominant strain varied significantly over the 5 year period. The results of the study indicated that G3P[8] strains predominated in 1994 to 1995. G9P[8] was detected in more than 50 % cases during rotavirus G9 strain outbreak in 1995 to 1996 rotavirus season. In both 1996 to 1997 and 1997 to 1998 period acute diarrhea in young children was caused by G1P[8] strain, and in 1998 to 1999 G1P[8] and G2P[4] were each detected in about 50 % cases of rotavirus infection.

Before the use of rotavirus vaccine for immunization of USA infants in 2006, Gentsch *et al* (2009) conducted rotavirus strain surveillance for 9 years: 1996 to 2005, with the aim to provide pre-vaccination data. A total of 13100 rotavirus strains from different USA regions were genotyped to determine the G-types and P-types using RT-PCR and nucleotide sequencing. In

USA the most prevalent strain each year, 1996-2005, was G1P[8] (78.5 %).

The G2P[4], G9P[8], G3P[8] and G4P[8] had frequencies of 9.2 %, 3.6 %, 1.7 % and 0.8 % respectively (Gentsch *et a*l, 2009). Rare genotypes such as G12P[6], G6P[9] and G3P[9] were observed during several rotavirus seasons in USA at low frequencies ranging from 0.5 % to 1.7 % (Gentsch *et a*l, 2009). The genotype G9P[6] emerged in 1995 and it was detected continuously for several seasons, from 1996-1997 and 2000-2001 but it was not detected in 2002-2005 rotavirus seasons. This observation necessitates monitoring of rotavirus strains after introduction of rotavirus vaccines (Gentsch *et a*l, 2009). About 85 % of rotavirus strains detected in USA infants during the pre-vaccine surveillance period, 1996-2005 had either a G or P antigen that is present in both rotavirus vaccines; RotaTeq and Rotarix (Gentsch *et a*l, 2009).

1.3.3 Circulating rotavirus genotypes in some African countries

According to Page and Steele (2004), the chance of a child born in Africa dying before the age of 5 years is one in six. The top three killers of children under 5 years in Africa include lower respiratory infections, diarrhoeal diseases, and perinatal disorders (Page and Steele, 2004).

In developed countries, diarrhoea is considered a minor illness rather than a life threatening disease (Page and Steele, 2004). Early microbial exposure and pediatric malnutrition result in an estimated 2.4 to 3.3.million childhood deaths due to diarrhoea per year in developing countries; rotavirus is responsible for about 25 % of all diarrhoeal deaths, 1 in 120 to 1 in 150 children will die by age of 5 years from rotavirus gastroenteritis in Africa (Cunliffe *et al*, 1998).

The typing of circulating rotavirus strains before 1990 was done for studies which were conducted in Gambia, Kenya , Malawi, Ghana, Guinea-Bissau, Central African Republic, Nigeria and South Africa, G1, G2, G3, and G4 were predominant, and 22 % of the EIA rotavirus

positive samples from these African settings were untypeable (Page and Steele, 2004). The improvement in molecular techniques allowing G-typing of circulating rotavirus strains resulted only in 12 % of the rotavirus positive samples being untypeable.

At the WHO sponsored African Rotavirus Network annual workshops held in 1998 to 2004 , genotypes were determined for rotavirus positive samples from : Zimbabwe, Zambia, Botswana, Tanzania, Kenya, Sudan, Tunisia, Namibia, Nigeria, Burkina Faso, Cote d' vore, Mauritius, and Ghana; the genotype G1-G4 were predominant although G9 genotype was detected in Ghana, Nigeria, Cameroon, Kenya and Botswana (Page and Steele , 2004). In a study by Cunnliffe *et al* (2010) in Malawi rotavirus detection rate in hospitalized children with gastroenteritis was 32.1 % which was slightly lower than the rate in outpatients, 34.0 %. In the same study 76.7 % of children with confirmed rotavirus gastroenteritis were younger than 12 months of age, 33.8 % were less than 6 months of age, and 7.6 % were younger than 3 months old. The seasonality of rotavirus infection in Malawi was noted during the dry season months of May to October during 1997 to 2007 study period (Cunnliffe *et al* 2010). The detected genotypes in Malawi included G1P[8], G8P[6], G8P[4], G1P[6], G3P[8] and G9P[6] which comprised 83 % of all typeable strains (Gentsch *et al*, 2005). The common rotavirus strains which caused gastroenteritis during 1998 – 1999 study period in Namibian children below 5 years were G1P[8], G1P[6], G1P[4] and G2P[4], and the children below 18 months of age were most affected (Page *et al*, 2010). The G9P[6] and G8P[4] genotypes were also identified in Namibia (Iturriza-Gómara *et al*, 2001). The rotavirus season in Namibia occurs during the cool dry winter months, May to October, a pattern similar to that observed in South Africa (Page *et al*, 2010). In South Africa a second rotavirus infection peak was observed during the rainfall months of October to December (Page *et al*,

2010).

In a study to investigate circulating rotavirus strains in the rural areas in South Africa by Potgieter, *et al* (2010); a total of 111 children who reported at the primary health care clinic with acute gastroenteritis, which was equivalent to 26.4 % of tested samples, were positive for group A rotavirus. The G1P[6] genotype was most predominant followed by G1P[8], G9P[6], G8P[4], G1P[4], G8P[6] and G2P[6] Potgieter, *et al* (2010). In this investigation gastroenteritis caused by rotavirus infection occurred more frequently in children below 2 years of age, predominantly in infants younger than one year old (Potgieter, *et al*, 2010). Similar trends were reported from many African countries, such as Egypt and Tunisia; Central African countries, such as Malawi; East African countries, such as Tanzania and Kenya; West African countries, such as Guinea-Bissau and Nigeria; and other Southern African such as Namibia and Botswana countries. Little is known about the circulating rotavirus strains in Zimbabwe, although the first study on rotavirus caused diarrhoea in children was carried out by Tswana *et al* in 1990.

1.3.4 Circulating rotavirus genotypes in Zimbabwe

In a study by Tswana et al (1990) the incidence of rotavirus infection in Zimbabwean children, younger than 24 months, from selected communal farming location and high density suburb was reported, but no genotyping was done on the EIA rotavirus positive faecal samples. There was association between presence of diarrhoea and detection of rotavirus in faecal samples, 23.6 % diarrhoeal faecal samples and 8.5 % control faecal samples were EIA rotavirus positive. The highest rotavirus infection incidence was found during the dry cool season, May to September months and the most affected children were below 6 months old. Rotavirus genotypes G1P[8],

G2P[4], G12P[6] and G9P[8] were identified from EIA rotavirus positive faecal samples which were collected from children with acute diarrhoea, in the 2 provinces in Zimbabwe during 2007 and 2008 (The Department of Medical Microbiology, College of Health Sciences, University of Zimbabwe: unpublished data). The year to year rotavirus genotypes circulating in Zimbabwe are not known, due to high variation in the rotavirus genome it is important that strain surveillance is carried out before introduction of a vaccine (Zhao-Yin Fung *et al*, 2002). In some countries where the rotavirus vaccine was introduced without adequate strain surveillance it was found out that the diversity of rotavirus strains was greater than was originally recognized. With the approval of RotaTeq and Rotarix vaccines by WHO rotavirus vaccination is now a reality. Since Zimbabwe is one of the African countries considering introduction of rotavirus vaccine it is crucial that proper rotavirus strain surveillance is carried out.

1.4 Project justification

The sentinel rotavirus surveillance in hospitalized children with gastroenteritis was conducted in Zimbabwe at Parirenyatwa Group Hospitals, Harare Central Hospital and Chitungwiza Central Hospital with the aim to determine the disease burden, to establish the characteristics of the current strains and to study rotavirus subtypes at the molecular level to obtain pre-vaccine introduction data and subsequent vaccine effectiveness studies (Bourdett-Stanziola *et al*, 2008). In order to be able to measure the benefits of rotavirus vaccination programme in Zimbabwe, there is need to detect and characterize the circulating strains before the introduction of the rotavirus vaccine. This will also assist the vaccination programme managers to procure a vaccine which gives appropriate protection against the predominant rotavirus strains which commonly cause gastroenteritis in Zimbabwe. When the rotavirus vaccine is introduced, surveillance of

circulating strains should be continued in order to detect the emergent strains because rotavirus has the ability to evolve through genetic drift or re-assortment (O'Ryan *et al*, 2009). The emergent rotavirus strains could result from naturally occurring re-assortment between human and animal strains or direct transmission from animal to humans (O'Ryan *et al*, 2009).
The findings of this study will determine the lineage of the circulating rotavirus in Zimbabwe, and this will provide important epidemiologic information for effective surveillance of rotavirus strains.

1. 5 Objectives of the study

1. 5. 1 Main objective

The main objective of this study is to determine the predominant rotavirus strain or strains which cause(s) severe diarrhoea requiring hospitalization of children younger than 5 years old in Zimbabwe. Furthermore, the data obtained will be considered as a baseline of circulating rotavirus strains in Zimbabwe, and this makes assessment of rotavirus vaccination benefits possible. Emergent rotavirus strains can also be detected in the post vaccine introduction era when the predominant strains are identified before vaccination.

1. 5. 2 Specific objectives:

- To determine the most affected age group which is admitted with severe diarrhoea caused by rotavirus in Zimbabwe.
- To detect common, uncommon and novel rotavirus strains which cause severe diarrhoea requiring hospitalization of children younger than five years old in Zimbabwe.
- To determine prevalence of rotavirus EIA antigen by gender (control and cases)

- To compare the association of PAGE (electropherotypes) results with genotypes.

CHAPTER 2

2 Materials and Methods

2.1 Ethical Issues

Since faecal specimens which were used in this study were submitted to the Lab by the Ministry of Health for laboratory diagnosis of rotavirus caused diarrhoea and AFP, the permission to use them was applied for and granted by the MOHCW. The letter granting authority is shown in Appendix 7.

2. 2 Study Setting

The study was carried out in the Department of Medical Microbiology, College of Health Sciences, University of Zimbabwe. As a designated WHO National Virology Laboratory, the department provides clinical laboratory diagnostic service for infectious diseases to the private and public patients as well as to research projects. It houses the WHO National Virology Laboratory whose role is to carry out laboratory diagnoses of Poliomyelitis, Measles, Rotavirus diarrhoea, and H1N1 Influenza viruses. The laboratory is well equipped with instruments to perform serological tests and molecular techniques. Characterization of influenza viruses, measles virus strains and intratypic differentiation of polioviruses procedures are performed in the virology laboratory using the traditional PCR and real time PCR. The virology laboratory also has the capacity to do cell culture techniques for the study of measles virus, influenza viruses polioviruses and enteroviruses.

As a WHO accredited laboratory the virology laboratory collaborates with the WHO regional and specialized laboratories and has access to the specialized facilities in these laboratories.

2.3 Specimens

2.3.1 Justification of sample size

Due to time constraint the rotavirus surveillance samples which were collected from January to July 2010 were analysed in this study. According to WHO recommendation 30% of the rotavirus EIA positive samples should be genotyped (World Health Organization, 2009). A total of 515 samples tested positive for rotavirus antigen detection by EIA but only 50 (9.7 %) of them were genotyped because of shortage of reagents.

2.3.2 Faecal specimens from gastroenteritis cases

This study is part of the on-going rotavirus surveillance programme conducted by the Ministry of Health and Child Welfare (MOHCW) in 3 sentinel sites: Parirenyatwa Group Hospitals, Harare Central Hospital and Chitungwiza Central Hospital. A gastroenteritis case was defined as a child less than 5 years of age who presented to a designated sentinel site for treatment of acute gastroenteritis as a primary illness (World Health Organization, 2009). Faecal specimens were collected within 48 hours of admission not more than 7 days after onset of acute diarrhea. Faecal specimens were sent from all the 3 sentinel sites and selection of the specimens for this study was based on the following criteria:

a) Inclusion criteria: (World Health Organization, 2009)

1. Child less than 5 years of age
2. Admitted for treatment of acute gastroenteritis as a primary illness
3. Gastroenteritis of less than or equal to 7 days
4. Admitted in a hospital ward

b) Exclusion criteria: (World Health Organization, 2009)

1. Child aged more than or equal to 5 years
2. Child with bloody diarrhoea
3. Child with symptoms for more than 7 days
4. Patient acquired gastroenteritis during hospitalization for treatment of other diseases.

All the case information required for application of the criteria to a given specimen were captured in the World Health Organization rotavirus surveillance form which is shown in Appendix 1. Faecal specimens were transported at 4ºC to the Department of Medical Microbiology, Virology Laboratory for detection of rotavirus antigen using enzyme-immunoassay (EIA) assay. Every faecal specimen received in the Virology Laboratory was accompanied by a properly filled-in rotavirus surveillance form. On receipt, 3 aliquots of each specimen were prepared in 2ml cryovials: one aliquot was used for detection of rotavirus antigen by EIA and, was then stored at 4ºC; the remaining 2 backup aliquots were stored at -20°C. From January 2010 to July 2010 a total of 784 faecal specimens were received and tested for rotavirus antigen using EIA. A total of 50 stored faecal specimens, positive for enzyme-immunoassay (EIA) rotavirus antigen test were genotyped and the EIA rotavirus antigen results were confirmed by PCR. Polyacrylamide gel electrophoresis (PAGE) was performed on 30 rotavirus antigen positive specimens.

2.3.3 Control: Faecal specimens from cases without gastroenteritis

Fifty non-diarrhoeal faecal specimens from children less than 5 years old admitted in a provincial and referral hospitals, with acute flaccid paralysis (AFP) were selected as controls for samples collected from children with gastroenteritis. The AFP surveillance programme of MOHCW was

designed to detect all AFP cases caused by the wild poliovirus strains and is on-going. The selected non-diarrhoeal faecal specimens were part of the faecal specimens which were sent at 4ºC to the Virology Laboratory for poliovirus isolation, from January to July 2010. On receipt, an aliquot of the specimen was processed for poliovirus isolation and the remaining original faecal specimen was stored, in its original container, at -20°C as a backup. The control faecal specimens were tested for rotavirus antigen using EIA ProSpecT™ Rotavirus Microplate Assay kit. The genotyping, PAGE and PCR were not done for control faecal specimens due to resources limitations.

2.4 Bio-safety issues

All specimens: faeces, amplicons and gels were treated as potentially infectious and were handled with care in a Class II safety cabinet. Gloved hands were used in all procedures. The detailed laboratory safety rules are shown in Appendix 2.

2.5 Methods

2.5.1 Rotavirus detection techniques:

a) Antigen detection using EIA

The faecal specimens were prepared and tested for rotavirus antigen according to instructions of the commercially-available, ProSpecT™ Rotavirus Microplate Assay, (Oxoid Ltd, UK). The assays were validated using the manufacturer's results validation criteria. For QA/QC purposes the only microtitre pipettes with a calibration certificate were used, and the in-house negative and positive controls were used in addition to the negative and positive controls from the kit. Results were read spectrophotometrically at 450nm with 620nm as reference filter. The optical

densities (ODs) were printed out and the cut-off value was calculated by adding 0.100 absorbance units to the OD of the negative control. Any OD value below the cut-off value was recorded on the worksheet as negative and the OD values above cut-off value were recorded as positive. For a valid assay the OD of the negative control must be < 0.150 and that of a positive control must be > 0.500.

b) Nucleic acid detection

i) Polyacrylamide gel electrophoresis (PAGE)

A total of 50 EIA rotavirus positive specimens were transported by air, at 4ºC, to the MRC Diarrhoeal Pathogens Research Unit (DPRU), University of Limpopo, Medunsa Campus (South Africa), where RNA-PAGE was performed on 30 specimens during the month of August 2010. The DPRU is a rotavirus regional reference laboratory for WHO Afro-Region. The reagent preparation for rotavirus dsRNA extraction, PAGE and silver staining methods was carried out according to SOP used in DPRU which is described in Appendix 3 (MRC Diarrhoeal Pathogens Research Unit, 2009 and WHO Rotavirus detection Manual, 2009). The rotavirus dsRNA was extracted from a 10 % faecal suspension using phenol/chloroform and the DPRU standard operating procedure (SOP) for extraction of RNA from faeces for PAGE, (MRC Diarrhoeal Pathogens Research Unit, 2009). The rotavirus RNA was precipitated with 1M sodium acetate containing 1 % sodium dodecyl sulphate using according to the SOP. The rotavirus RNA segments were separated by PAGE as per the method described in the SOP (MRC Diarrhoeal Pathogens Research Unit, 2009). The separated rotavirus RNA segments on the gel were stained with silver nitrate stain for visualization of separated rotavirus RNA segments using the staining (MRC Diarrhoeal Pathogens Research Unit, 2009 and WHO Rotavirus detection Manual, 2009).

The migration pattern of the rotavirus RNA segments from the faecal specimen was compared to the migration pattern of a known group A rotavirus strain, controls. The migration pattern of the rotavirus RNA segments on the gel identified the rotavirus strain as either "short" or "long" long electropherotype. Results were recorded as "short" for rotavirus RNA segment migration pattern similar to that of the control rotavirus strain with "short" electropherotype (DS1-like strains), and "long" for rotavirus RNA segment migration pattern similar to that of the control rotavirus strain with "long" electropherotype (WA-like strains) (MRC Diarrhoeal Pathogens Research Unit, 2009 and WHO Rotavirus detection manual, 2009). The QA/QC was achieved by inclusion of control rotavirus strains in the assay and, use of regularly serviced equipment.

ii) Reverse transcription polymerase chain reaction (RT-PCR)

The same 50 EIA rotavirus positive faecal specimens which were transported at 4ºC to the MRC Diarrhoeal Pathogens Research Unit, University of Limpopo, Medunsa Campus (South Africa), for PAGE technique were also tested for rotavirus RNA using RT-PCR method. All the 50 specimens were tested during the month of August 2010. The rotavirus RNA extraction from faecal specimens for detection of rotavirus using reverse transcription-polymerase chain reaction (RT-PCR) was carried out using a commercially available kit, the QIAamp® viral RNA extraction kit (Qiagen, Germany). The RT-PCR was performed using several primer pairs designed to target highly conserved regions of the RNA genome. Con2 and Con3 primers were used to amplify the VP4 gene (Gentsch *et al*, 1994), and the End9 and sBeg9 primers were used to amplify the VP7 gene (Gouvea *et al*, 1990). Sequences of the primers used are shown in Appendix 4. The RT-PCR was performed according to the SOP used in DPRU (MRC Diarrhoeal Pathogens Research Unit, 2009). The preparation of RT-PCR reagents and RT-PCR method is

shown in Appendix 5. The amplicons and a 100bp molecular size marker were electrophoresed on the 1 % agarose TAE gel and viewed under UV light for VP4 or VP7 gene-specific amplicons. The amplicons were compared to those of a molecular marker and the positive control (Africa rota 2009) and, the results were recorded as negative, 1+ intensity, 2+ intensity, 3+ intensity or 4+ intensity. The amplicons were stored at -20°C for use in the genotyping PCR. The QA/QC was considered by the inclusion of the molecular marker, negative and positive extraction controls and regular maintenance of equipment.

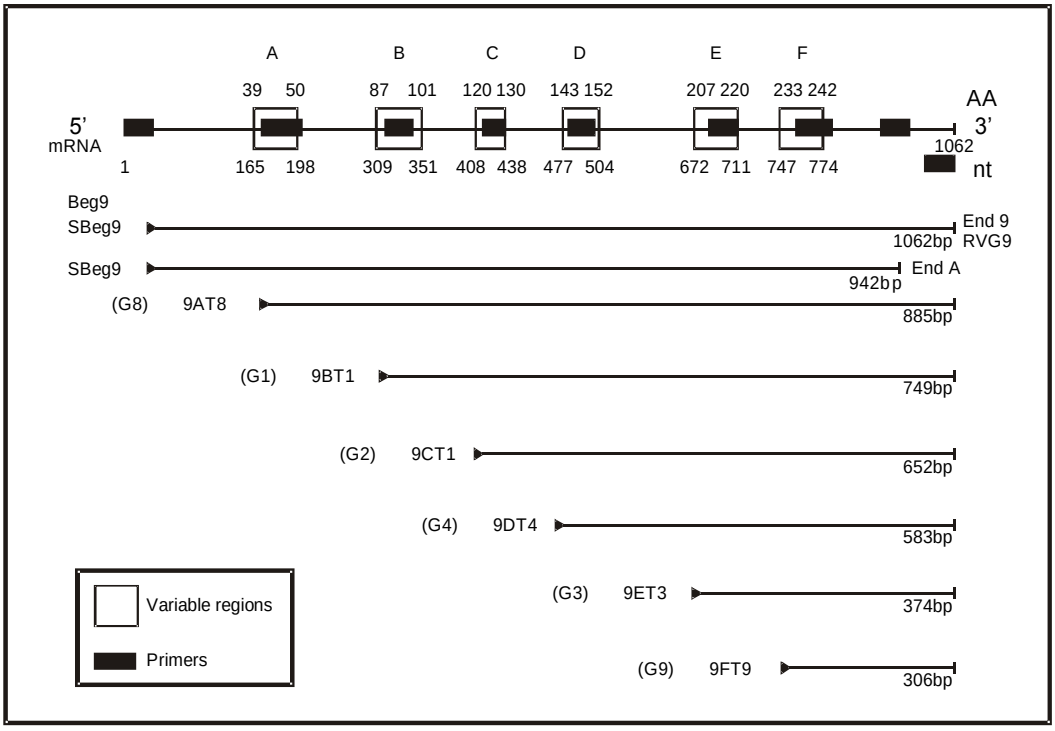
2.5.2 Rotavirus genotyping

The stored amplicons, 50 specimens, from the above method were used for genotyping of both VP7 and VP4 genes. The genotyping PCR was performed in accordance with the SOP used by the DPRU which is shown in Appendix 5 (MRC Diarrhoeal Pathogens Research Unit, 2009). Precautions were taken not to use excessive RT-PCR reactions for those amplicons which were 3+ or 4+ for both VP7 and VP4 gene amplifications. This was done in order to avoid formation of non specific PCR products during electrophoresis of the genotyping PCR products. A cocktail of genotype specific primers was used. The VP7 genotyping was carried out using genotype-specific primers for G1, G2, G3, G4, G8 and G9, the primers used were 9BT1, 9CT1, 9ET3, 9DT4, 9AT8 and 9FT9 respectively. Genotypic-specific primers which were used to genotype VP4 were; Con3, dP[8], 2T-1, 3T-1, 4T-1, 5T-1, mP[11] and P[14] (SE-1). All the primers used for genotyping of VP7 and VP4 are shown in Appendix 4 with their sequences. The amplicons were electrophoresed together with a 100bp molecular weight marker in a 2 % agarose TAE gel at 90 volts according to the method used in DPRU which is shown in Appendix 5 (MRC Diarrhoeal Pathogens Research Unit, 2009). The bands were compared with the molecular

weight marker and the known genotypes. The expected VP7 genotypes PCR sizes are shown in Figure 4 and the expected VP4 genotypes PCR sizes are shown in Figure 5.

The results of controls were used to validate the assay and the results were recorded on a spreadsheet with all the patient's details. QA/QC procedures included the dedication of specific rooms for; specimen preparation, reagent preparation, PCR and gel electrophoresis. Working areas were disinfected with both hypochlorite and 70 % alcohol in order to avoid contamination of the work and other lab workers.

Figure 4. Schematic illustration of the VP7 genotyping PCR



Source: Adopted from MRC DPRU Manual, 2009

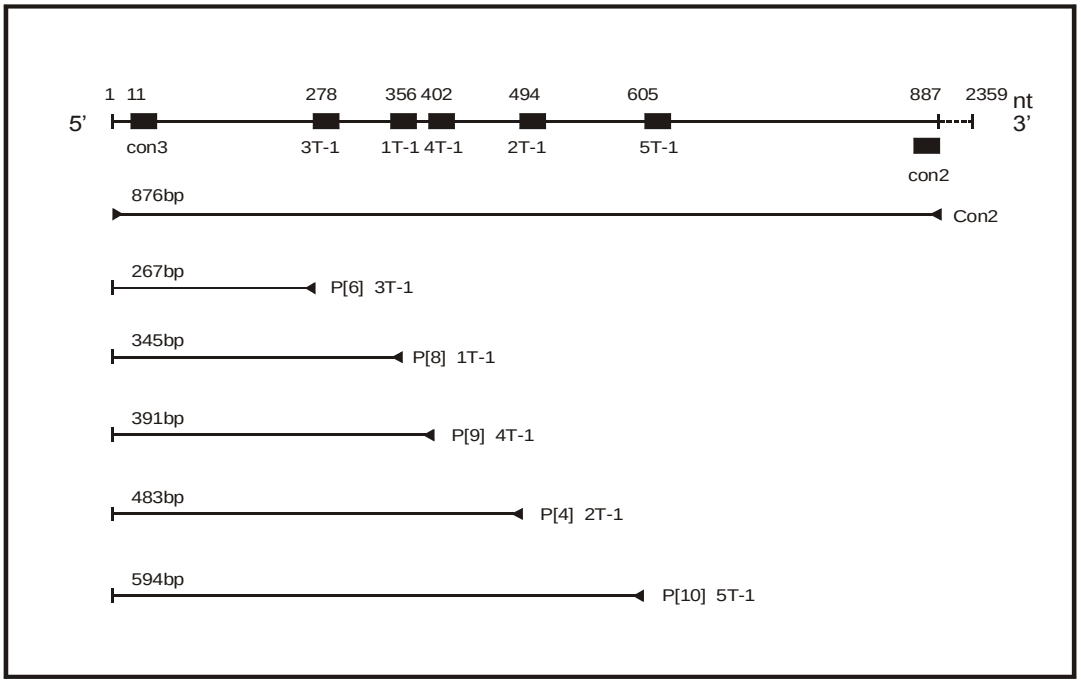
Key: sBeg9/Beg9 and End9 products 1062bp

Human rotavirus VP7 genotypes (3' end primer):

VP7 genotype G1 749bp

VP7 genotype G2	652bp
VP7 genotype G3	374bp
VP7 genotype G4	583bp
VP7 genotype G8	885bp
VP7 genotype G9	306bp

Figure 5. Schematic illustration of the VP4 genotyping PCR using Gentsch primers



Source: Adopted from MRC DPRU Manual, 2009

Key: Con2/Con3 products 876bp

Human rotavirus VP4 genotypes:

VP4 genotype P[8]	345bp
VP4 genotype P[4]	483bp
VP4 genotype P[6]	267bp
VP4 genotype P[9]	391bp
VP4 genotype P[10]	594bp

2.6 Analysis of Results

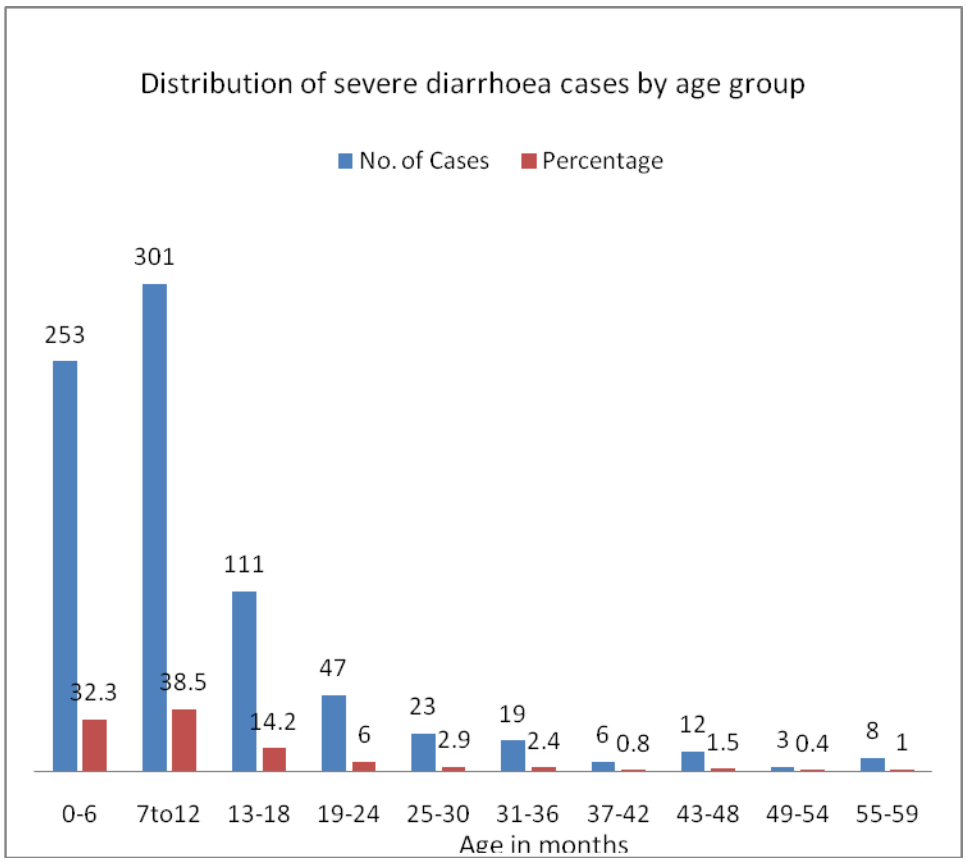
The statistical analysis of the data was done using STATA 10.1 software programme. Prevalence of EIA rotavirus antigen positive specimens collected from acute diarrhoea cases was compared to the prevalence of EIA rotavirus antigen positive specimens from non-diarrhoeal patients. The prevalence of electropherotypes in rotavirus diarrhoea cases was determined. The commonly affected age group and frequency was determined. The prevalence of EIA rotavirus antigen in male and female cases was compared. The prevalence of the separate G genotypes, P genotypes and the combination genotypes were calculated. The prevalences of the genotypes were tabulated. Summary of the raw data is shown in Appendix 6.

CHAPTER 3

3. Results

A total of 783 faecal samples collected from hospitalized children < 5 years old with severe diarrhoea were analyzed by rotavirus antigen detection EIA. Analysis of surveillance data forms showed that the diarrhoea cases comprised of 303 females and 480 males. The incidence of severe diarrhoea which required hospitalization was highest (90.9 %) in children less than 25 months old. Distribution of severe diarrhoea by age group is shown in Figure 6. The incidence of severe diarrhoea which required hospitalization of the patient was reduced in children > 24 months old (9.1 %). The total number of EIA analyzed faecal samples from non-diarrhoea cases aged less than 5 years (controls) was 50, with 19 females and 31 males.

Figure 6: Distribution of severe diarrhoea cases by age group in the study

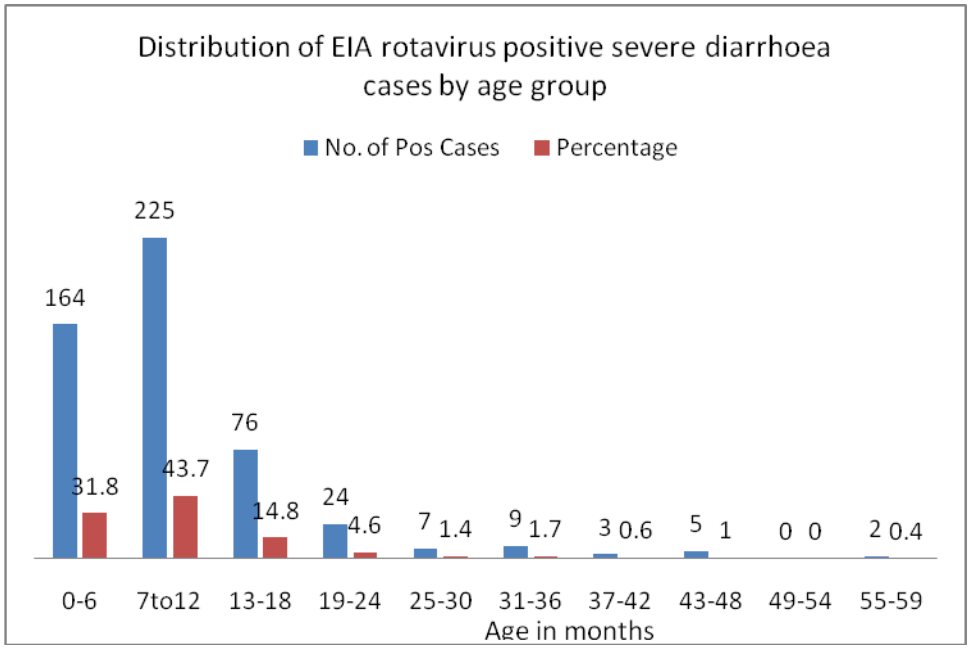


3.1. Detection of rotavirus antigen in severe diarrhoea cases and controls by EIA kit method

A total of 515 (65.7 %) and 16 (32 %) controls (non-diarrhoea patients) tested positive for rotavirus antigen. The result showed an association between severe diarrhoea and rotavirus infection, odds ratio of 4.083, with $p<0.0001$ and 95 % CI (2.22-7.53). The rotavirus positive cases in males constituted 63.7 % (n=328) of the total rotavirus positive cases which compares to 36.3 % of cases in females (n=187). Although the odds ratio showed that male cases had 1.3 chances of being infected by rotavirus than female cases, the difference is not significant, odds

ratio was 1.9 with p=0.058, 95 % CI (0.94-1.8). The risk of control males and females for infection by rotavirus was analyzed.

Figure 7: Distribution of EIA rotavirus positive severe diarrhoea cases by age group in the study



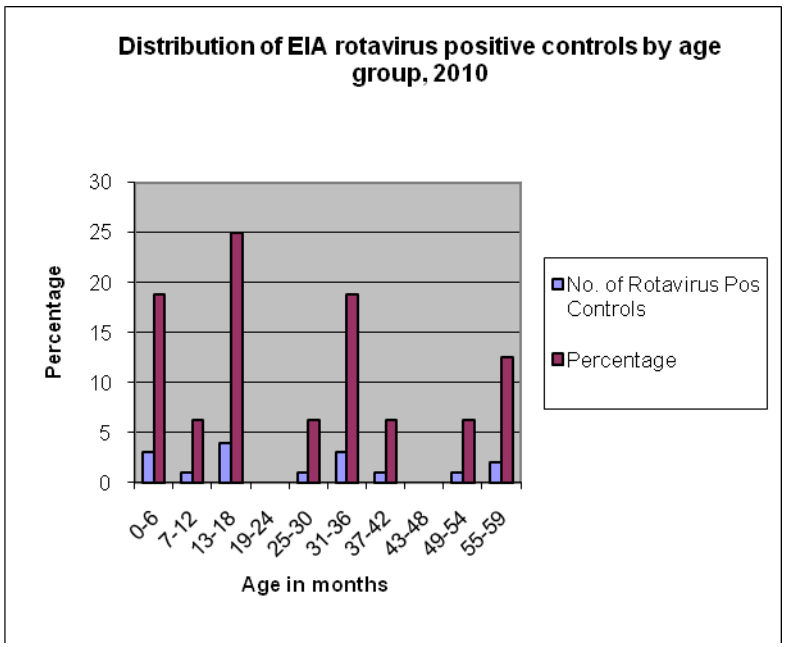
Distribution of EIA rotavirus positive cases shows that the children ≤18 months old had high prevalence of rotavirus infection and the older age groups showed reduced prevalence (Figure 7). Children below 18 months had high risk of being infected by rotaviruses compared to older children; this was statistically significant, 3.80 with p<0.0001, 95 % CI (2.28-6.31).

Table 1: Distribution of EIA rotavirus positive severe diarrhoea cases by month in 2010

Month	No. of Pos Cases	Percentage
January	3	0.6
February	0	0
March	0	0
April	0	0
May	21	4.1
June	300	58.2
July	191	37.1
Total	515	100

All the acute gastroenteritis cases which were detected during the study period January to July 2010 were tested for rotavirus antigen by EIA. The prevalence of cases with confirmed rotavirus diarrhoea was determined and recorded for each month. Rotavirus prevalence was highest during the dry and cool month of May, June and July 2010. Largest number of severe rotavirus diarrhoea cases was detected during the month of June 2010. Frequency of rotavirus acute gastroenteritis cases was lowest during relatively wet months of January to April 2010.

Figure 8: Distribution of EIA rotavirus positive controls by age group



The controls which tested positive for EIA rotavirus antigen were evenly distributed throughout all the age groups, with slightly high incidence in ≤ 18 months old children (Figure 8). Both sexes in the controls showed the equal number, 50 % (n=8) with rotavirus positive EIA results. Control males had 1.1 chances of getting rotavirus infection as compared to females but this was not significant, odds ratio was 0.19 (p=0.846), 95 % CI (0.34-3.70).

3.2 Rotavirus genotyping

3.2.1 VP7 genotyping

A total of 50 samples were selected from the rotavirus antigen positive faecal specimens and subjected to genotyping using RT-PCR methods. After the first amplification step, VP7 RT-PCR products were obtained for 42 (84 %) of the selected 50 samples. The remaining 8 (16 %) did not yield any visible first-round products. For the 42 samples with visible first-round amplification

products, VP7 genotypes were determined in 25 (59.5 %), 12 (28.6 %) were non-typeable and 9 (11.9 %) produced non-specific results. A total of 7 different rotavirus VP7 genotypes were observed. The strains included the common G1-G3 and G9 strains and unusual human rotavirus strains with G8, G10 and G12 VP7 genes (Table 2). The predominant G-genotypes observed were G9 (9 [21.4 %] of 42 samples) followed by G2 (4 [9.5 %]), G1 (2[4.8 %]) and G3 (1 [2.4 %]). The unusual human rotavirus strains were identified as mixtures at low levels. Mixed infections of G1/G10, G10/G12, G8/G12 and G3/G10 genotype specificity were observed in 9 specimens 21.4 % (Table 2). High prevalence of the common human rotavirus strains and mixed infections occurred in children ≤ 12 months old.

Table 2: Distribution of rotavirus G-types by age group, January to July 2010

G-type	<12 months No. (%) (n=37)	12-59 months No. (%) (n=37)
G1	2 (5.4)	0 (0)
G2	3 (8.1)	1 (2.7)
G3	1 (2.7)	0 (0)
G4	0 (0)	0 (0)
G9	5 (13.5)	4 (10.8)
G1/G10	1 (2.7)	2 (5.4)
G10/G12	1 (2.7)	0 (0)
G8/G12	3 (8.1)	0 (0)
G3/G10	2 (5.4)	0 (0)
Untypeable	9 (24.3)	3 (8.1)
Total G-types	27 (73)	10 (27)

3.2.2 VP4 Genotyping

Forty-four (88 %) samples out of 50 selected from rotavirus EIA positive samples yielded visible first round amplification products for VP8 portion of the VP4 gene, when genotyped by RT-PCR. Six (12 %) samples did not yield visible-first round amplification products. A total of 23 (52.3 %) samples, out of 44, had VP4 genotypes assigned and 19 (43.2 %) were non-typeable. Two (4.5 %) samples produced non-specific results. A total of 3 human rotavirus P-types such as P[4], P[6] and P[14] were observed. Some rotavirus strains with mixed P-types were observed (P[4]/P[6] and P[4]/P[14]) (Table 3). The predominant P genotypes in children ≤ 12 months old were P[6] 11 (26.2 %) followed by P[4] 4 (9.5 %) and P[14] 2 (4.8 %). The human rotavirus strains with mixed P genotypes were circulating at lower level

Table 3: Distribution of rotavirus P-types by age group, in January to July 2010

P-type	<12 months No. (%) (n=42)	12-59 months No. (%) (n=42)
P4	4 (9.5)	2 (4.8)
P6	11 (26.2)	2 (4.8)
P14	2 (4.8)	0 (0)
P4/P6	1 (2.4)	0 (0)
P4/P14	1 (2.4)	0 (0)
Untypeable	10 (23.8)	9 (21.4)
Total P-types	29 (69)	13 (31)

3.2.3 G and P combination

Combination of G and P genotype specificities was possible for 15 rotavirus positive strains (Table 4). The predominant G and P type combinations found were; G2P[6] accounting for 7.1 % (n=3) of the 42 fully characterized rotavirus strains and G9P[6] 3(7.1 %) of the fully typeable strains). Other G and P combinations observed were G1P[4] 1(2.4 %), G1P[6] 1(2.4 %) and G2P[4] 1(2.4 %). Almost all of these G and P combination strains were found in children ≤ 12 months old (Table 4). No globally common G and P combination strains were identified; only less common genotypes and possible reassortants ones. Mixed infections were found in 6 samples (14.3 %) and they were associated with G1/G10, G8/G12, P[4]/P[6] and P[4]/P[14].

Table 4: Distribution of rotavirus G and P type combination by age group, January to July 2010

G-P type	<12 months No. (%) (n=15)	12-59 months No. (%) (n=15)
G1P[4]	1 (6.7)	0 (0)
G1P[6]	1 (6.7)	0 (0)
G2P[4]	0 (0)	1 (6.7)
G2P[6]	3 (20)	0 (0)
G9P[6]	2 (13.3)	1 (6.7)
G1/G10P[4]P[14]	1 (.67)	0 (0)
G1/G10P[4]	0 (0)	1 (6.7)
G8/G12P[4]	3 (20)	0 (0)
G12P[4]P[6]	1 (6.7)	0 (0)
Total G andP type combinations	12 (80)	3 (20)

3.3 Polyacrylamide gel electrophoresis (PAGE).

Thirty samples out of the 50 genotyped EIA rotavirus positive samples were analyzed by RNA PAGE. A total of 27 (90 % of 30 samples) samples showed RNA migration pattern typical of group A rotavirus strains. No migration pattern was observed for 3 (10 %) samples. Both ‘long’ and ‘short’ electropherotypes were obtained. A total of 19 (70.4 % of 27 PAGE positive samples) were ‘long’ electropherotype, 6 (22.2 %) samples were ‘short’ electropherotype and heterogeneity was observed in 2 (7.4 % samples which showed both long and ‘short’ electropherotypes (Table 5). Group A genotypes could have either ‘long’ or ‘short’ electropherotype, no specific genotype could be associated with a particular electropherotype. However, majority of the circulating rotavirus which caused acute gastroenteritis had long electropherotypes.

Table 5: Association of electropherotypes and genotypes of some rotaviruses circulating in Zimbabwe, January to July 2010

Genotype	Electropherotype (L-long or S-short)	Total
G2P[6]	S	2
G2P[6]	L/S	1
G9P[6]	L	1
P[14]	L	2
G3/G10	L	1
G3/G10	S	1
G1P[4]	L	1
P[4]/P[6]	S	1
G1/G10P[4]	S	1
G1/G10P[4]//P[14]	L/S	1
P[6]	L	4
G10/G12	L	1
G2P[4]	S	1

CHAPTER 4

4. Discussion

This study used stool samples collected from children with severe diarrhoea who were hospitalized at the 3 referral hospitals situated in the urban areas of Harare and Chitungwiza. Although no data was gathered about the place of residence for the hospitalized children, most of the severe diarrhoea cases were probably coming from the urban locations. To our knowledge, no previous study was carried out to address the problem of childhood gastroenteritis in urban areas of Zimbabwe. Only one study was conducted for the purpose of documenting the incidence of rotavirus infection in rural communities (Tswana *et al*, 1990). This study was designed to provide valuable data on the circulating rotavirus strains in the urban communities of Zimbabwe. Investigations into the human rotavirus types circulating in other African countries, such as Egypt, Tunisia, Guinea-Bissau, Nigeria, Kenya, Tanzania and Malawi, also focused predominantly on diarrhoeal faecal specimens from urban and periurban communities (Potgieter *et al*, 2010). In this January to July 2010 (7 month) study; children below the age of 25 months were the most severely affected by severe gastroenteritis. This finding was similar to findings from other African countries (Nyangao *et al*, 2010). In this study rotavirus was identified as the major cause of severe diarrhoea in children < 59 months old requiring hospitalization. The 65.7 % rotavirus detection rate determined in this study was more than double that reported (23.6% incidence) in a study carried out in one of the provinces of Zimbabwe by Tswana *et al*, (1990). The detection rate of rotavirus in children with acute gastroenteritis was higher than those reported in other African countries which were about 36 %. Analysis of rotavirus antigen detection in both the diarrhoea cases and controls shows that the difference in detection rates is

significant ($p<0.0001$) and rotavirus is the major cause of severe diarrhoea in children less than 5 years old.

As has been reported elsewhere for urban and periurban children in Africa (Potgieter *et al*, 2010), this investigation found that rotavirus diarrhoea occurred more frequently in chidren < 2 years old, predominantly in infants < 1 year of age. Similar trends have been reported from many developing countries, namely India, North African countries (Egypt and Tunisia), Central African countries (Malawi), East African countries, (Tanzania and Kenya), West African countries, (Guinea-Bissau and Nigeria) and other Southern African countries (Namibia and South Africa) (Nyangao *et al*, 2010). This is the age group which is targeted to receive the available rotavirus vaccines, Rotarix and RotaTeq, all doses of the vaccines should be administered within the first 9 months of life (WHO Introduction of Rotavirus Manual, 2009). Now that vaccines are a reality it is important to know the baselines before introducing their use for effective monitoring of the benefits. Some studies should be carried out in order to determine rotavirus disease burden and identify the circulating rotavirus strains before introduction of the vaccination programme. The identification of unusual P and G combinations in Zimbabwe may affect the efficacy of currently available rotavirus vaccine formulations and may contribute to the design and development of a broadly reactive rotavirus vaccine for use in African countries.

The rotavirus infection was higher in males (63.3 %) than in females (36.3 %) but the difference was not statically significant, the observation was similar to the findings reported previously (Zuridah *et al*, 2004). High rotavirus detection rate during dry, cool months of May to July is a finding which is consistent with studies from elsewhere in Sub-Saharan Africa, rotavirus

detection rates are increased during the dry cool months and wet months (Potgieter *et al*, 2010). The detection rate for the wet and warm months was not determined in this study because it was carried out during the complete dry cool period of the year and only 3 samples were collected for the few wet months. The high detection rate during the study period also suggests that this was the peak period for rotavirus gastroenteritis.

The main objective of this study was to establish which G and P genotypes of circulating rotavirus strains are present in hospitalized children with acute gastroenteritis in urban areas of Zimbabwe. The prevalence of rotavirus G and P genotypes has been monitored worldwide, and types G1, G2, G3, G4, G9 combined with P[8], P[4] and P[6] are the most frequently identified genotypes (Esona *et al*, 2010). This study shows that G9 (24.3%) was the most prevalent G type followed by G2 (10.1%), G1 (5.4%) and G3 (2.7), no G4 genotype was detected, this was in agreement with findings reported from Ghana, Nigeria and India where G1, G2, G3 and G9 were detected but not G4 (Potgieter *et al*, 2010). This highlights the need for continued surveillance to establish which rotavirus strains are circulating in a country each year. The relatively high prevalence of the G2 genotype in children below the age of 24 months is similar to the findings of a study conducted by Armah *et al* (2001) in Ghana. The detection of a globally uncommon G8 genotype suggests occurrence of zoonotic transmission at some time in the past, because G8 is a recognized rotavirus genotype circulating in cattle (Cunliffe *et al*, 2010). Genotype G10 detection in children with acute gastroenteritis also suggests zoonotic transmission because G10 rotavirus strains are important gastroenteritis pathogens in calves, horses, pigs and lambs (Esona *et al*, 2010). They have also been reported in humans in Asia, South America and Europe.

Identification of the G12 genotype suggests probable reassortment with other co-circulating rotavirus strains: G12 genotype was first identified in Malawi in 2005 (Cunliffe *et al*, 2010).

In this study P[6] genotype was the predominant P genotype (31 %) which occurred mainly in < 24 months in children and occasionally in older children with acute gastroenteritis. This finding is similar to that reported in South Africa (Potgieter *et al*, 2010). No P[8] genotype was identified in this investigation although it is prevalent in most African countries. This observation further justifies the need to conduct yearly rotavirus surveillance in order to determine which rotaviruses are circulating in the country. The P[4] genotype had a prevalence of 14.3 % and mixed P genotypes infections such as P[4]/P[6] displayed an incidence of 2.7 %, and is similar to the findings reported elsewhere in Africa (Nyangao *et al*, 2010). The detection of single P[14] genotype at a prevalence of 4.8 % and as a mixed infection, P[4]/P[14] (2.4 %) in this investigation, to our knowledge, has not been reported anywhere in the African setting. The genotype was detected from hospitalized infants below 12 months old who were suffering from acute gastroenteritis.

Unusual genotype combinations and mixed infection were found circulating in urban areas in Zimbabwe although at low levels. Unusual strains such as G1P[4], G1P[6], G2P[6] and G9P[6] accounted for 21.4 %, the mixed G and P combinations such as G1/G10P[4]/P[14], G1/G10P[4], G8/G12P[4] and G12P[4]/P[6] comprised 14.3 % of circulating strains in this study. In Africa, a few studies have reported the predominance of unusual genotype combination: G3P[4] and G2P[6] as the most important strains causing acute gastroenteritis in 1998 and 1999 respectively (Armah *et al*, 2001). In this study the G3P[4] and G2P[6] caused a few cases of acute

gastroenteritis. A number of unusual strains have also been reported worldwide (Gentsch *et al*, 2005). The appearance of the unusual genotypes may be due to possible genetic reassortments events during natural infections. The identification of unusual P and G combinations in Zimbabwe may affect the efficacy of currently available rotavirus vaccine formulations and may contribute to the design and development of a broadly reactive rotavirus vaccine for use in African countries.

Regardless of primers used, the occurrence of non-typeable (NT) rotavirus strains are expected in low percentage up to >50 % of all samples which give visible amount of first round amplification PCR products. Sequencing of the VP4 or VP7 genes of these non-typeable strains show that they contained several sequence changes in the region corresponding to the P[8] or G1 primer binding site that prevented amplification with the original genotyping primer. Prevalence of (NT) rotavirus in this study was 19 (45.2 %) which was within the expected range. According to some studies reported elsewhere in Africa, if the NT isolates are sequenced they can be assigned the appropriate G genotype or P genotype (Esona *et al*, 2010). In this study due to limited resources, the NT isolates were not sequenced but they were part of the denominator for calculation of the prevalence of a given genotype.

The long RNA electropherotype patten was predominant in gastroenteritis cases with typeable rotavirus isolates. Some studies reported specific association between electropherotypes and particular rotavirus genotype, no such association was observed in this study. Another study reported the association of short electropherotype with hypotonic dehydration and the long electropherotype with lethargy gastroenteritis signs (Zuridah *et al*, 2004). Association between

the age of a child with gastroenteritis electropherotype was reported.The short electropherotype infected older children than those infected by the long electropherotype (Zuridah *et al*, 2004). No such similar association was observed in this study; both the short and long electropherotypes were identified in all age groups. In a study by Potgieter *et al* (2010), the long RNA electropherotype was associated with new human genotypes and the finding substantiated the evidence of continued genetic interaction and elution of rotavirus strains in communities living in close association with domestic animals and cattle. In this study rotavirus genotypes such as G1P[4], G9P[6], G2P[6] and G1/G10P[4]/P[14] could be ascribed to close human – animal association (Potgieter *et al* 2010). People staying in the rural area are more closely associated with cattle and domestic animals than those in the urban area but due to high level of rural-urban migration some animal rotavirus strains can be transmitted to urban children.

CHAPTER 5

5. Conclusion

Overally, rotavirus was detected in 515 (65.8 %) of 783 specimens from rotavirus surveillance programme, which were obtained from Harare, Parirenyatwa and Chitungwiza Referral Hospitals from January to July 2011. Analysis of the age distribution of rotavirus-positive specimens revealed that the majority of children affected were <24 months of age. Analysis of the monthly distribution of rotavirus-positive specimens revealed that rotavirus season occurred mainly in dry cool winter months of May to August. The results of this study show that the most predominant combined G and P genotypes which cause gastroenteritis requiring hospitalization in children < 5 years old are G2P[6] (20 %), G9P[6] (20 %) and G8/G12P[4] (20 %). The most prevalent single G genotype is G9 (24.3 % and P genotype is P[6] (20 %). All these strains are novel and therefore are not included in the available rotavirus vaccines. The only common worldwide rotavirus genotype identified in this study was G2P[4] which constituted 6.7 % and the rest were novel strains. This indicates that there is extraordinary diversity of rotaviruses circulating in Zimbabwe. The predominant rotavirus strains were identified in children under 12 months old. The findings of this study suggest that the potential impact of rotavirus vaccines should be evaluated against the large number of circulating strain types, specifically against these unusual rotavirus strain combinations lacking the common rotavirus VP4 and VP7 proteins, which have been the focus of rotavirus vaccine development. The inclusion of comprehensive cross-reactive strains in a successful rotavirus vaccine will prevent infection of rotavirus in the first years of life and reduce the mortality associated with diarrhea in children. The identification of unusual P and G combinations in Zimbabwe may affect the efficacy of currently available rotavirus vaccine

formulations and may contribute to the design and development of a broadly reactive rotavirus vaccine for use in African countries.

The detection and characterization of rotavirus strains circulating in Zimbabwe between January to July 2010 adds to the baseline data on the molecular epidemiology of rotaviruses in Zimbabwe.

5.1 Recommendations

Similar studies should be conducted for at least 5 consecutive years in order to know all the circulating strains in the country. There is need to carry out a study to determine the mortality rate of rotavirus diarrhoea in children. Surveillance of animal rotaviruses and their P and G combinations should be carried out in Zimbabwe so that new reassortants that contain both the gene segments of animal and human rotavirus strains can be identified when they arise. Gene sequencing should be performed for all non-typeable rotavirus strains. A multivalent rotavirus strain should be developed so that immunity against the unusual rotavirus genotypes can be induced.

5.2 Limitations

The main limitation encountered when conducting this study was lack of adequate resources. Due to shortage of reagents, the rotavirus positive samples which produced invalid (production of smears) results when genotyping PCR was done were supposed to be repeated using less of the first PCR product (based on this scale: for high concentration of the product 4+ use 0.5 µl, 3+ use 0.8µl, 2+ use 1µl, 1+ use 2µl). A large number of samples were non-typeable (NT), 45.2 % which could have affected the prevalence rate of the overall rotavirus genotypes, these NT were supposed to be sequenced. Sequencing was not done because of shortage of adequate funds. In order to increase the power of this study more rotavirus isolates were supposed to be characterised but due to shortage of funds the number of isolates was limited to 50 only. Furthermore, the rotavirus genotypes circulating in non diarrhoeal controls were supposed to be determined in order to evaluate their association with asymptomatic rotavirus infection but lack of resources did not allow this. The study period was less than 12 months so a comprehensive seasonality pattern of rotavirus infection was not observed.

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APPENDIX 1

ROTAVIRUS DIARRHOEA CASE INVESTIGATION FORM (CIF)

Patient Name: _____ Admission Date ____ /____ /_____ PBM ID_____

Medical Record # (or folder #) if available _____ Hospital Name: _____

PART 1: CASE INFORMATION

Sex: Male ☐ **Female** ☐ **Age** M D **Date of Birth** Y M D

Father's/Mother's Name: _____

District of Residence: _____

Province/Region:_____ City/Town: _____ Neighborhood: _____

Physical location: _____ Mobile phone:_____

PART 2: CLINICAL INFORMATION

Treated at: Emergency Room ☐ **Duration in hours: _____**

Admitted to ward ☐ **Duration in days: _____**

Date of onset of illness: _____/_____/_____ Duration of gastroenteritis symptoms: ____ (in days)

Temperature at admission:_____ ^{o}C (axillary) **History of Fever: _____** (y/n/unknown)

Vomiting: _____ (y/n/unknown) **Diarrhoea: _____** (y/n/unknown) **Treatment:** (check one)

No. of episodes/24 hr: _____ **No. of episodes/24 hr: _____** ORT ☐

Duration: ____ (in days) **Duration: ____** (in days) IVF ☐

Clinical status: (check all that apply)

Lethargy/unconscious ☐ Sunken eyes ☐

Skin tugor impaired ☐ Temperature ☐

Drinking poorly ☐

Dehydration status: (check one)

Severe ☐ Shock ☐

Some ☐ None ☐

Stool specimen collected? ___ (y/n/unknown) **If yes, date collected: _____/_____/_____**

Date sent to laboratory: _____/_____/_____

PART 3: VACCINATION HISTORY

Has patient received Rotavirus vaccine? _____ (y/n/unknown) **If yes, # of doses: _____**

(unknown = 9)

Type of vaccine:

Rotarix ☐ Rotateq ☐ Other ☐ **If other type, specify:**

_____

Date of Rotavirus vaccination: dose #1___/____/___ dose #2 ____/___/____ dose #3 ____/____/____

Source of information? Vaccination card ☐ Caregiver recall ☐ Unknown ☐

Patient Name: _____ Admission Date ____ /____ /_____ PBM ID_____

Medical Record # (or folder #) if available _____ Hospital Name: _____

PART 4: PATIENT OUTCOME

Outcome at discharge: Recovered ☐ **Date of discharge** (or death or transfer): _____/_____/_____

(please tick (√) Died ☐ Discharged against medical advice ☐ Transferred ☐

Final diagnosis at discharge?

Rotavirus ☐ Non-specified infection ☐ Other infection: _____

PART 5: LABORATORY RESULTS

Date stool specimen received in laboratory: ____/____/____

Stool adequacy: (check one)

Good adequacy ☐

Inadequate ☐

Unknown ☐

Kit type: (check one)

Dako ☐

Rotackne ☐

Other _____

Rotavirus ELISA test Result:

Rotavirus + ☐

Indeterminate ☐

Negative ☐

Other test results? _____

Date results sent to ward: ____/____/_____

Was specimen sent for genotyping? _____ (y/n/unknown)

PART 6: REGIONAL REFERENCE LABORATORY

Was stool specimen sent to RRL? ____(y/n) **If yes, date sent: _____/_____/_____**

CASE NOTES:

_____

_____

_____

_____

APPENDIX 2

THE LABORATORY SAFETY RULES

(FROM MRC DPRU MANUAL, 2010)

Personal Safety

- No eating, drinking or smoking allowed in the laboratory.
- Don't touch eyes, nose, mouth, face or hair while working in the laboratory.
- Don't put pens, pipettes or other objects into your mouth.

  No mouth pipetting allowed. Use pipette-aids or teats.
- Laboratory coats or gowns must be worn over street clothes when in the laboratory and white coats must be buttoned. These laboratory coats are **not** to be worn outside the laboratory.
- Gloves must be worn when working with specimens and reagents.
- Gloves should be used for a specific task only and removed upon completion. Don't walk around touching doorknobs, telephones, computer sets and surfaces with contaminated gloves. Discard contaminated gloves in an autoclavable bag.
- All accidents must be reported immediately to a senior staff member and the incident recorded in the accident book.
- New staff members must be tested for immunity to relevant viruses.
- ***All cell phones must be switched off at all times in the laboratory.***

Personal Hygiene

- Wash hands before and after each procedure, and before leaving the laboratory.

- Tie long hair back and away from your face.
- Remove all dangling jewellery and rings.

Laboratory Hygiene and Access to Laboratories

- Keep laboratory clean and tidy.
- Turn off all unnecessary equipment upon leaving the laboratory.
- No unauthorised persons are allowed in the laboratory.
- Keep the laboratory doors closed.

Laboratory Ethics

- Results must be given to authorised persons only. Rather refer the doctor to an experienced person or pathologist.
- Results are never given to a patient, even if the "patient" is a doctor.
- Results of a particular patient are never discussed outside the laboratory.
- Do not try to interpret the results for clinicians.

Laboratory Procedures

- Specimens should be received in plastic specimen holders and kept in a compartment separate from the request form.
- If the request form is contaminated with specimen material, it should be placed inside a plastic bag and photocopied. The contaminated original is then discarded.
- Avoid aerosols by:

i. Allowing pipetted liquid to slide down the side of the vessel and do not dispense in a drop wise fashion

ii. Properly capping any material to be shaken or centrifuged;

iii. Using a biohazard hood when performing procedures that create aerosols, such as grinding or homogenising tissues.

- Decontaminate working surfaces after use.
- Wipe up all spillage immediately using the recommended disinfectant.

  NB: If unsure of the danger involved, call a senior immediately.
- Do not dispose any biological material into the drainage system.
- Do not remove any biological material from the laboratory.

Sterility Checking

- Check the date and sterility tape on each reagent or item before using it.
- Sterilise again if the date is more than three months old.

  Resterilise containers holding pipettes, tips etc after use.

Equipment Hazards

- Read the instructions before using any equipment or request assistance.
- During centrifugation ensure:

i. That the rotor is capable of the speed required;

ii. That the centrifuge tubes can withstand the gravitational force and use plastic tubes for high-speed centrifugation;

iii. That the load is balanced;

iv. That the tubes are sealed.

Reagent Hazards

- Lock up alcohols, acids and other dangerous reagents.
- Keep flammable reagents in a fireproof cupboard.
- Store acids and alkalis on low or floor-level shelves.
- Dispose of chemicals into bottles marked: **Waste Chemicals**.

  NB: Do not pour any reagents down the drains.
- Autoclave all used glassware and equipment before disposal or washing.
- Discard plastic items into autoclavable plastic bags or boxes, autoclave and incinerate.
- Loosen the caps of bottles before autoclaving.
- Pull out the plunger of syringes before disposal.
- Discard all needles, scalpel blades, glass Pasteur pipettes and broken glass into the "sharps" containers and incinerate when full.

APPENDIX 3

THE RNA ELECTROPHORESIS

A. EXTRACTION OF RNA FROM STOOL FOR POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) (FROM MRC DPRU MANUAL, 2010)

i) Detailed method

1. Before beginning the extraction remember to place the 1M sodium acetate (NaAc) containing 1% sodium dodecyl sulphate (SDS) pH 5.0 in the 37°C water bath. This dissolves or prevents the SDS from precipitating out.
2. Make a 10% stool suspension by adding 0.1 g of solid stool or 100 µl of liquid stool to 900 µl distilled water in a screw cap test tube and mix well.
3. Place 450 µl of the 10% stool suspension in an eppendorf tube. Add 50 µl of the pre-warmed 1M NaAc containing 1% SDS, pH 5.0 to the suspension and vortex 10 seconds. Incubate in a 37°C water bath for 15 minutes.
4. Add 500 µl phenol/chloroform (1:1) to the eppendorf and **NB!! Vortex for 1 minute**. Incubate for a further 15 minutes in a 56°C water bath.
5. Open and immediately reseal tubes prior to vortexing. This reduces the air pressure within the tube and prevents the tubes popping open during mixing.
6. Vortex for 1 minute. Centrifuge for 3 minutes at 12 000 rpm.
7. Carefully remove the upper aqueous phase, containing the dsRNA, and pipette in a new clean eppendorf tube.

NB: Avoid any interface material as this contains protein and DNA that will contaminate your extraction and potentially degrade your RNA.

Repeat steps 5-7 (phenol extraction) if the interface shows a lot of protein/fatty material by using $^{1}/_{2}$ volume (250 µl) of phenol/chloroform mix.

8. Add 1/10 volume (approximately 40 µl) 3M NaAc and fill the tube (1-1.5 ml) with ice-cold (-20°C) absolute ethanol.

   Mix gently by turning the tube over and over for 4-6 times.

9. Incubate tubes at -20°C for at least 2 hours/overnight or at -70°C for 30 minutes (if you are in a hurry) to allow the dsRNA to precipitate.

10. Centrifuge tubes at 4°C for 15-20 minutes at 12 000 rpm to pellet the dsRNA. Take tubes carefully out and pour off the supernatant immediately, allow the tube containing the RNA to air-dry upside down on a piece of paper towel.

10. Resuspend the pellet in 30 µl PAGE sample dye before loading on a PAGE gel. DO NOT STORE the pellet in running dye, use 10 µl dH_20 / 1xTE buffer.

ii) Preparation of Reagents for Extraction of RNA from Stool

a. 10% Sodium dodecyl sulphate (SDS) stock

Add 1 g SDS in

10 ml distilled water.

Dissolve in a 65°C water bath.

b. 1M Sodium acetate (NaAc) containing 1% SDS

Dissolve 8.2 g sodium acetate in

60 ml distilled water.

Add 10 ml 10% SDS stock and mix.

Adjust pH to 5.0 with glacial acetic acid and make up to 100 ml with sterile distilled water. Heat solution to 42°C if precipitation is noticed.

c. Phenol/chloroform (1:1)

Mix equal volumes of saturated phenol (pH 4.3) and chloroform. Place in a dark or foil-covered bottle, as the solution is light sensitive.

Store at 4°C.

d. 3M Sodium acetate (NaAc) pH 5.0

Dissolve 4.92 g sodium acetate in

10 ml distilled water.

Make up to 20 ml with distilled water.

e. PAGE sample dye

Bromophenol Blue	10 mg (0.01 g)
Spacer gel buffer	5 ml
Glycerol	1 ml

Dissolve and make up to 10 ml with distilled water.

f. Phenol/Chloroform (using Phenol crystals)

Dissolve 14.74 g phenol in

10 ml chloroform.

C. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

i) Pouring of gels- Take note of the following:

- Carefully clean glass plates with 96% ethanol. Assemble equipment for gel casting and mark level of resolving gel.
- The recipe for 1.5 mm gels requires the thick spacers
- Gels must be allowed to polymerise for **at least two hours** prior to loading of samples.

Gels are poured according to the recipes below.

1. 10% Resolving Gel

Reagents	**1.5mm Gel**	
	1 X	**2 X**
dH_2O	15.8 ml	31.6 ml
30% Acrylamide Stock	10.0 ml	20.0 ml
Resolving Buffer (pH 8.9)	3.75 ml	7.5 ml
TEMED	15 µl	30 µl
10% Ammonium persulphate	450 µl	900 µl

2. 3% Spacer Gel

	1.5mm Gel	
	1 X	**2 X**
dH_2O	6.8 ml	13.6 ml
30% Acrylamide Stock	1.6 ml	3.2 ml
Spacer Buffer (pH 6.7)	1.25 ml	2.5 ml
TEMED	5 µl	10 µl
10% Ammonium persulphate	150 µl	300 µl

1) Prepare the resolving gel according to the recipe above.

Pipette the acrylamide solution immediately between the glass plates up to the spacer gel mark and overlay the gel before polymerization with 1 ml layer of water to ensure the formation of an even interface and exclusion of oxygen.

2) Depending on the size and the temperature, allow the gel to polymerize for at least 60 min - 2 h, or until the interface between the gel and the overlay is visible.

3) Pour the water from the top of the resolving gel, and allowing the excess liquid to absorb into tissue paper

4) Place the gel apparatus upright, prepare the spacer gel, and load it on top of the resolving gel, immediately positioning the comb without any air bubbles.

5) Allow the gel to polymerize for at least 45 min-1 h before loading the samples.

6) Remove the comb, and assemble the glass plates onto the electrophoresis apparatus.

7) Add 1x Glycine running buffer to the bottom reservoir, and insert the glass plates into the electrophoresis tank remove air from under the gel bottom. Fill the upper tank back and front separately checking for leakage of buffer before filling these upper tanks to the top.

Rinse the wells with the electrophoresis buffer.

8) Load each dsRNA sample diluted in PAGE buffer into the designated gel wells according to labelling on worksheet.

Electrophoresis of samples at 100V or 20 mA for 16-20 h.

9) Stop electrophoresis and continue with Silver nitrate staining process.

i) Reagents for PAGE

a. 30% Acrylamide stock

Acrylamide 30 g

N, N' methylene bis-acrylamide 0.8 g

Dissolve in 100 ml distilled water.

Filter before use. (Optional)

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

Caution!!!!! Acrylamide is a potent neurotoxin and is absorbed through the skin.

Always wear gloves when working with acrylamide and bis-acrylamide.

1N Hydrochloric acid (HCl)

Add 8,6 ml concentrated HCl to

91,4 ml sterile distilled water.

b. Resolving gel buffer - pH 8.9

Tris Base 36.3 g

1N HCl 48 ml

Dissolve and adjust the pH to 8.9 with HCl or 3M sodium hydroxide (NaOH). Make up to 100 ml with distilled water.

c. Spacer gel buffer - pH 6.7

Tris Base 5.98g

Distilled water 50 ml

Adjust the pH to 6.7 with 1N HCl.

Make up to 100 ml with sterile distilled water.

d. 10% (w/v) Ammonium persulphate (APS)

Dissolve 0.1 g ammonium persulphate in 1 ml distilled water just prior to use.

f. 5 x Tris-glycine Running Buffer

25 mM Tris Base 15.1 g

250 mM Glycine 94 g

Dissolve and make up to 1000 ml with distilled water.

g. 1 x Tris-glycine running buffer

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

Use to run PAGE gels.

D. SILVER NITRATE ($AgNO_3$) STAINING

(FROM MRC DPRU MANUAL, 2010)

i) Detailed method:

1. Remove the gel from the glass plates and cut the bottom right hand corner for gel orientation and discard the spacing gel.
2. Add 200 ml fixing solution 1 to each gel and incubate for 30 minutes on an orbital shaker.
3. Drain off fixing solution 1 and replace with 200 ml fixing solution 2. Incubate for 30 minutes on the orbital shaker.

   NB! Prepare silver nitrate ($AGNO_3$) solution just before use. **Please work carefully as $AgNO_3$ stains hands, bench tops etc. black.**
4. Drain off fixing solution 2 and add silver nitrate staining solution. Incubate for 30 minutes on the orbital shaker.

5. Prepare developing solution by adding the NaOH to previously prepared formaldehyde and water solution.
6. Drain the silver nitrate staining solution and wash the gel twice (2x) with distilled water for 2 minutes each time.

   NB: Rinsing time is very important, too long washing- zero results.
7. Add approximately 50 ml of developing solution to the gel and agitate by hand for 30 seconds. This removes any black precipitate.
9. Drain off and add remaining developing solution (approximately 200 ml). Incubate for approximately 5 minutes or until 11 rotavirus dsRNA segments can be seen.
10. Drain off the developing solution and add the stopping solution to prevent further colour development.

    Incubate for 10 minutes before rinsing gel in distilled water.
11. Label and Cover gel with cellophane sheets and dry on the Easy Breeze Gel Dryer overnight at room temperature.

    ii) Preparation of Reagents for Silver Nitrate Staining

1 Gel	**2 Gels**
Fixing Solution 1	**Fixing Solution 1**
80 ml Ethanol	160 ml Ethanol
110 ml dH_20	220 ml dH20
10 ml Acetic Acid	20 ml Acetic Acid
Fixing Solution 2	**Fixing Solution 2**
20 ml Ethanol	40 ml Ethanol

180 ml dH_20	360 ml dH_20
1 ml Acetic Acid	2 ml Acetic Acid
Silver Nitrate Staining Sol.	**Silver Nitrate Staining Sol.**
0.37 g AgN03	0.74 g AgN03
200 ml dH_20	400 ml dH_20
NB!!! Rinsing	**NB!!!! Rinsing**
2 x 2 minutes in	2 x 2 minutes in
200 ml dH_20	400 ml dH_20
Developing Solution	**Developing Solution**
7.5 g NaOH	15 g NaOH
250 ml dH_20	500 ml dH_20
2 ml 36% Formaldehyde	4 ml 36% Formaldehyde
Stopping Solution	**Stopping Solution**
10 ml Acetic Acid	20 ml Acetic Acid
200 ml dH_20	400 ml dH_20

Add dH_2O to gels and leave until ready to dry

APPENDIX 4

THE PRIMER LIST AND SEQUENCES

Rotavirus VP7 Primer list (FROM MRC DPRU MANUAL, 2010)

Table 1. Gouvea / Miren Iturriza-Gómara and Das / Cunliffe

Oligonucleotide primers as used for VP7 [G]-genotyping.

Primer	Sequence (5'-3')	Position (nt)	Strain (genotype)
	Gouvea / Miren Primers		
sBeg9	GGCTTTAAAAGAGAGAATTTC	1-21	5'
Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	5'
End9	GGTCACATCATACAATTCTAATCTAAG	1062-1036	3'
dAnEnd9 *animal*	GGTCACATCAAACAATTCTA TT GC	1062-1045	3'
RVG9	GGTCACATCATACAATTCT	1062-1044	3'
EndA	ATAGTATAAAATACTTGCCACCA	922-944	3'
aAT8 **aAT8v**	GTCACACCATTTGTAAATTCG GTCACACCATTTGTAAAYTCAC	178-198	69M (G8) 885bp
aBT1	CAAGTACTCAAATCAATGATGG	314-335	Wa (G1) 749bp
aCT2	CAATGATATTAACACATTTTCTGTG	411-435	DS-1 (G2) 652bp
aDT4	CGTTTCTGGTGAGGAGTTG	480-498	ST-3 (G4) 583bp
aET3 **mG3**	CGTTTGAAGAAGTTGCAACAG ACGAACTCAACACGAGAGG	689-709 250-269	P 374bp(G3) 812 bp
aFT9	CTAGATGTAACTACAACTAC	757-776	306bp(G9)W161

mG9	CTTGATGTGACTAYAAATAC	757-776	305 bp
mG10	ATGTCAGACTACARATACTGG	666-687	396bp (G10)
mG12 **G12b**	CCG ATG GAC GTA ACG TTG TA GGT TAT GTA ATC CGA TGG ACG	548-567 504-524	514 bp (G12) 558 bp (G12)
	DAS / Cunliffe Primers **5'.....3'**		
9Con1	TAGCTCCTTTTAATGTATGG	37 - 56	5' + sense
9T1-1	TCTTGTCAAAGCAAATAATG	176 - 195	Wa (G1)
9T1-2	GTTAGAAATGATTCTCCACT	262 - 281	S2 (G2)
9T-3P	GTCCAGTTGCAGTGTTAGC	484 - 503	107E1B (G3)
9T-4	GGGTCGATGGAAAATTCT	423 - 440	ST3 (G4)
9T-9B	TATAAAGTCCATTGCAC	131 - 147	116E (G9)
MW-8	TCT TCA AAA GTC GTA GTG	670 - 688	MW8 (G8)

Y=C/T R=A/G N=A/G/C/T

Rotavirus VP7 Animal primer set

Primer	**Animal-Sequence (5'-3')**	**Position nt**	**Strain/ Genotype**
aFT5	GACGTAACAACGAGTACATG	779 - 760	OSU G5
aDT6	GATTCTACACAGGAACTAG	499 - 481	UK G6
aHT8	GTGTCTAATCCGGAACCG	273 - 256	B37 G8

aET10	GAAGTCGCAACGGCTGAA	714 - 697	B223 G10
aBT11	GCAACTCAGATTGCTGATGAC	336 - 316	YM G11
sBEG	GGCTTTAAAAGAGAGAATTTC	1-21	5’

Rotavirus VP4 Human and Animal Primer lists

Table 2. Gentsch / Miren Iturriza-Gómara Primers used for VP8* gene amplification and human and animal [P]-genotyping

Primer	**Human -Sequence (5’-3’)**	**Position nt**	**Strain Genotype**
Con2	ATTTCGGACCATTTATAACC	868-887	3’
Con3	TGGCTTCGCTCATTTATAGACA	11-32	5’
1T-1	TCT ACT TGG ATA ACG TGC	339-356	KU **P[8]**
1T-1D	TCT ACT GGR TTR ACN TGC	339-356	**dP[8]**
2T-1	CTATTGTTAGAGGTTAGAGTC	474-494	RV5 **P[4]**
3T-1	TGTTGATTAGTTGGATTCAA	259-278	1076 **P[6]**
4T-1	TGAGACATGCAATTGGAC	385-402	K8 **P[9]**
5T-1	ATCATAGTTAGTAGTCGG	575-594	69M **P[10]**
mP11	GTAAACATCCAGAATGTG	305-323	MC435 **P[11]** 312bp
SE-1	CTCTGCTACTCTACCTATTTG	271-291	280bp **P[14]**
4943	GGTGTAGTTCCTGCGTA	538-544	533bp **P[14]**
	Animal – Sequence (5’-3’)		
pGott	GCTTCAACGTCCTTTAACATCAG	465-487	Gottfried **P[6]** 423bp

pOSU	CTTTATCGGTGGAGAATACGTCAC	389-412	OSU **P[7]** 502bp
pUK	GCCAGGTGTCGCATCAGAG	336-354	UK **P[5]** 555bp
pNCDV	CGAACGCGGGGGTGGTAGTTG	269-289	NCDV **P[1]** 622bp
pB223	GGAACGTATTCTAATCCGGTG	574-594	B223 **P[11]** 314bp

R=A/G N=A/C/G/T

APENDIX 5

THE REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

A) QIAamp Viral RNA Mini Extraction Kit Method

(FROM MRC DPRU MANUAL, 2010)

Handling of QIAamp Mini spin columns – Wear Gloves

Owing to the sensitivity of nucleic acid amplification technologies, the following precautions are necessary when handling QIAamp Mini spin columns to avoid cross-contamination between sample preparations:

- Carefully apply the sample or solution to the QIAamp Mini spin columns. Pipet the sample into the QIAamp Mini spin column without wetting the rim of the column.
- Change pipet tips between all liquid transfer steps. The use of aerosol-barrier tips is recommended.
- Avoid touching the QIAamp membrane with the pipet tip.
- After all pulse-vortexing steps, briefly centrifuge 1.5 ml microcentrifuge tubes to remove drops from the inside of the lid.
- Wear gloves throughout the procedure. In case of contact between gloves and samples, change gloves immediately.

Spin protocol

- Close the QIAamp Mini spin columns before placing in the microcentrifuge. Discard the filtrate and the old collection tube. Please note that the filtrate may contain hazardous waste and should be disposed off properly.
- Open only one QIAamp Mini spin column at a time, and take care to avoid generating aerosols.

- For efficient parallel processing of multiple samples, it is recommended to fill a rack with collection tubes to which the QIAamp Mini spin columns can be transferred after centrifugation.

Protocol: Purification of Viral RNA (Spin Protocol)

This protocol is for purification of viral RNA from 140µl plasma, serum, urine, cell-culture media, or cell-free body fluids using a microcentrifuge. Larger starting volumes, up to 560 µl (in multiples of 140µl), can be processed by increasing the initial volumes proportionally and loading the QIAamp Mini spin columns multiple times, as described below in the protocol.

Important points before starting

- All centrifugation steps are carried out at room temperature (15-25˚C).

Things to do before starting

- Equilibrate samples to room temperature (15-25˚C).
- Equilibrate Buffer AVE to room temperature for elution in step 11.
- Check that Buffer AW1 and Buffer AW2 have been prepared according to the instructions.
- Add carrier RNA reconstituted in Buffer AVE to Buffer AVL according to instructions.

A) Detailed Extraction Procedure of total RNA (from QIAgen Handbook 12/2005)

1. Pipet 560 µl of prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.

   If the sample volume is larger than 140 µl, increase that amount of Buffer AVL-carrier RNA proportionally (e.g., a 280 µl sample will require 1120 µl Buffer AVL-carrier RNA) and use a larger tube.

2. Add 140 µl supernatant to the Buffer AVL-carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.

   To ensure efficiency lysis, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogeneous solution. Frozen samples that have only been thawed once can also be used.

3. Incubate at room temperature (15-25˚C) for 10min.

   Larger incubation times have no effect on the yield or quality of the purified RNA. Potentially infectious agents and RNases are inactivated in Buffer AVL.

4. Briefly centrifuge the tube to remove drops from the inside of the lid.
5. Add 560 µl of ethanol (96-100%) to the sample, and mix by pulse-vortexing for 15 seconds. After mixing, briefly centrifuge the tube to remove drops from inside the lid.

   Only ethanol should be used since other alcohols may result in reduced RNA yield and purify. If the sample volume is greater than 140 µl, increase the amount of ethanol proportionally (e.g., a 280 µl sample will require 1120 µl of ethanol). In order to ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogeneous solution.

6. Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini spin columns (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp spin columns into a clean 2 ml collection tube, and discard the tube containing the filtrate.

   Close each spin column in order to avoid cross-contamination during centrifugation.

   Centrifugation at full speed will not affect the yield or purify of the viral RNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all of the solution has passed through.

6. Carefully open the QIAamp Mini spin columns, and repeat step 6.
   If the sample volume was greater than 140 µl, repeat this step until all of the lysate has been loaded onto the spin column.

7. Carefully open the QIAamp Mini spin columns, and add 500 µl of Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1min. Place the QIAamp Mini spin columns in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
   It is not necessary to increase the volume of Buffer AW1 even if the original sample volume was larger than 140 µl.

8. Carefully open the QIAamp Mini spin columns, and add 500 µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000rpm) for 3 min. Continue directly with step 11, or to eliminate any chance of possible AW2 carryover, perform step 10, and then continue with step 11.

Note: residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in flow-through, containing Buffer AW2, contacting the QIAamp Mini spin column. Removing the QIAamp Mini spin column and collection tube from the rotor may also cause flow-through to come into contact with QIAamp Mini spin column. In these cases, the optional step 10 should be performed.

9. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
10. Place the QIAamp Mini spin column in a sterile clean labelled 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate.
11. Carefully open the QIAamp spin column and add 60 µl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1- 5 min. Centrifuge at 6000 x g (8000 rpm) for 1 min.

    Discard the column and keep the 1.5 ml eppie with eluate containing the viral RNA. Store at -20˚C or -70˚C.

    A single elution with 60 µl of Buffer AVE is sufficient to elute at least 90% of the viral RNA from QIAamp Mini spin column. Performing a double elution using 2 x 40 µl of Buffer AVE will increase yield by up to 10%. Elution with volumes of less than 30 µl will lead to reduced yields and will not increase the final concentration of RNA in the eluate.

RT-PCR (REVERSE-TRANSCRIPTASE – POLYMERASE CHAIN REACTION)

(FROM MRC DPRU MANUAL, 2010)

a. PRINCIPLES OF PCR AMPLIFICATION

RT-PCR *of rotavirus dsRNA involves three steps:*

1. Denaturation of dsRNA
2. Reverse transcription of dsRNA
3. PCR amplification of cDNA

PCR *involves the following steps:*

1. The DNA to be reproduced is heated to separate the two template strands.
2. Two primers, which are complimentary to the region to be amplified, are added. A heat-stable DNA *polymerase* enzyme is also added. The enzyme catalyses the extension of the primers, using the DNA strand as template.
3. The cycle is repeated, with the newly synthesised cDNA being heat- denatured and the enzymes extending the primers attached to the liberated single DNA strands.

Preparation of Master Mix

The Master Mix contains all of the components necessary to make new strands of DNA in the PCR process. The Master Mix reagents include:

Final Conc.	Component	Purpose
	Water	Provides a diluent for reagents
1 X	Buffer	Keeps the master mix at the proper pH So the PCR reaction can take place.

200 uM	Deoxy nucleotides	Provide both the energy and nucleosides for the synthesis of DNA. It is important to add equal amounts of each nucleotide (dATP, dTTP, dCTP, dGTP) to the master mix to prevent mismatches of bases.
0.2-1.0 uM	Primers	Short pieces of DNA (20-30 bases) that bind to the DNA template allowing Taq DNA polymerase enzyme to initiate incorporation of the deoxynucleotides.
2.5U/100 ul	*Taq* polymerase	A heat stable enzyme that adds the deoxy-nucleotides to the DNA template.
0.05-1.0 ug	Template	The cDNA which will be amplified by the PCR DNA reaction

→ **Master Cycler / PCR machine**

94ºC x 1 min

94ºC x 1 min

42ºC x 1 min } **30 cycles** → **Run 1% AGAROSE GEL**

72ºC x 1 min

72ºC x 1 min

4ºC Hold

d.i) Detailed Method for RT-PCR

1. Label a 1.5 ml eppendorf tube for the reverse transcription (RT) master mix and also for the amplification master mix (PCR).

1. Label 0.5 ml or 0.2 ml thin walled tubes for each amplification reaction and include a positive and negative control. Write on top of lid; sample number, primers used and date.
2. Prepare RT-master mix according to worksheet and place on ice. Add AMV enzyme prior to use. Vortex and spin 10 seconds.
3. Add primers and dsRNA to 0.5 ml amplification tubes as labelled and mix by pipetting.
4. Denature for 5 minutes at 94ºC and transfer all the sample tubes immediately to an ice-bath (maximum of 5 minutes).
5. Add 3.2 µl of RT-master mix to each tube and mix well. Spin for 10 seconds and incubate tubes at 42ºC for 30 minutes.

   NB!!!!!! Make sure the RT-mix is added within 5 minutes.
6. Prepare the amplification master mix according to worksheet and place on ice. Add *Taq* Polymerase 5 minutes before use.
7. Centrifuge sample tubes for 10 seconds after incubation at 42ºC and place in sample rack. Add 40 µl of amplification master mix to each tube, mix and KEEP on ICE.
8. Add 2-3 drops of mineral oil to each tube, only when master-cycler is without a heated lid (105ºC).

9. Run PCR fragments and a 100 bp molecular weight marker on a 1% TAE- agarose gel.

d.ii) Detailed Method for Genotype Amplification

1. Prepare a 0.2 or 0.5 ml PCR tube for each reaction and label as previously: Number, primers and date. Add cDNA (0.1 µl to 6 µl depends on concentration of RT-PCR amplicons).
2. Prepare genotype master-mix according to worksheet in a 1.5 ml eppendorf tube.
3. Add master mix to each tube and mix well; add 2-3 drops of mineral oil if needed.
4. Place tubes in master cycler for 30 cycles as previously described.
5. Run PCR fragments on a 2% agarose gel at 80-90 volts with appropriate molecular weight marker.

e) Preparation of reagents for running RT-PCR products

1. 20 x Tris-Acetic acid-EDTA buffer (TAE) pH 7.9

0.4M Tris base	48.44 g
0.05M NaCl	6.81 g
0.01M EDTA	3.72 g

Dissolve in distilled water and adjust pH to 7.9 with glacial acetic acid. Make up to 1000 ml with distilled water.

2. 1 x TAE (pH 7.9)

To make 1000 ml. Add 50 ml of 20 X TAE to 950 ml of distilled water.

3. Ethidium bromide stock (10 mg/ml)

Dissolve one 100 mg ethidium bromide (EtBr) tablet in 10 ml distilled water.

Store in a foil-covered bottle as EtBr is light sensitive.

Caution: EtBr is a powerful mutagen and is moderately toxic. Always wear gloves when working with solutions or gels containing this dye.

4. 1% Agarose gel

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

Heat in the microwave until agarose is completely dissolved.

Make solution up to 30 ml with distilled water.

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

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

NB: 1-2% Agarose gels are electrophoresed at 90V in 1 X TAE.

Remember to include an appropriate molecular weight marker.

5. Bromophenol blue tracking dye

Dissolve 0.001 g bromophenol blue in 1 ml distilled water.

Add 9 ml 40% sucrose solution made in 1 x TAE.

The final solution is filtered prior to use.

Store in a foil-covered bottle as dye is light sensitive.

6. 5 x TBE Buffer (Tris-borate EDTA Buffer) pH 7.5-7.8

54 g Tris Base

27.5 g Boric Acid

Dissolve in 800 ml distilled water and add 20 ml 0.5M EDTA (pH 8.0)

7. 0.5M EDTA (pH 8.0)

Dissolve 18.61 g EDTA.$2H_20$ (mw 372.24) in 70 ml dH_20, adjust pH and make final volume to 100ml with dH_20.

8. 0.5 x TBE working strength for agarose gel electrophoresis:

Add 100 ml 5 x TBE to 900 ml sterile distilled water and mix.

f. Detailed Method for VP7 and VP4 amplification

f.i) RT-PCR and Genotyping Protocol of the VP7 gene using Vera Gouvea and Miren Iturriza-Gómara primers

(ref: Gouvea *et al*., 1990; Miren Iturriza-Gómara *et al*., 2004)

Step 1: **RT-PCR** of the VP7 gene using outer primers, sBeg9 + End9/ (Fig 1).

Step 2: **Genotyping** of human rotaviruses using a cocktail of *Gouvea/Iturriza* primers (Table 1 and Fig 2).

RVG9 or EndA + aAT8, aBT1, aCT2, aDT4, mG3, mG9, mG10 and G12 to determine G1 to G4 and G8, G9 and G10 genotypes.

(NOTE:Amplicon sizes are smaller when making use of primer EndA step

f.ii)RT-PCR and Genotyping of the VP7 gene using Das and Cunliffe primers

(ref: Das *et al*., 1994; Cunliffe et al., 1999)

Step 1: RT-PCR of the VP7 gene using primers

9Con1 + 9Con2 = 903bp

9Con1 + End9 = 1025 bp (Fig 3)

Step 2: Genotyping of the VP7 gene using the following cocktail of primers:

9Con1 + 9T1-1, 2, 3, 4, MW8 and 9B for genotypes G1-G4, and

G8 and G9 (Fig 3 and 4).

Step 3: Amplification for both sets of primers is done by:

Denaturing 94°C x 2 min

30 cycles of 94°C x 1 min., 42°C x 2 min., 72°C x 3 min.

or

Denaturing	94°C x 2 min
30 cycles of	94°C x 1 min., 42°C x 1 min., 72°C x 1min.
extension	72°C x 7 min.
HOLD	4°C

(NOTE: When RT-PCR negative samples are genotyped the amplification cycles can be increased to 40 cycles to obtain better results from very low positive results)

USE MINERAL OIL ON MASTER CYCLERS WITHOUT A HEATED LID

WORKSHEETS

PAGE Worksheet (Polyacrylamide Gel Electrophoresis)

Date:……

Name:……

Country/Hosp:……

Run started at: ……

Volts:…… Lab.Signature:…….

Gel …….	**LAB No**	**Electropherotype identification**
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		

RT-PCR amplification of Rotavirus (dsRNA)

dsRNA: _____ Country _____

_____

Method of Extraction: _____ Date: _____

A: DENATURE (dsRNA)

_____µl dsRNA

_____µl primer 1 VP7____ VP4 _____ Other_____

_____µl primer 2 VP7____ VP4 _____ Other_____

6-10 µl Total

Denature for 5 minutes in boiling water bath.

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

B: REVERSE TRANSCRIPTION (dsRNA)

1x reaction		**_____ x reactions**
o.25µl	10mM dATP	_____µl
o.25µl	10mM dCTP	_____µl
o.25µl	10mM dGTP	_____µl
o.25µl	10mM dTTP	_____µl
0.2 µl	(AMV) RTase	_____µl Roche Boehr.(23U/µl)
2.0 µl	AMV Buffer	_____µl

Work on ice. Add 3.2 µl of RT- master mix to each tube and mix with pipette. Quick spin. Incubate 25-30 minutes at 42°C.

(NOTE: Check manufacturers' concentration of AMV. Use 3-5U of RTase per reaction.)

C: PCR AMPLIFICATION (cDNA)

1 X reaction		**_____x reactions**
1 µl	dATP	_____µl
1 µl	dCTP	_____µl
1 µl	dGTP	_____µl
1 µl	dTTP	_____µl
5 µl	10 x *Taq* Buffer	_____µl (BIOLINE-Celtic)
1.2µl	50 mM $MgCl_2$	_____µl
29.5µl	ddH_2O	_____µl
0.3µl	*Taq* Polymerase	_____µl(5U/µl)

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

Keep on ice!

D. AMPLIFICATION CYCLES used:

……..°C ……min

……°C……min ……°C ……min ……°C……min **x 30 cycles**

……..°C…….min

Genotyping or Re-Amplification of cDNA

Samples:_____ Country:_____

_____

Primers:_____ Date:_____

1x Reaction		**_____xReactions _____**	**x Reactions**
0.1-6 µl	cDNA	_____µl	_____µl
1 µl	10mM dATP	_____µl	_____µl
1 µl	10mM dCTP	_____µl	_____µl
1 µl	10mM dGTP	_____µl	_____µl
1 µl	10mM dTTP	_____µl	_____µl
1 µl	10pmol Primer 1	_____µl…….	_____µl.....
1 µl	10pmol Primer 2	_____µl.....	_____µl.....
5 µl	10 x *Taq* Buffer	_____µl	_____µl (BIOLINE-Celtic)
1.2 µl	50 $MgCl_2$	_____µl	_____µl
25 µl	sterile dH_2O	_____µl	_____µl (depends on amount of primers used)
0.3 µl	*Taq* Pol. (5U/µl)	_____µl	_____µl
	TOTAL	_____µl	_____µl

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

Keep on ice!

Genotyping or re-amplification of cDNA

1 x reaction		**____ x reactions**
0.1-6 µl	cDNA	_____µl
1 µl	10mM dATP	_____µl
1 µl	10mM dCTP	_____µl
1 µl	10mM dGTP	_____µl
1 µl	10mM dTTP	_____µl
1 µl	10pmol Primer 1	_____µl……
1 µl	10pmol Primer 2	_____µl.....
5 µl	10 x Buffer	_____µl
1.2 µl	50mM $MgCl_2$	_____µl
26 µl	ddH_2O	_____µl
0.3µl	*Taq* Polymerase (5U/µl)	_____µl

AMPLIFICATION CYCLES used:

……°C……min ……°C……min ☐

……°C……..min ……..°C……..min x30

☐

x40…….°C……..min

RT-PCR and GENOTYPING Results (FROM MRC DPRU MANUAL, 2010)

VP7 RT-PCR FULL GENE RESULTS:

Beg9/sBeg9 + End9/RVG = 1062 bp (Gouvea)
Beg9/sBeg9 + EndA = 944 bp (Gouvea)
9Con1 + EndA = 904 bp (Das & Gouvea)

Human VP7 genotypes:

	VG/Miren + RVG	VG/M + End A	DAS/Cunliffe + 9Con1
G1	**749 bp**	**631 bp**	**158 bp**
G2	**652 bp**	**534 bp**	**244 bp**
*G3	374 bp	256 bp	**466 bp**
G4	**583 bp**	**465 bp**	**403 bp**
G8	**885 bp**	**767 bp**	**651 bp**
*G9	306 bp	187 bp	**110 bp**
mG9	**305 bp**	**192 bp**	
mG3	**812 bp**	**694 bp**	
mG10	**396 bp**	**278 bp**	
mG12	**514 bp**	**396 bp**	
G12new	**558 bp**	**440 bp**	

*primers not included in master-mix for genotyping

VP4 (VP8*) RT-PCR RESULTS:

Con2 + Con3 = 876 bp **VP4F + VP4R = 663bp**

Human VP4 genotypes

Con3 + Gentsch/Miren		**VP4F + Gentsch/Miren**
dP[8] = 345 bp		**dP[8] = 224 bp**
P[4] = 483 bp		**P[4] = 362 bp**
P[6] = 267 bp		**P[6] = 146 bp**
P[9] = 391 bp		**P[9] = 270 bp**
P[10] = 583 bp		**P[10] = 462 bp**
P[14] = 533 bp	4943/PA169	**P[14] = 412 bp**
P[14] = 280 bp	SE-1	**P[14] = 159 bp**
P[11] = 312 bp		**P[11] = 191 bp**

Animal VP4 genotypes:

Con2 +Gouvea

P[1]	**622 bp**	NCDV
P[5]	**555 bp**	UK
P[6]	**423 bp**	Gott
P[7]	**502 bp**	OSU
P[11]	**314 bp**	B223

Animal VP7 genotypes:

sBeg + Vera Gouvea

G5	**780 bp**	Sb-2 pig
G6	**500 bp**	NCDV/UK
G8	**274 bp**	A5 cow
G10	**715 bp**	B223 cow
G11	**337 bp**	YM pig

APPENDIX 6

SUMMARY OF RAW DATA

THE ZIMBABWE ROTAVIRUS EIA, PAGE AND GENOTYPING RESULTS; AUGUST 2010

	MRC Lab N0	Zimbabwe Reference N0	Date	Sex	Age (month)	EIA	PAGE	PCR RT	PCR G	PCR RT	PCR P
1	3340	R1629/10	26/06/10	F	16	POS	L1	3+	repeat	3+	repeat
2	3341	R1634/10	24/06/10	M	1	POS	L1	2+	repeat	3+	repeat
3	3342	R1336/10	6/6/2010	F	11	POS	L1/S	3+	G1/G10	1+	P2/P14
4	3343	R1636/10	24/06/10	M	15	POS	L1	1+	repeat	1+	NEG
5	3344	R1654/10	26/06/10	M	12	POS	NEG	NEG	G3	NEG	repeat
6	3345	R1651/10	26/06/10	M	20	POS	NEG	1+	G1/G10	NEG	NEG
7	3346	R1650/10	26/06/10	F	7	POS	L1	1+	repeat	1+	NEG
8	3347	R1314/10	1/6/2010	M	9	POS	S1	1+	G3/G10	1+	NEG
9	3348	R1611/10	24/06/10	M	9	POS	L2	NEG	repeat	1+	repeat
10	3349	R1610/10	24/06/10	F	25	POS	S1	2+	G1/G10	1+	P4
11	3350	R1609/10	23/06/10	F	15	POS	L1	1+	repeat	3+	NEG
12	3351	R1394/10	22/06/10	M	12	POS	L1	4+	NEG	1+	NEG
13	3352	R1608/10	22/06/10	M	3	POS	L1/S	4+	G2	4+	P6
14	3353	R1649/10	23/06/10	F	3	POS	L3	4+	G10/G12	4+	repeat
15	3354	R1635/10	26/06/10	F	15	POS	S2	4+	G2	4+	P4
16	3355	R1652/10	27/06/10	M	22	POS	L1	4+	repeat	4+	NEG
17	3356	R1637/10	23/06/10	F	1	POS	S3	4+	G2	4+	P6
18	3357	R1638/10	4/2/2010	M	11	POS	NEG	NEG	NEG	3+	P6
19	3358	R1335/10	4/2/2010	M	11	POS	S2	3+	G2	3+	P6
20	3359	R1332/10	5/6/2010	M	9	POS	L1	1+	NEG	NEG	P6
21	3360	R1395/10	10/6/2010	F	11	POS	L1	1+	NEG	2+	P6
22	3361	R1475/10	16/06/10	F	7	POS	L4	2+	NEG	4+	P6
23	3362	R1399/10	11/6/2010	M	9	POS	L1	4+	G9	3+	P6

24	3363	R1473/10	15/06/10	M	6	POS	L1	4+	repeat	3+	P14
25	3364	R1478/10	16/06/10	M	5	POS	L1	4+	repeat	3+	P14
26	3365	R1474/10	10/6/2010	F	7	POS	L3	4+	G3/G10	3+	NEG
27	3366	R1337/10	2/6/2010	F	21	POS	L1	4+	repeat	4+	NEG
28	3367	R1475/10	15/06/10	F	7	POS	L1	4+	G1	3+	P4
29	3368	R1397/10	11/6/2010	M	13	POS	S1	4+	NEG	3+	P6/P4
30	3369	R1457/10	16/06/10	M	17	POS	L1	4+	NEG	3+	P6
31	3370	R1432/10	15/06/10	M	3	POS	Not done	4+	G1	2+	P6
32	3371	R1479/10	16/06/10	F	9	POS	Not done	NEG	NEG	2+	repeat
33	3372	R1398/10	11/6/2010	M	12	POS	Not done	NEG	NEG	NEG	repeat
34	3373	R1439/10	13/06/10	M	22	POS	Not done	3+	G9	3+	NEG
35	3374	R1383/10	7/6/2010	F	11	POS	Not done	NEG	NEG	NEG	P6
36	3375	R1459/10	16/06/10	M	9	POS	Not done	2+	G9	2+	P6
37	3376	R1460/10	16/06/10	F	17	POS	Not done	2+	G9	2+	P6
38	3377	R1462/10	16/06/10	M	11	POS	Not done	2+	G8/G12	2+	P4
39	3378	R1467/10	15/06/10	M	7	POS	Not done	2+	G8/G12	3+	P4
40	3379	R1509/10	18/06/10	F	2	POS	Not done	2+	G9	4+	NEG
41	3380	R1526/10	17/06/10	M	11	POS	Not done	4+	G9	4+	NEG
42	3381	R1416/10	21/06/10	F	13	POS	Not done	NEG	G9	2+	NEG

43	3382	R1461/10	15/06/10	F	12	POS	Not done	4+	NEG	NEG	NEG
44	3383	R1505/10	18/06/10	F	10	POS	Not done	2+	repeat	2+	NEG
45	3384	R1508/10	18/06/10	M	1	POS	Not done	2+	G12	1+	P4/P6
46	3385	R1589/10	21/06/10	M	13	POS	Not done	NEG	G9	4+	NEG
47	3386	R1527/10	7/6/2010	M	13	POS	Not done	1+	NEG	1+	NEG
48	3387	R1594/10	23/06/10	F	8	POS	Not done	3+	G9	3+	NEG
49	3388	R1593/10	22/06/10	M	12	POS	Not done	3+	repeat	1+	NEG
50	3389	R1592/10	23/06/10	M	6	POS	Not done	2+	G8/G12	1+	P4

ELISA ROTAVIRUS ANTIGEN RESULTS FOR 50 CONTROL STOOLS (2010 afp CASES)

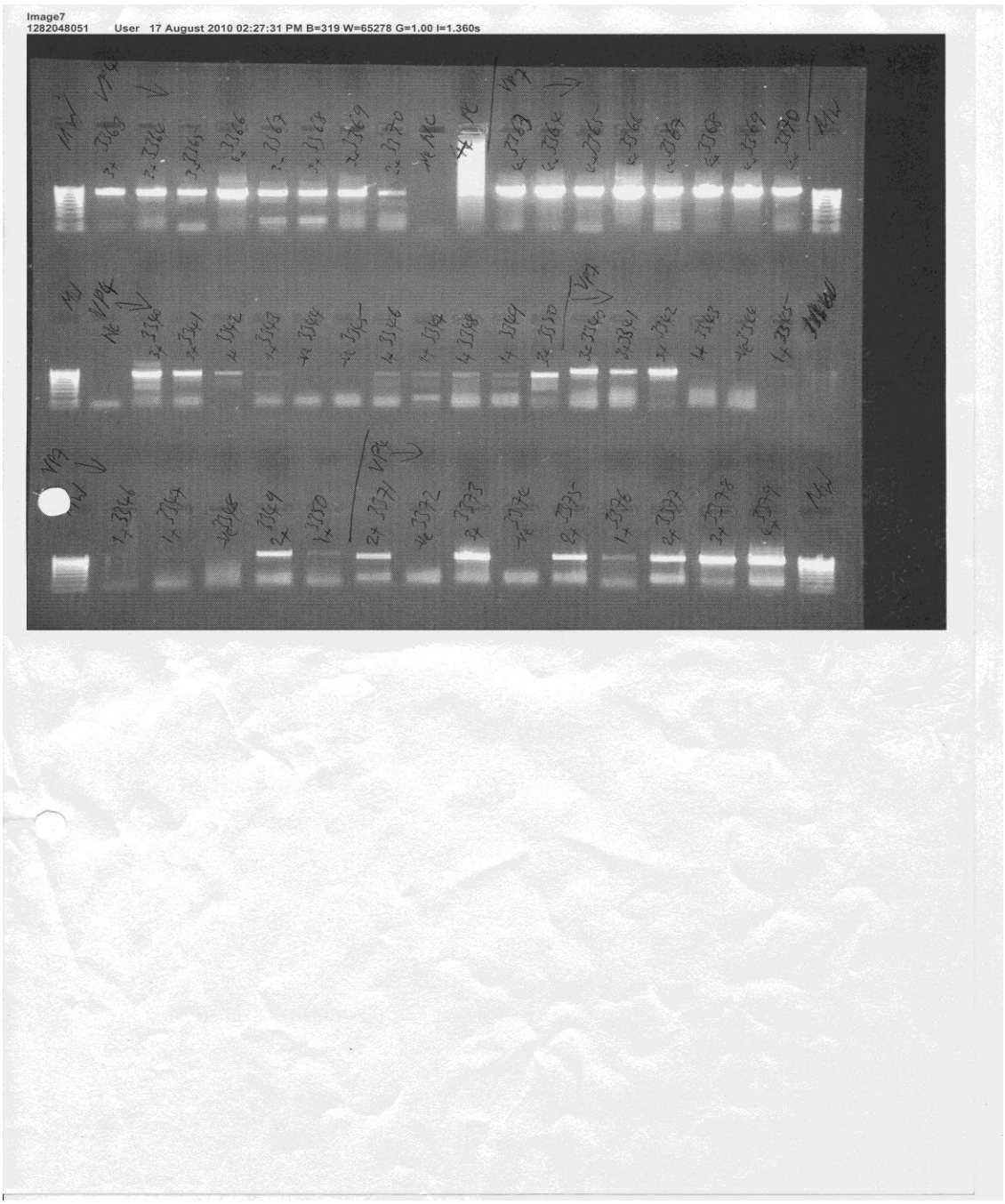
Lab Number	**Age in Months**	**Sex**	**Date Onset**	**Date Stool Collected**	**Cell Culture Result**	**Rotavirus Ag ELISA**	ORIGIN
2571/10	58	F	5/1/2010	5/4/2010	No virus	NEG	Chivi
2629/10	16	M	4/15/2010	4/28/2010	No virus	NEG	Hwange
2630/10	17	M	4/20/2010	4/28/2010	No virus	POS	Hwange
2647/10	44	M	5/6/2010	5/7/2010	No virus	NEG	Harare
2840/10	55	F	5/13/2010	5/15/2010	No virus	NEG	Rushinga
2855/10	20	M	5/1/2010	5/17/2010	No virus	NEG	Mt Darwin
2953/10	35	M	5/16/2010	5/17/2010	NPEV	NEG	UMP
3030/10	27	M	5/18/2010	5/27/2010	No virus	NEG	Harare
3106/10	27	M	6/3/2010	6/8/2010	No virus	POS	Harare

3114/10	23	M	5/6/2010	5/19/2010	No virus	NEG	Chipinge
3133/10	14	M	5/24/2010	6/10/2010	No virus	NEG	Mudzi
3144/10	58	M	6/5/2010	6/7/2010	No virus	NEG	Hwange
3171/10	59	M	6/6/2010	6/14/2010	No virus	NEG	Plumtree
3173/10	26	M	6/7/2010	6/12/2010	No virus	NEG	UMP
3314/10	1	M	6/11/2010	6/21/2010	No virus	NEG	Makonde
3316/10	28	M	6/19/2010	6/27/2010	No virus	NEG	Makonde
3318/10	6	M	6/21/2010	6/21/2010	No virus	NEG	Makonde
3320/10	18	M	6/14/2010	6/14/2010	No virus	NEG	Makonde
3380/10	19	M	6/28/2010	7/6/2010	Polivirus, V1&V3	NEG	Rushinga
3382/10	20	F	7/5/2010	7/10/2010	No virus	NEG	Mutare
3470/10	9	F	7/10/2010	7/20/2010	No virus	NEG	Mudzi
3482/10	54	M	7/6/2010	7/16/2010	No virus	NEG	Buhera
3497/10	29	F	7/23/2010	7/23/2010	No virus	NEG	Gokwe Nort
3544/10	33	F	7/23/2010	7/26/2010	No virus	POS	Buhera
3549/10	4	F	7/29/2010	7/29/2010	No virus	POS	Gokwe Sout
3551/10	17	M	7/24/2010	7/24/2010	No virus	POS	Gokwe Sout
3740/10	31	F	8/24/2010	8/30/2010	No virus	POS	Zvishavane
3745/10	34	F	8/12/2010	8/14/2010	No virus	POS	Mwenezi
3811/10	50	M	9/4/2010	9/10/2010	No virus	NEG	Bulawayo
3813/10	30	M	9/4/2010	9/12/2010	No virus	NEG	Zvishavane
3858/10	28	M	9/11/2010	9/16/2010	No virus	NEG	Chivi
3922/10	58	M	9/16/2010	9/24/2010	NPEV	NEG	Banket
3902/10	19	M	9/21/2010	9/22/2010	No virus	NEG	Bulawayo
4069/10	50	M	10/16/2010	10/20/2010	No virus	POS	Hwedza
4071/10	13	M	10/18/2010	10/20/2010	No virus	POS	Buwayo
4072/10	56	F	10/12/2010	10/15/2010	No virus	POS	Buhera
4085/10	15	F	10/13/2010	10/21/2010	No virus	NEG	Bulawayo
3630/10	40	F	8/12/2010	8/12/2010	No virus	NEG	Mazowe
3632/10	4	F	7/18/2010	7/20/2010	No virus	POS	Kwekwe
3696/10	4	F	8/15/2010	8/20/2010	No virus	NEG	Zvishavane

3698/10	13	F	8/15/2010	8/23/2010	Polivirus, V2	POS	Gokwe Sout
3707/10	55	F	8/17/2010	8/22/2010	No virus	POS	Shurugwi
3715/10	41	M	8/21/2010	8/24/2010	No virus	POS	Chipinge
3719/10	12	M	8/20/2010	8/24/2010	No virus	POS	Chipinge
3735/10	20	F	8/22/2010	8/25/2010	No virus	NEG	Makonde
3737/10	4	M	8/8/2010	8/12/2010	No virus	POS	Makonde
4154/10	36	F	10/13/2010	10/17/2010	No virus	NEG	Mal-Ntcheu
4156/10	5	M	10/22/2010	10/26/2010	No virus	NEG	Mberengwa
4246/10	11	M	10/26/2010	11/2/2010	No virus	NEG	Harare
4312/10	34	F	10/30/2010	11/2/2010	No virus	NEG	Mt Darwin

EIA Rotavirus Test Results for gastroenteritis cases tested routinely.

Total received and tested: Jan to July 2010 = 783, (EIA Pos = 515, Neg = 268)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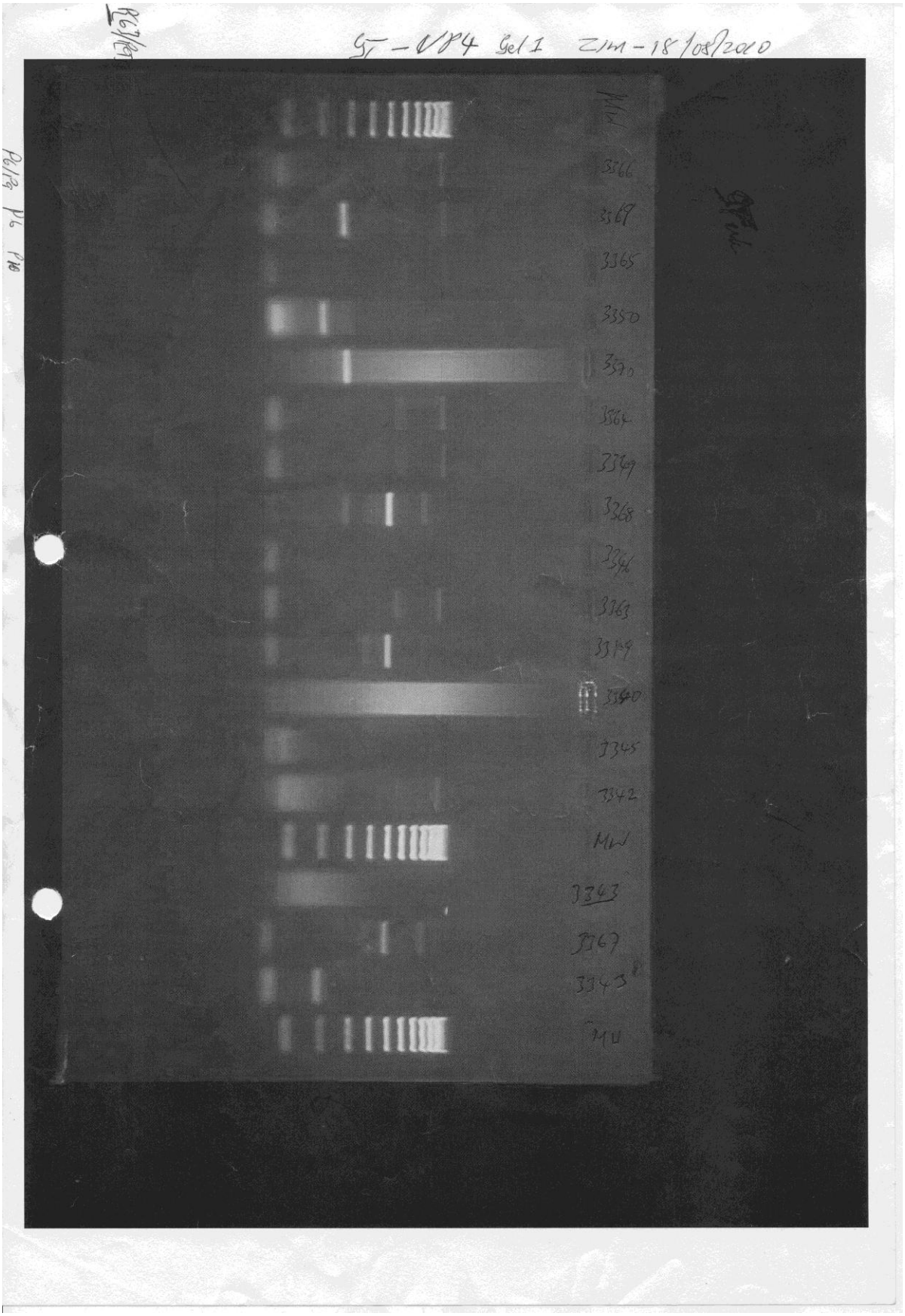
GT-V84 Gel 1 ZM-18/08/2020
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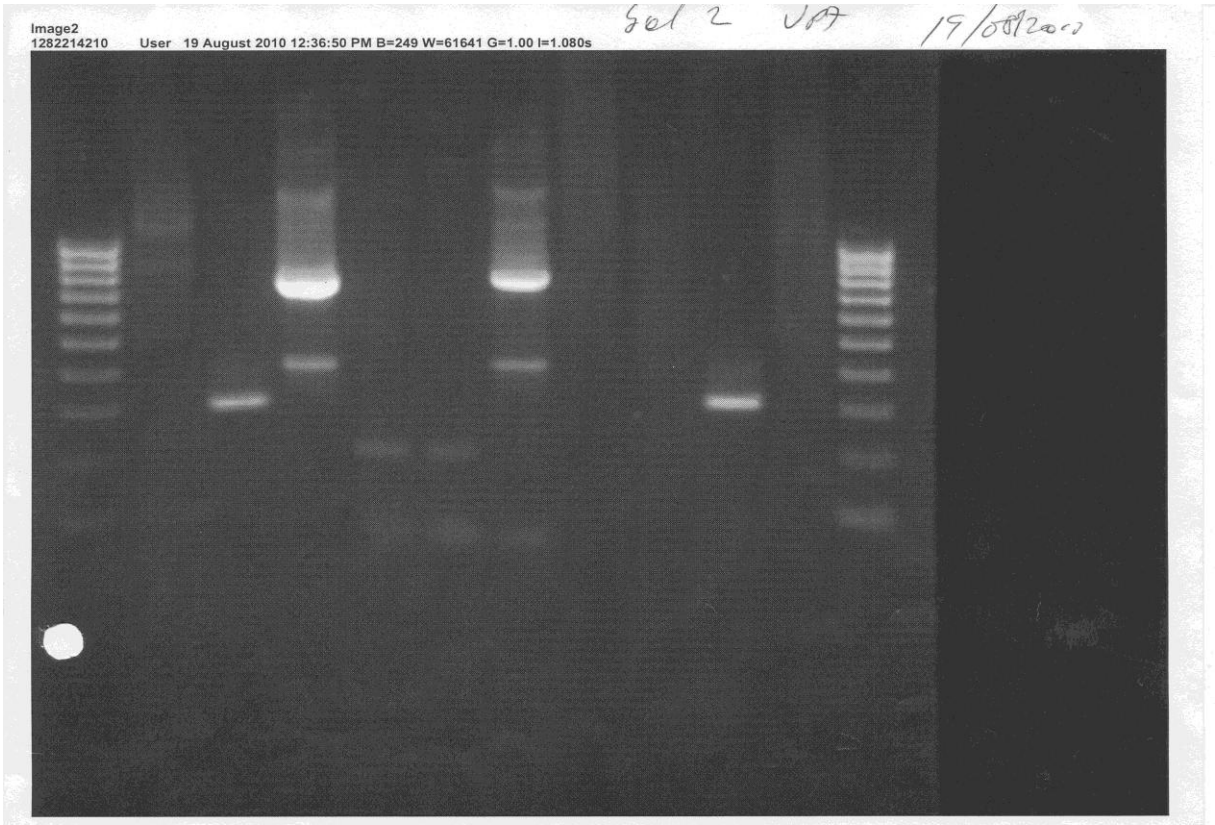
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1282214210 User 19 August 2010 12:36:50 PM B=249 W=61641 G=1.00 I=1.080s
Gel 2 VA 19/08/2010

APPENDIX 7

Telephone: +263-4-798620
Telegraphic Address:
"MEDICUS", Harare
Fax: +263-4-720119/702293
Telex: MEDICUS 22211ZW

ZIMBABWE

Reference:
Ministry of Health and Child Welfare
P O Box CY1122
Causeway
HARARE

The Chairman
Department of Medical Microbiology
University of Zimbabwe

Attention: Dr P Nziramasanga
(Head WHO – National Polio Laboratory)

RE: APPLICATION REQUESTING TO USE STORED STOOL SAMPLES: MR ARNOLD MUKARATIRWA

On the 16th of November 2010 you wrote to the Ministry of Health and Child Welfare in support of Mr Arnold Mukaratirwa's request to use stored stool samples of national rotavirus surveillance and Flaccid Paralysis Surveillance programmes in his research/study project.

Authority is hereby granted for use by the applicant of the stored stool samples.

Brigadier General (Dr) G Gwinji
SECRETARY FOR HEALTH AND CHILD WELFARE

SECRETARY FOR HEALTH AND CHILD WELFARE
SECRETARY'S OFFICE (01)
03 DEC 2010
P.O. BOX CY 1122, CAUSEWAY
ZIMBABWE