DETECTION AND GENETIC TYPING OF ROTAVIRUS IN SOME AVIAN SPECIES FROM SOUTHWESTERN NIGERIA

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CHAPTER ONE

1.0 INTRODUCTION

Agriculture continues to be the most important sector of the Nigerian economy in terms of provision of employment in spite of its declining contribution to the nation's foreign exchange earnings. About 65% of Nigerians are estimated to depend on agriculture for their livelihood while 34.8% of the GDP and over 38% of the non-oil foreign exchange earnings, are contributed by the agricultural sector (Adene and Oguntade, 2006). The sector is being transformed by commercialization at the small, medium and large-scale enterprise levels (Olomola, 2007). The poultry sub-sector is the most commercialised of all the sub-sectors of Nigeria's agriculture. There is however no comprehensive data on the sub-sector; thus making proactive intervention and planning impossible (Adene and Oguntade, 2006).

1.1 OVERVIEW OF POULTRY PRODUCTION IN NIGERIA

The types of poultry that are commonly reared in Nigeria are chickens, ducks, guinea fowls, turkeys, ostriches and more recently quails. Those that are of commercial or economic importance in Nigerian poultry industry, however, are chickens, guinea fowls and turkeys, amongst which the chickens predominate.

1.2 DESCRIPTION OF THE MAIN CHARACTERISTICS OF THE DIFFERENT PRODUCTION SYSTEMS

There are two distinct poultry production systems in Nigeria, as in most developing countries of Africa and Asia. Each of these two systems is associated with features of scale, stock, husbandry and productivity that therefore define the two distinct production systems. The two systems are conventionally referred to as the commercial poultry and the rural poultry, respectively. The commercial production system as the name implies is industrial in its prototype and therefore based on large, dense and uniform stocks of modern poultry hybrids. It is capital and labour intensive as well as inputs and technology demanding. On the other hand, the rural poultry is by convention a subsistence system

which comprises stocks of non-standard breeds or mixed strain, types and ages. It is generally of small scale, associated with household or grass root tenure and with little or no veterinary inputs. The rural poultry sector is therefore in its original sense a village-based, household or individual holding and occupation which has however been extended to non-village settings in peri-urban localities, mainly by the middle class dwellers. The common features to all these intermediate grades are in their subsistence scale generally, with minimal or no inputs and labour overheads (Adene and Oguntade, 2006).

1.3 BASIS AND STRUCTURE OF DISEASE PROBLEMS IN THE POULTRY INDUSTRY

One of the side effects of intensive poultry production is the creation of a ready habitat for the proliferation of disease causing infectious agents such as viruses, bacteria, parasites and fungi. The control of the introduction and multiplication of these agents has remained one of the greatest challenges to poultry producers. A few decades ago, the major diseases of poultry which were recognized, were due to a few bacterial, parasitic and viral agents. Most of the diseases caused by bacterial and parasitic agents have been controlled or in some cases eradicated in developed agricultural countries where more and newer viral diseases have subsequently emerged.

Viral diseases are less readily amenable to common therapeutic and other control measures. In Nigeria as an example of a developing economy, the poultry stocks not only share in the worldwide disease epidemic pool but are unfortunately, at a greater risk of exposure to viral, bacterial and even parasitic diseases because of the rather more auspicious climate for the proliferation of the organisms as well as the generally prevalent sub-standard prophylactic and husbandry inputs (Adene, 2004).

1.4 EMERGENT DISEASES

The poultry industry in the developing countries has continued to benefit from advances in poultry health management technologies in the developed countries. Also, import dependent poultry industry in the developing countries has inevitably acquired some new problems in the process. It is however, arguable that some of the so-called emergent diseases of poultry are indeed exotic to Africa, as believed in some quarters. Findings in the past showed that many of our native chickens and guinea fowls, having little or no contacts with imported chickens, do possess antibodies (Adene, 2004). There could

therefore be many other diseases which exist undetected in our poultry stocks and continue to wreck untold damages due to lack of adequate facilities to monitor and control them. In Nigeria the list surprisingly includes the not too new entities like infectious bronchitis and egg drop syndrome ’76 (EDS ’76) as well as the newly emergent ones caused by reoviruses, rotaviruses and adenoviruses (Adene, 2004).

1.5 ROTAVIRUS IN THE POULTRY INDUSTRY

In 1963 Adams and colleagues observed virus-like particles in intestinal tissue from mice infected with epizootic diarrhoea of infant mice virus (Adams and Kraft, 1963). Ten years after Bishop and colleagues reported the discovery of human rotavirus and its association with severe endemic diarrhoea in infants and young children (Bishop *et al.*, 1973). The identification of rotavirus in mucosa of infected children was achieved without the benefit of tissue culture technology and relied only on visualization by electron microscopy (EM). After this, rotavirus was also identified in faeces by EM by several studies (Flewett *et al.*, 1973; Bishop *et al.*, 1974). Using EM, the viral particles observed above resembled a short-spiked wheel, which led to the name rotavirus (from the latin *rota*, meaning wheel) (Flewett *et al.*, 1974). After its discovery, it became obvious that rotavirus was an important etiologic agent of diarrhoea of infants and young children, causing about 35% to 50% of the hospitalizations for severe diarrhoea during the first 2 years of life (Kapikian, 1993).

Rotavirus infection in avian species was first reported by Bergeland *et al.* (1977) who found particles morphologically indistinguishable from rotavirus in intestinal contents of poults with watery droppings and increased mortality. Since then it has become apparent that rotaviruses infect many species of domestic birds. Rotavirus infection in avian species is frequently associated with outbreaks of diarrhoea. Symptoms of rotavirus infection may vary from a mild disease in young chickens to a more severe manifestation in 12 to 21-day-old chickens, characterised by unrest, litter ingestion, watery faeces, wet litter, and severe diarrhoea (Barnes, 1997). Few studies have been published related to enteric viruses in Nigerian poultry. Such studies demonstrate significant occurrence and the importance of enteric viruses in poultry presenting enteric problems.

The rotaviruses belong to the family Reoviridae and contain a genome of 11 segments of double stranded RNA (dsRNA), which can be separated into distinct bands by

electrophoresis. The migration pattern of the 11 genome segments following electrophoresis of the viral RNA in polyacrylamide gel is called the RNA electropherotype (Estes *et al.*, 1984). Rotavirus in birds belongs to groups A, D, F and G (Saif *et al.*, 1985). Detailed studies on the epidemiology of rotavirus associated diarrhoea in poultry have been performed in advanced countries but none has been reported in Nigeria. It has however been reported in children (Adah *et al.*, 2001) in Nigeria.

Today, rotaviruses are recognized as the single most significant cause of gastroenteritis, malnutrition and diarrhoea in nearly 40% of hospitalized young children under 5 years of age in animals (Estes and Cohen, 1989; Kapikian and Chanock, 1990) and also avian species (Minamoto *et al.*, 1988). It has been suggested that interspecies transmission from birds to cattle had occurred in nature (Brussow *et al.*, 1992). Avian rotavirus has also been shown to be transmissible to mammalian species (Mori *et al.*, 2001).

Mortality rates are low in developed countries where illness is usually self limiting (Sudhir and Gagandeep, 2012). However, each year more than 600,000 young children die in developing countries throughout the world including Nigeria (Sudhir and Gagandeep, 2012). Rotavirus infection in children is mainly restricted to the small intestinal villus epithelium, resulting in the occurrence of total villus atrophy (Bishop *et al.*, 1973). Although rotavirus can infect older children and adults, diarrhoeal disease is primarily observed in children under 2 years of age (Estes and Cohen, 1989; Estes, 2001).

Rotavirus induced diarrhoea is thought to be caused by a combination of factors (Salim *et al.*, 1990), which include a reduction in epithelial surface area, replacement of mature enterocytes by immature (crypt-like) cells (Osborne *et al.*, 1988), an osmotic effect resulting from incomplete absorption of carbohydrates from the intestinal lumen in combination with bacterial fermentation of these non-absorbed compounds, secretion of intestinal fluid and electrolytes through activation of the enteric nervous system (ENS)(Lundgren *et al.*, 2000), and the effect of the rotavirus non-structural protein 4 (NSP4), which is thought to act as a viral enterotoxin (Ball *et al.*, 1996).

In poultry, both in layer and broiler birds, rotavirus has been established as a pathogen that manifests diarrhoea, dehydration, anorexia, loss of weight and nutrient malabsorption. Cumulatively, all these can lead to huge economic losses to poultry production systems (McNulty 2003; Villarreal *et al.*, 2006). Rotavirus infection is commonly observed in

chickens and turkeys, but avian rotaviruses (ARVs) have been isolated from other birds also (Yason and Schat 1985; McNulty, 2003). Recently, it has been suggested that ARVs can also cause runting and stunting syndrome in poultry (Otto *et al.*, 2006). Typically, ARVs belong to groups D, F, and G (McNulty 2003; Villarreal *et al.*, 2006) but those belonging to group A have been considered a major threat (Brussow *et al.*, 1992; Sugiyama *et al.*, 2004; Villarreal *et al.*, 2006). In contrast to the disease in domestic animals, the older birds can also be susceptible like the young ones, and turkeys have been the major sufferer when compared to chickens (Yason and Schat, 1987). In birds, the *in-vitro* cytotoxic activity studies have suggested a prominent role for natural killer (NK) cell activity against rotavirus infections (Myers and Schat, 1990). Also, the role of maternally derived immunoglobulins in protecting the intestinal mucosa during early life has been documented (Shawky *et al.*, 1993; McNulty 2003). For detection of ARVs, isolation in MA104 cell line, polyacrylamide gel electrophoresis (PAGE), EM or enzyme linked immunosorbent assay (ELISA) have to be performed (Yason and Schat 1985; McNulty 2003; Villarreal *et al.*, 2006). Recently, a multiplex RT-PCR assay for ARVs has been developed for use in poultry flocks (Day *et al.*, 2007). For genomic characterisation of ARVs, PAGE, northern hybridization or sequencing of VP4 and VP7 genes are usually performed but limited information exist regarding the serotypes; although there are reports of presence of serotype G7 in birds (McNulty, 2003). For control of infection, secondary bacterial enteritis has to be checked, for which antimicrobial medication is essential, and dehydration has to be countered by implementing fluid and electrolyte therapy.

1.6 INTER-SPECIES TRANSMISSION OF ROTAVIRUSES

The inter-species transmission abilities of mammalian rotaviruses have been demonstrated by assessing the presence of non-genogroup specific antibodies or by challenge studies with different rotaviruses (Sato *et al.*, 1981a; Heinrich *et al.*, 1983; Castrucci *et al.*, 1984; Castrucci *et al.*, 1985). Calves inoculated with equine or human rotaviruses have been protected against bovine rotavirus (BRV) challenge, showing their close relationship (Woode *et al.*, 1978). It has also been suggested that porcine, murine, simian and equine rotaviruses are antigenically related (Mebus *et al.*, 1977; Castrucci *et al.*, 1985). The capability of human rotaviruses (HRVs) to affect calves or piglets has been reported, and it has been found that piglets excreted the virus without clinical signs while calves produced

intestinal lesions (Bridger *et al.*, 1975; Mebus *et al.*, 1977). Furthermore the presence of HRV antibodies has been detected in milk of cows (Yolken *et al.*, 1985). In another study, calves were reported to be susceptible to rabbit rotaviruses and the rabbits also became infected, when inoculated with a BRV strain (Castrucci *et al.*, 1984). Also, it has been suggested that infection can occur in calves from rotaviruses of simian, porcine or lapine origin (Castrucci *et al.*, 1985). Similarly, pet animals like cats and dogs may also excrete BRV, and are thought of having a role in propagation of BRV (Schwers *et al.*, 1982). Much later, in an interesting observation, a BRV isolate has been noticed to differ from other mammalian isolates during probe analysis, but got hybridized to genome of an ARV strain, thus representing a classical candidate for a natural interspecies transmission between different classes of vertebrates (Brussow *et al.*, 1992). Apart from bovines, interspecies transmission of ARVs to experimental animals has also been reported (Rohwedder *et al.*, 1995; Mori *et al.*, 2001). Similarly, instances of transmission of mammalian rotaviruses to avian species have also been documented (Wani *et al.*, 2003). Similarly equine rotaviruses may also have close serological and genomic relations with HRV and porcine rotaviruses. On sequence comparison of NSP4 of the rotavirus strains belonging to equine and swine, it has been observed that the equine rotavirus strains may represent an excellent example of interspecies transmission from swine to equines (Ciarlet *et al.*, 2001). Further, hybridization experiments with probes prepared from different geno-groups and sequence analysis have given ample evidence for interspecies transmission during the evolutionary process of rotaviruses (Palombo, 2002). It has also been suggested that rotaviruses circulating in one animal species can pose a risk to another by re-emerging as a reassortant virus (Khamrin *et al.*, 2006). Very recently, the porcine G4/G5, G6/G8 serotypes have been found to be closely related to rotaviruses circulating in humans, cattle and camelids, respectively; and this could suggest that swine might play a crucial role as reservoir and generator of newly adapted emerging strains for human beings and other animal species (Duan *et al.*, 2007; Parra *et al.*, 2008).

1.7 ZOONOTIC SIGNIFICANCE OF ANIMAL ROTAVIRUSES

Animal rotaviruses could be considered as a potential threat to humans due to the possibility of genetic reassortment, through exchange of gene segments. Infections by bovine-human reassortants and the presence of several unusual strains in cases of infant

diarrhoea suggest that animal rotaviruses could be considered as having significant zoonotic impact (Ramani and Kang, 2007). Increasing evidences have been obtained regarding direct transmission; or animal rotaviruses contributing one or several genes to make animal-human reassortant viruses (Nakagomi and Nakagomi, 1991; Adah *et al.*, 2003; Malik *et al.*, 2005; Muller and Johne, 2007; Ramani and Kang, 2007). Further, the surveillance of circulating rotaviruses in the human population has revealed the presence of uncommon serotypes that are commonly found in domestic animals (Cook *et al.*, 2004; Malik *et al.*, 2005). In infants generally, the rotavirus infection is characterised by watery diarrhoea and severe dehydration while the infection in adults is often subclinical (Malik *et al.*, 2005; Ramani and Kang, 2007). The main reason for a zoonotic transmission is the close contact between humans and domestic animals as seen in backyard poultry in Nigeria, promoting exposure to rotaviruses, especially in geographical regions where there may be intermittent floods or torrential rains. Potential also exists for contamination of water bodies and food crops with animal rotaviruses, via animal excreta. Similarly, animal rotaviruses could also be transmitted by food materials which are eaten raw, especially vegetables (Svensson, 2000; Malik *et al.*, 2005). Further, rotavirus strains such as G3 (commonly seen in cats, dogs, pigs and horse), G5 (pigs and horses), G6 and G8 (cattle), G9 (pigs and lambs) and G10 (cattle) have been isolated from the human populations from different parts of the world (Desselberger *et al.*, 2001a; Malik *et al.*, 2005; Ramani and Kang, 2007). Studies have also given numerous clues regarding HRVs deriving the surface proteins from animal rotaviruses. To further understand the epidemiological and genetic basis for the origin of HRV strains, relative frequencies of different serotypes of BRVs have been analyzed (Varshney *et al.*, 2002). Based on the sequence analysis of VP4 and VP7 genes, it is presumed that there is predominant association of BRV G10 serotype-derived reassortant strains causing asymptomatic infections in newborn infants. It has now been well proven that the HRVs can acquire genomic segments from BRV strains through gene reassortment (Adah *et al.*, 2003). Similarly, hybridization experiments with HRVs have provided the evidence for the close relationship with feline and canine rotaviruses (Nakagomi *et al.*, 1990). In Italy, the sequence analysis of VP6, VP7, VP4 and NSP4 genes of human rotaviruses has given vital clues regarding the role of canine rotaviruses in contributing gene segments for reassortment with human viruses (De Grazia *et al.*, 2007).

Also, genetic relatedness has been identified in case of porcine rotaviruses, when VP1, VP2, VP3, VP4, VP7 and NSP4 genes were analyzed with those of recent HRV strains (Teodoroff *et al.*, 2005; Varghese *et al.*, 2006; Mascarenhas *et al.*, 2007). Cumulatively, all these research findings are pointing to the fact that such events may lead to evolution of novel reassortant viruses during mixed infections that could further complicate the infection in humans and animals. The rapid evolution often seen with rotaviruses and the emergence of novel strains warrant an intensive serotype-specific surveillance in order to control the infection.

1.8 DIARRHOEA

Diarrhoea is the condition of having three or more loose or liquid bowel movements per day. Alongside respiratory diseases, diarrhoea is one of the most common conditions that affect chickens. Similar to domestic animals, rotaviruses have been identified as one of the major etiological agents of diarrhoea and enteritis in avian species (McNulty 2003; Pantin-Jackwood *et al.*, 2007). Huge economic losses are associated with diarrhoeal syndrome in birds, making this a major concern in the poultry industry (Niture *et al.*, 2010). Normal chicken droppings should be firm and brown with a white part on the top which is made from urates, as chickens urinate and defecate in one motion. Any yellow foamy droppings or bloody droppings are abnormal. There are a number of possible causes of diarrhoea in poultry which include: coccidiosis, worms, viruses such as rotavirus, bacterial diarrhoea, kidney damage and a feed too high in protein (www.chickenvet.co.uk; 2010-2013).

Since the intestine is the primary target of rotavirus infection, epithelial dysfunction plays an important role in rotavirus pathogenesis. However thus far, *in-vivo* data concerning specific epithelial responses are rather limited (Collins *et al.*, 1988; Katyal *et al.*, 1999; Rollo *et al.*, 1999; Halaihel *et al.*, 2000a). Studies on the effect of infection on epithelial homeostasis *in-vivo* are mainly restricted to studies in piglets, young rabbits and mice (Collins *et al.*, 1988; Shepherd *et al.*, 1979; Katyal *et al.*, 1999; Halaihel *et al.*, 2000b).

1.9 JUSTIFICATION OF THE PRESENT STUDY

Numerous viruses, including astroviruses, reoviruses, rotaviruses, coronaviruses, and adenoviruses, have been implicated as causative agents for enteric disease in poultry because they have been isolated from or identified in the intestines and intestinal contents of affected poultry flocks (Baxendale and Mebatsion, 2004; Cattoli *et al.*, 2005). Enteric

diseases cause substantial economic losses to the poultry industry because they cause decreased weight gain, increased morbidity, increased mortality, and increased production costs due to poor feed conversions. Avian rotaviruses are broadly distributed among birds, but only scarcely characterised at the molecular level. There is also increasing evidence that transmission of rotaviruses from animals to humans occurs and that it significantly contributes to genetic variability of human rotaviruses (Palombo, 2002; Cook *et al.*, 2004; Müller and Johne, 2007).

Avian rotavirus causes enteritis of variable severity in poultry especially during the early stage of life (Tamehiro *et al.*, 2003). Huge economic losses are associated with diarrhoeal syndrome in birds, making this a major concern to poultry industry due to a drop in production (egg and weight gain) and mortality. Interestingly, rotavirus strains or rotavirus genome segments of avian origin have been identified in calves (Brussow *et al.*, 1992), suggesting that interspecies transmission of group A rotaviruses from birds to mammals may occur in nature. Thus, gathering sequence information on avian rotaviruses is pivotal not only for investigating the genetic diversity of ARVs but also for understanding the origin of unusual strains. Serotypes, genotypes and clinical signs (which might have been mistaken for other disease) of rotavirus infection in poultry flocks in Nigeria has not been documented. Although diarrhoea occurs in poultry flocks, none has been linked to rotavirus infection. However, rotavirus infection has been reported in humans in Nigeria. Due to the close proximity of man and the avian species in Nigeria, it is not impossible that ARVs could reassort with the HRVs.

Runting and stunting syndrome (RSS), also called malabsorption syndrome, is the major enteric disease complex in broiler chickens with rotavirus implications. Since 2004, the broiler industry in the southeastern United States, and more recently in other regions of the world, has experienced severe outbreaks of RSS. Since our breeder stocks in Nigeria are sourced from different parts the world, the poultry industry would thus be at risk of exposure to different micro-organisms including the ARVs which have been implicated in RSS.

The major enteric disease complex in turkeys is poult enteritis complex (PEC) (Barnes *et al.*, 2000), also known as poult enteritis mortality syndrome (PEMS) in its more severe presentation (Barnes and Guy, 2003). To understand and control enteric disease in poultry,

more information on the prevalence and epidemiology of enteric viruses is essential and periodic surveys are necessary to develop valuable long term data.

Despite a great variety of rotaviruses detected in avian species (McNulty *et al.*, 1980; Otto *et al.*, 2006; Otto *et al.*, 2007), data on genome sequences are scarce. Rotaviruses of groups A, D, F and G have been detected in different avian species, but their causative role for diarrhoea and a chronic disease designated as runting and stunting syndrome in chickens or turkeys is not completely understood (Day *et al.*, 2007; McNulty, 2003; Otto *et al.*, 2006). Chick hood mortalities and diarrhoea in Nigerian poultry have previously been linked to various factors including disease; none however has been related to rotavirus infection. This study serves as the first report of rotavirus infection of poultry in Nigeria.

Since the potential economic resources of the poultry industry may not be fully utilized until the etiological agent of diseases are recognized and possibly controlled, there was thus the need to conduct epidemiological studies to determine prevalence of rotavirus in Nigerian poultry with subsequent genetic characterisation of the virus. This study was designed to detect the presence of rotavirus in poultry in south-western Nigeria using molecular methods. To accomplish this, a survey of the virus was conducted, nucleotide and amino-acid sequences were analyzed and phylogenetic analysis performed in order to provide epidemiological information.

1.10 STUDY OBJECTIVES

1.10.1 General objectives: To investigate the presence and genotype of avian rotavirus, its possible role in interspecies transmission and reassortment among avian or mammalian rotaviruses.

1.10.2 Specific objectives

1. To design primers for optimization of the most suitable PCR protocol for this study.

2. To detect avian rotavirus using molecular techniques.

3. To characterise the gene segments of avian rotavirus strains present in samples by nucleotide and amino acid sequence analysis.

4. To determine the genetic relatedness of the various rotavirus strains by construction of phylogenetic trees.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 INTRODUCTION

Rotaviruses are now established as a major cause of enteritis and diarrhoea in a wide range of mammalian species, including humans (Estes, 2001). Rotavirus infection in avian species was first reported by Bergeland *et al.* (1977), who found particles morphologically indistinguishable from rotaviruses in intestinal contents of poults with watery droppings and increased mortality. Since then, it has become apparent that rotaviruses infect many species of domesticated birds. As in mammals, rotavirus infection in avian species is frequently associated with outbreaks of diarrhoea. The economic significance of rotaviral enteritis to the poultry industry has not yet been defined, but by analogy with the situation in mammals, it is likely to be significant. Some mammalian rotaviruses have limited ability to infect other mammalian species, and rotaviruses from turkeys and pheasants can infect chickens (Yason and Schat, 1986a).

2.2 ETIOLOGY

2.2.1 Classification of Rotaviruses

Rotaviruses are classified as a genus in the family Reoviridae. This family contains 9 genera, members of which infect vertebrates, invertebrates, and plants (Estes and Kapikian, 2007). Rotaviruses infect only vertebrates and are transmitted by the faecal-oral route. They differ in morphology from other genera within the family. Rotaviruses possess 11 segments of double-stranded RNA. Their morphogenesis involves the temporary acquisition of a lipid envelope and the deposition of viral-coded glycoprotein in the endoplasmic reticulum (Mertens *et al.*, 2000). Rotaviruses readily undergo genetic reassortment (i.e., when two different rotaviruses belonging to the same serogroup infect the same cell, hybrid viruses containing mixtures of the genome segments from each parent are generated). Recently, a classification system was proposed for rotaviruses in which all the 11 genomic RNA segments are used (Matthijnssens *et al.*, 2008a). Based on nucleotide identity cut-off percentages, different genotypes were defined for each

genome segment. A nomenclature for the comparison of complete rotavirus genomes was considered in which the notations Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx are used for the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 encoding genes, respectively. This classification system is an extension of the previously applied genotype-based system which made use of the rotavirus gene segments encoding VP4, VP7, VP6, and NSP4. As more human and animal rotavirus genomes will be completely sequenced, new genotypes for each of the 11 gene segments may be identified. A Rotavirus Classification Working Group (RCWG) including specialists in molecular virology, infectious diseases, epidemiology, and public health was formed, which can assist in the appropriate delineation of new genotypes, thus avoiding duplications and helping minimize errors (Matthijnssens *et al.*, 2008b).

Historically, VP6 was the first rotavirus protein used for classification. Both VP2 and VP6 are highly immunogenic proteins in the virion (Ciarlet and Estes, 2002). After infection, antibodies to VP6 are easily detected (Ciarlet and Estes, 2002), and the most sensitive immunologic diagnostic assays are based on detection of this protein. VP6 bears different epitopes which allow differentiating different subgroup (SG) specificities of group A rotaviruses: SG I, SG II, SG I + II, or SG non-I, non-II viruses can be distinguished according to reactivities with two monoclonal antibodies (MAbs) (Ciarlet and Estes, 2002). More recently, based on molecular characterisation, only two groups (termed genogroups) were distinguished among group A human rotaviruses (genogroup I: SGI; genogroup II: SGII, SGI + II, and SG non-I, non-II) (Iturriza *et al.*, 2002). In 1985, a classification scheme for rotaviruses was proposed to allow for the presence of multiple ‘‘groups’’ of rotaviruses and for the existence of ‘‘serotypes’’ which crossed species (Graham and Estes, 1985) and later in 1989, a binary classification system reminiscent of the one used for the classification of influenza viruses was established, derived from immunological reactivities and gene structures of the two outer capsid proteins, VP4 and VP7, that independently elicit neutralizing antibodies (Ciarlet and Estes, 2002). Thus, rotavirus strains were classified into VP4 or P serotypes (P for protease-sensitive) and VP7 or G (G for glycoprotein) serotypes (Estes and Kapikian, 2007). Classification of rotaviruses into VP4 or VP7 serotypes is performed by cross-neutralization assays using hyperimmune sera raised to prototype viruses and/or to laboratory-engineered mono-

reassortants. Since antigenic characterisation is time-consuming and requires virus collections and proper immunological reagents that are not available in all the laboratories, and due to the increasing ease of sequencing, the antigenic classification has slowly been replaced by a classification system of rotaviruses into VP4 or VP7 genotypes, performed by sequence analyses and based on identities between sequences of cognate rotavirus gene segments. So far, 19 G genotypes and 27 different P genotypes have been identified (Matthijnssens *et al.*, 2008a). In contrast, a dual nomenclature has been adopted for the VP4 antigenic and genetic classification (Estes and Kapikian, 2007). The P serotype (when known) is denoted by an Arabic number (sometimes followed by a capital letter) and the P genotype is denoted immediately after the P serotype number by an Arabic number within squared brackets. Rotavirus strains belonging to 11 G types (G1– G6, G8–G12) and 12 P types (P1A[8], P1B[4], P2A[6],P2C[6], P3[9], P4[10], P5A[3], P6[1], P8[11], P11[14], P12[19], P[25]) have been isolated from humans (Ciarlet and Estes, 2002; Gentsch *et al.*, 2005; Rahman *et al.*, 2005; Desselberger *et al.*, 2006; Matthijnssens *et al.*, 2008a). During the late 1990s, sequence analyses of the rotavirus enterotoxin NSP4 gene from human and animal rotavirus strains revealed the presence of six (A-F) distinct NSP4 genotypes (Ciarlet *et al.*, 2000; Estes and Kapikian, 2007). The complete open reading frames (ORF) sequences of all 11 genome segments of 53 rotavirus strains have also been determined (human Wa, DS-1, AU-1, D, KU, P, TB-Chen, ST3, IAL28, Se584, 69M, WI61, A64, L26, T152, B1711, B3458, B10925-97, 111/05-27, B4633-03, RV161-00, RV176-00, N26-02, Dhaka12-03, Dhaka16-03, Matlab13- 03, Dhaka25-02, Dhaka6, DRC86, DRC88, Hun5, MG6, PA169, KTM368, B4106, simian SA11, RRV, TUCH, bovine B383, UK, RF, WC3, BRV033, porcine A131, A253, YM, OSU, Gottfried, avian PO-13, ovine OVR762, guanaco Chubut, Rı´o Negro, and lapine 30/96) (Ito *et al.*, 2001; Matthijnssens *et al.*, 2006; Matthijnssens *et al.*, 2008a).

2.2.2 Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments

In order to render the classification system of rotavirus A useful and to explore its biological significance in full, a set of recommendations is provided. The new classification system (Matthijnssens *et al.*, 2008a) is based on the nucleotide (nt) sequences of complete ORF, and therefore the nt sequence of the entire ORFs of all genes

of a new strain should preferentially be obtained in order to unequivocally assign it to one of the known/established genotypes or to a new genotype.

2.2.3 Classification strategy

2.2.3.1 Complete open reading frame analysis

Once the complete ORF of a rotavirus gene under investigation has been determined, it will be compared to other complete ORFs of cognate genes available in the GenBank database through a basic local alignment search tool (BLAST) search (http://www.ncbi.nlm.nih.gov/BLAST/).

If pairwise nucleotide identities between the gene of the novel strain under investigation (strain X) and strains belonging to an established genotype A are above the cutoff value of that gene segment (Table 1), strain X can be assigned to genotype A. The exact relationship between the gene of strain X and cognate genes of all established genotypes has to be obtained phylogenetically. When all the pairwise nucleotide identities between a gene of the new strain Y,

and the cognate genes of all the established genotypes are below the cut-off value for that gene segment (Table 1), strain Y may be the prototype of a new genotype (Matthijnssens *et al.*, 2008a).

Table 1: Nucleotide percentage identity cut-off values defining genotypes for 11 rotavirus gene segments (Matthijnssens *et al.*, 2008b)

Gene product	Percentage identity cut-off values (%)	Genotypes	Name of genotypes
VP7	80	19G	**G**lycosylated
VP4	80	27P	**P**rotease-sensitive
VP6	85	11I	**I**nner capsid
VP1	83	4R	**R**NA-dependent RNA Polymerase
VP2	84	5C	**C**ore protein
VP3	81	6M	**M**ethyltransferase
NSP1	79	14A	Interferon **A**ntagonist
NSP2	85	5N	**N**TPase
NSP3	85	7T	**T**ranslation enhancer
NSP4	85	11E	**E**nterotoxin
NSP5	91	6H	p**H**osphoprotein

2.2.3.2 Partial open reading frame analysis

If only the partial ORF sequence of a rotavirus genome segment is available, assigning it to a certain genotype is less certain because the genotypic diversity across the ORF is not a constant value. Some regions of the ORF may be highly variable, while others may be more conserved. Since the cut-off percentage values for each of the 11 genome segments has been calculated based on entire ORFs, applying these cut-off percentages to only a part of the ORF might lead to erroneous conclusions. However, a partial gene sequence might be used to assign a rotavirus gene to an established genotype, only under certain circumstances when all three of the following restrictions are obeyed:

1. At least 50% of the ORF sequence should be determined.
2. At least 500 nt of the ORF should be determined.
3. Identity between strain X and strains belonging to an established genotype A should be at least 2% above the appropriate cut-off sequence (Table 1), before strain X can be assigned to genotype A.

To assign a genotype to a new ORF sequence, whether complete or partial, the comparison should only be done to strains for which the genotype has been established based on the entire ORF analysis, and not with other partial ORF sequences. Due to intra-segmental recombination (Parra *et al.*, 2004; Phan *et al.*, 2007a; Phan *et al.*, 2007b) or to different rates of diversification throughout a genome segment, classification assignments based on partial ORFs may yield misleading or incorrect results.

2.3 MORPHOLOGY

Intact rotavirus virions appear to be approximately 70-75 nm in diameter when visualized by negative contrast electron microscopy (figure 1). They are composed of 3 concentric protein layers, comprising a core about 50 nm in diameter, enclosed within a double-shelled capsid made up of inner and outer shells. They have been described as reovirus like but can be distinguished from reoviruses by their more clearly defined smooth outer edge. Some negatively stained intact rotavirus particles resemble a wheel with short spokes radiating from a wide hub, hence the derivation of the name rotavirus from the Latin word rota, which means wheel. The outer capsid shell may be lost, producing noninfectious or poorly infectious so-called single-shelled particles that resemble orbiviruses and are about 10 nm smaller than intact double-shelled virions. Intact and

single-shelled particles of turkey rotavirus had densities in cesium chloride of 1.34 and 1.36 g/ml respectively (Kang *et al*, 1988). Intact and single-shelled group D rotavirus particles from a pheasant were reported to be larger, at 80 nm and 70 nm, respectively, than those of an avian group A rotavirus with densities of 1.347 and 1.365 g/mL, respectively (Devitt *et al*, 1993). Advances in our understanding of the structure of the rotavirus virion have revealed that the so-called single-shelled particles are in fact double-layered. Similarly, intact virions, previously referred to as double-shelled particles, are triple layered. Both inner and outer capsids have a T =13(l) icosahedral surface lattice, with 132 channels spanning both capsid shells and extending inwards from the virion surface to the core; 60 short spikes extend 12 nm from the smooth surface of the outer capsid shell (Merten *et al*, 2000).

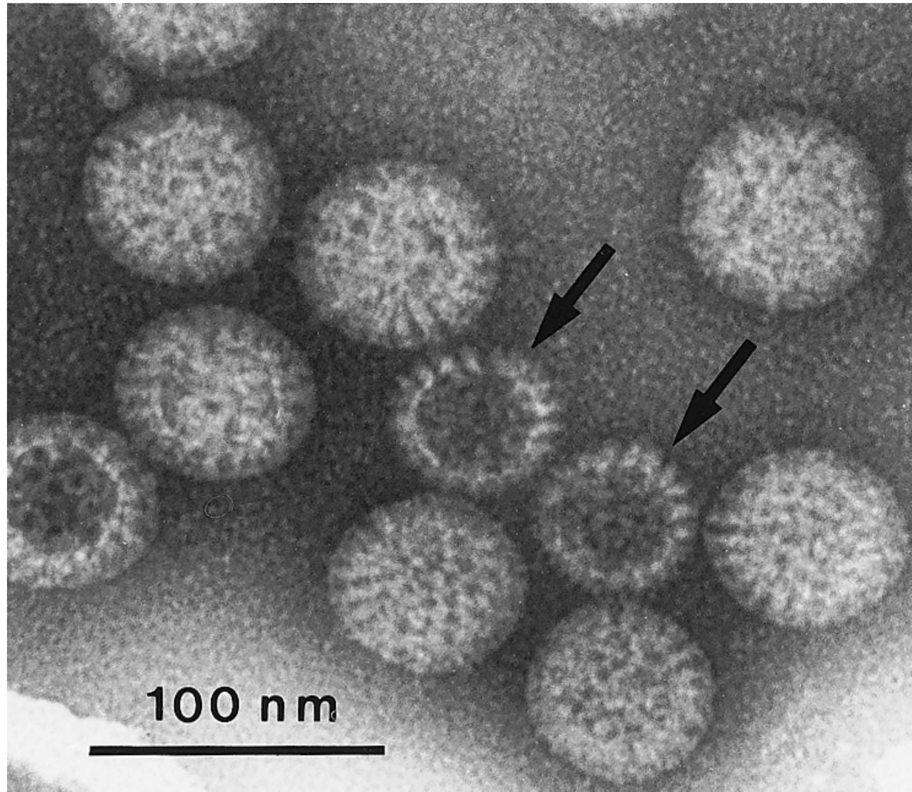


Figure 1: EM image of rotavirus particles in chicken faeces showing intact particles with smooth outer edge and particles with serrated edges (arrows), lacking outer capsid shell (McNulty, 2003).

2.3.1 Virion structure

The morphologic appearance of rotavirus particles is distinctive. Intact virus particles resemble a wheel, with short spokes and a well-defined rim, when examined by negative-stain electron microscopy (EM). The name rotavirus was suggested on the basis of this characteristic. Three types of particles (double-shelled, single-shelled, and core) are often observed by EM. SAl1 double-shelled particles are 76.5 nm in diameter, single-shelled particles are 70.5 nm in diameter, and cores are 50 nm in diameter. Single-shelled particles and cores can be produced by chemical disruption of double shelled or single-shelled particles, respectively. It is unknown whether the single-shelled or core particles are identical to subviral particles synthesized during virus replication. Early structural studies of rotavirus particles agreed that these particles possessed icosahedral symmetry, but the ultra structure of the virus was controversial. For example, the triangulation number (Aboudy *et al.*, 1988), which designates the relationship between the neighboring fivefold axes of the icosahedral surfaces, was initially reported to vary from 3 to 16 (Esparza and Gil, 1978). The application of new techniques (which avoided double-sided images produced by negative staining) to the study of rotavirus structure has recently resolved these earlier discrepancies. Roseto *et al.* (1979) studied the structure of single-shelled rotaviruses by using a freeze-drying technique and reported the existence of 132 capsomeres arranged in a skew symmetry with T = 13. They also showed that the outer layer contained small holes that corresponded one-to-one with holes in the inner capsid. This structure was subsequently confirmed for the inner capsid by Ludert *et al.* (1996), using the same technique and chemically disrupted particles. The three-dimensional structure of double- and single shelled rotavirus particles, determined at a 4-nm resolution by using cryoelectron microscopy and image-processing techniques (Prasad *et al.*, 1988), provides new insights into the virion structure. These studies have unequivocally established a T = 13 1 (levo) icosahedral surface lattice for the two outer layers. A distinctive feature of the virus structure is the presence of 132 large channels spanning both shells and linking the outer surface with the inner core; 120 channels are along the six-coordinated centers and 12 are along the five-coordinated centers.

Three types of channels can be distinguished on the basis of their positions and sizes. The type I channels are those running down the icosahedral fivefold axes, the type II channels

are those on the six-coordinated positions surrounding the fivefold axes, and the type III channels are those on the six-coordinated positions around the icosahedral threefold axes. Type III channels are about 14 nm in depth, and they are about 5.5 nm wide at the outer surface of the virus. Going into the particle, these channels constrict and then widen, and their maximum width is close to the surface of the inner-shell proteins. Similar features and dimensions are seen in the other two types of channels, except that type I channels have a narrower (ca. 4.0-nm) opening at the outer surface of the virus. The biological role of these channels is not yet clear, but it is likely that they are involved in importing the metabolites required for RNA transcription and exporting the nascent RNA transcripts for subsequent virus replication processes (Estes and Kapikian, 2007).

2.3.2 Genome organisation

The viral genome of 11 segments of dsRNA is contained within the virus core capsid. The segments range in size from 667 (segment 11) to 3,302 base pairs (segment 1), with the total genome containing approximately 19,000 base pairs (Table 2) (Trojnar *et al.*, 2009). This number, compiled from sequence data of segments from different virus strains, agrees closely with the genome size (18,680 base pairs) determined by EM measurements (Rixon *et al.*, 1984).

The simian rotavirus SA11 strain is considered the type species of group A rotaviruses, and its genome segments range in size from 3,302 (segment 1) to 667 (segment 11) base pairs (bp) (Ramig *et al.*, 2005; Estes and Kapikian, 2007). Sequences from different rotavirus strains show that each RNA segment starts with two 5'guanines followed by the 5'end non-coding sequences

[UTR = untranslated region] [9–49 nucleotides (nt)], an open reading frame (or two in the case of genome segment 11), the 30 UTRs (17–182 nt), and ends with two 3' cytidines. In addition, the plus-stranded RNA is capped at the 5' end with m7GpppG(m)Gpy, but there are no polyadenylated sequences at the 3' end (Estes and Kapikian, 2007).

Table 2:Comparison of genome segment sizes and sequence similarities among rotaviruses isolated from chicken (Ch-2G3), pigeon (PO-13) and monkey (SA11) (Trojnar *et al.*, 2009)

Segment number/encoded protein	Nucleotides/amino acids (genotype)		
	Ch-2G3	PO-13	SA11
1/VP1	3305/1089 (R6)	3302/1088 (R4)	3302/1088 (R2)
2/VP2	2732/895 (C6)	2738/897 (C4)	2690/882 (C5)
3/VP3	2583/829 (M7)	2583/829 (M4)	2591/835 (M5)
4/VP4	2354/770 (P[30])	2349/770 (P[17])	2362/776 (P[2])
5/NSP1	2122/577 (A16)	1870/576 (A4)	1581/496 (A5)
6/VP6	1348/397 (I11)	1348/397 (I4)	1356/397 (I2)
7/NSP3	1089/304 (T8)	1092/306 (T4)	1105/312 (T5)
8/NSP2	1042/315 (N6)	1042/315 (N4)	1059/317 (N5)
9/VP7	1066/329 (G19)	1065/329 (G18)	1062/326 (G3)
10/NSP4	724/168 (E10)	727/169 (E4)	751/175 (E2)
11/NSP5, NSP6	699/208, - (H8)	729/218, 96 (H4)	667/198, 92 (H5)
Genome	19,064	18,845	18,526

Hydrodynamic studies of the flexibility or stiffness of isolated rotavirus RNA segments in solution have indicated that these RNA segments cannot be packaged into the rotavirus capsid unless intimate protein-RNA interactions take place. In solution, these RNA molecules possess a "wormlike" or flexible cylinder structure; as an example, RNA segment 1 (3,302 base pairs and a contour length of 928 nm) theoretically cannot be bent into a capsid of 50 nm as a free molecule because the persistence length is 112.5 nm (Kapahnke *et al*, 1986). Therefore, to obtain RNA flexibility, one has to assume that intimate protein-RNA interactions occur in the virion to induce the needed bending and packaging of the dsRNA segments into the virus capsid. The proteins directly responsible for segment packaging remain unclear. The structural proteins present in core particles (VP1, VP2, and VP3) are obvious candidates, but nonstructural proteins may also play a scaffolding role. Deproteinized, purified rotavirus dsRNAs are not infectious, reflecting the fact that virus particles contain their own RNA-dependent RNA polymerase required to transcribe the individual RNA segments into active messenger RNAs (mRNAs) (Cohen, 1977).

The nucleotide sequences of the 11 RNA segments from different virus strains are now known, and the complete nucleotide sequence of one group A rotavirus (SA1l) was the first sequenced established. The sequences from different rotavirus strains show general features of the structure of each of the genome segments. Each positive sense RNA segment starts with a 5' guanidine followed by a set of conserved sequences that are part of the 5' non-coding sequences. An open reading frame (ORF) coding for the protein product and ending with the stop codon follows, and the another set of noncoding sequences is found containing a subset of conserved terminal 3' sequences and ending with two 3'-terminal cytidines. Almost all mRNAs end with the consensus sequence 5'-UGUGACC-3', and these sequences contain important signals for gene expression and genome replication. The last four nucleotides of the mRNAs can function as translation enhancers (Chizhikov and Patton, 2000).

All of the rotavirus gene sequences are A+T rich (58 to 67%), and the codon usage is biased against CGN and NCC codons, as in many eukaryotic and other viral genes (Estes and Kapikian, 2007). The RNA segments are base paired end-to-end, and the plus-sense strand of the genomic dsRNA contains the capped 5' sequence m7GpppG(m)GPy (Imai *et*

al., 1983). Similar features of the RNA termini (capped structures and 5' and 3' conserved sequences) are found in the primary structures of the genome segments of other viruses (e.g., reovirus, cytoplasmic polyhedrosis virus, and orbivirus) in the family Reoviridae (Estes and Kapikian, 2007), and 5' and 3' conserved terminal sequences are also found in other virus families with segmented genomes (Orthomyxoviridae, Arenaviridae, and Bunyaviridae). Therefore, these terminal sequences are thought to contain signals important for genome transcription, replication, and possibly assembly of the viral genome segments.

2.3.3 Structural proteins

The inner core consists of the structural proteins VP1 and VP3 and is surrounded by a layer of VP2 (figure 2). These proteins have affinity for ssRNA, and play a role in the processes of RNA transcription and replication. VP1 is thought to be the viral RNA polymerase and functions as both the viral transcriptase and replicase (Patton, 1996). For replicase activity however, VP1 requires VP2 and the vital role of VP2 in replication is linked to its ability to bind the mRNA template for minus strand synthesis (Patton *et al.*, 1997). In contrast to VP1, VP2 binds RNA nonspecifically (Pizarro *et al.*, 1991) and interacts with the dsRNA lining the VP2 shell (Prasad *et al.*, 1996). VP3 can bind to the N-terminus of VP2, involving a region of the innershell protein to which VP1 and ssRNA also bind and is required for RNA replication (Zeng *et al.*, 1998). VP3 also binds [α-32P] GTP, indicating that VP3 is the viral guanylyltransferase (Liu *et al.*, 1992) and VP3 was identified as a methyltransferase (Chen *et al.*, 1999), indicating that VP3 is a multifunctional capping enzyme. VP6 is the major component of the mature virus particle. VP6 forms the middle shell or intermediate layer of the virus particle and is found in trimers, which it forms spontaneously *in-vitro* (Estes *et al.*, 1987). VP6 plays a key role in virion strucure, since it interacts with the outer capsid (VP4, VP7) and the inner layer (VP2) (Estes *et al.*, 1987; Estes, 2001). The fact that VP6 is extremely stable and contains epitopes that are conserved in many virus strains makes it the most important antigen used in diagnostic assays. VP6 is probably also involved in polymerase activity as removal of VP6 from double-layered particles results in a loss of viral polymerase activity (Sandino *et al.*, 1986). It is however unclear whether VP6 plays a structural or functional role in this process.

Viral protein 4 (VP4) the product of gene segment 4, is a nonglycosilated outer capsid protein which forms 60 homodimeric spikes that project from the surface of the virus (Yeager, 1994). VP4 is also responsible for rotaviral hemagglutinin activity (Kalica *et al.*, 1983) and it is the protein responsible for cell attachment (Crawford *et al.*, 1994). VP4 has been implicated as a virulence determinant in mice, calves, piglets, and humans (Mota-Hernandez *et al.*, 2003). VP4 is comprised of about 775 amino acids and it is proteolytically cleaved into two polypeptides VP5* (~60KD) and VP8* (~28KD) (Estes *et al.*, 1981). Cleavage of VP4 results in an increase of viral infectivity (Estes *et al.*, 1981) and enhances penetration of virus into cells (Kaljot *et al.*, 1988). Cell binding however is not dependent upon cleavage of VP4 (Espejo *et al.*, 1981). For the SA11 strain, the cleavage sites have been located at two arginine residues (Arg 241 and Arg 247), creating VP8* from the N-terminal portion, and VP5* from the C-terminal portion of the primary VP4 protein (Lopez *et al.*, 1985). In some cases, a third cleavage may occur at arginine 231 (Arias *et al.*, 1996). The mechanism underlying cleavage-induced activation of infectivity is not apparent, but it is assumed that cleaved VP4 activates an early step of viral replication that may be triggered by one or both terminal regions generated by cleavage (Estes, 2001). Recently it was shown that cleavage of VP4 confers correct conformation of the VP4 spikes, which is essential for the virus to enter the cell (Crawford *et al.*, 2001) Why the virus produces a single translate, which is subsequently cleaved is not known. It has been postulated that the full length VP4 is more stable upon entry into the host (Nibert *et al.*, 1991). However, it was shown that infectivity using cleaved virus was not significantly different from uncleaved virus (Ludert *et al.*, 1996). Therefore, it seems unlikely that greater stability in the external environment explains the need to translate VP5* and VP8* as the single peptide VP4. Ludert *et al.*, showed that VP4 is already cleaved upon release from the intestinal cells and that virus shed into the lumen of the gastrointestinal tract of infected mice do not contain intact VP4 (Ludert *et al.*, 1996). Therefore, a hypothesis in which an intact VP4 peptide is needed as the outer capsid is assembled or as the virus particles are transported intracellularly, seems more feasible. Another explanation may be that the individual VP5* and VP8* proteins are somehow toxic for the host cell. Prasad and coworkers localized the VP5* and VP8* proteins by three dimensional cryo-electron microscopy using neutralizing antibodies that recognize

different epitopes of VP4 (Prasad *et al.*, 1990). It appeared that part of VP8* may interact with VP6, while VP5* seemed to be more externally exposed on the virus particle near the tips of the spikes (Estes, 2001). Hemagglutination activity has been mapped to VP8* (Fiore *et al.*, 1991). Monoclonal antibodies directed against VP8* more efficiently prevent binding of rhesus rotavirus to target cells than do antibodies against VP5* or VP7 (Ruggeri and Greenberg, 1991). This indicates an important role for VP8* in cell binding. In contrast, monoclonal antibodies directed against VP5*, but not VP8*, prevent release of the fluorophore CF (6-carboxyfluorescein) from isolated pig intestinal membrane vesicles. Recent studies have shown that VP5* can permeabilize lipid membrane vesicles and that there are at least two domains within VP5* required for pore formation. An N-terminal basic domain permits VP5* to associate with membranes and an internal hydrophobic domain that is essential for altering membrane permeability (Golantsova *et al.*, 2004). These findings point to a prominent role for VP5* in cell penetration and membrane destabilization rather that cell binding. The glycoprotein VP7, the product of gene segment 9, is the major outer capsid protein and it forms trimers on the viral surface (Estes, 2001). During morphogenesis, VP7 is directed to the ER by a cleavable signal peptide. As it is inserted in the membrane of the ER, VP7 is cotranslationally glycosylated (Kabcenell and Atkinson, 1985). VP7 only contains N-linked high mannose oligosaccharides that are processed by trimming in the ER (Kabcenell and Atkinson, 1985). VP7 does not contain the sequence KDEL found to confer retention for other ER proteins (Estes, 2001). Instead, two hydrophobic domains that are only present in the pre-mature VP7 are thought to act as the signal sequence that directs VP7 to and across the ER membrane (Estes, 2001). So far, two regions within VP7 (amino acids 51-61, and 61-111) are known to mediate retention in the ER (Poruchynsky *et al.*, 1985). VP7 is processed exclusively in the ER, and rotaviruses are not transported to the Golgi apparatus during morphogenesis. Deletion of the two retention regions of VP7 however results in transport of VP7 through the Golgi and into the extra cellular medium (Poruchynsky *et al.*, 1985). VP7 is resistant to proteolytic enzymes after insertion into membranes and remains membrane associated after high salt treatment or at alkaline pH (Kabcenell and Atkinson, 1985). This suggests that VP7 is not a membrane spanning protein but an integral membrane protein. VP7 also forms oligomer complexes with other proteins (VP4 and NSP4) in infected cells (Maass

and Atkinson, 1990). These interactions together with the interaction of VP7 with calcium appear to be important in the assembly of VP7 into the outer capsid (Dormitzer and Greenberg, 1992).

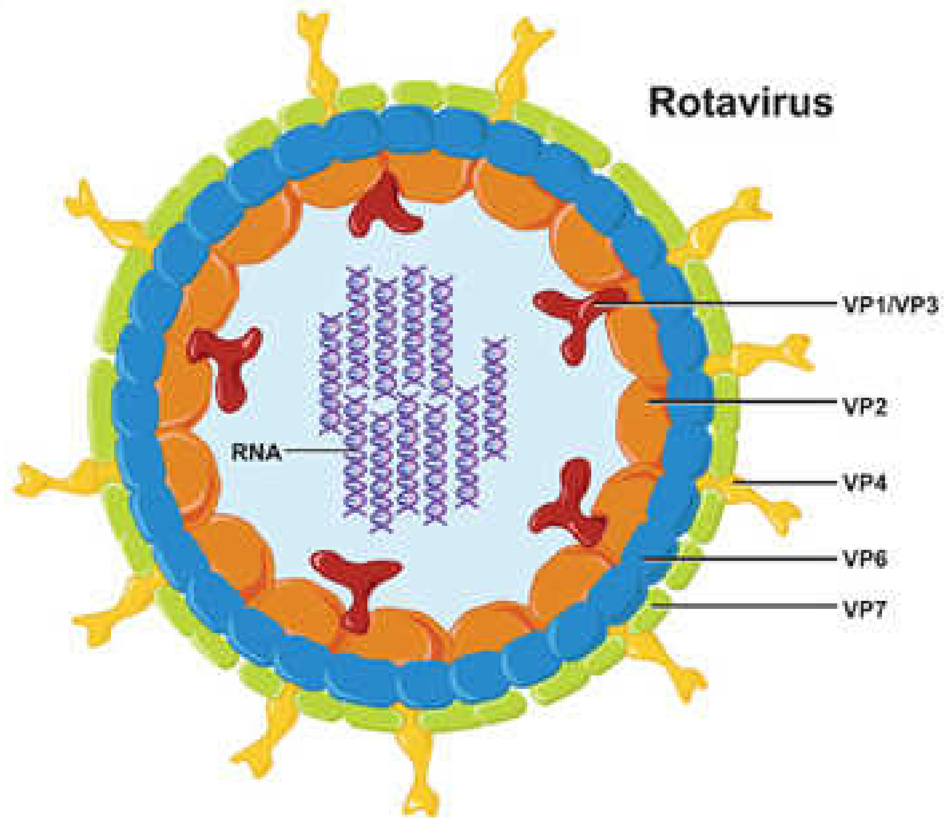


Figure 2: Image showing RNA and viral proteins on rotavirus particle (www.niaid.nih.gov/topics/rotavirus).

2.3.4 Nonstructural proteins

The rotavirus genome codes for six nonstructural proteins (NSP1-6). The proteins are involved in replication (NSP1, 3, 5, 6) and morphogenesis (NSP4) and except for NSP4, interact with nucleic acid (Estes, 2001). NSP1 is the least conserved of the rotavirus proteins. It seems to be nonessential for virus replication, although it was recognized as a virulence factor in mice (Broome *et al.*, 1993), but not in piglets or rabbits (Bridger *et al.*, 1998). NSP1 contains a relatively conserved zinc finger domain, but this domain is missing in some virus strains, showing it is not essential for virus replication and genome segment reassortment (Okada *et al.*, 1999). NSP2 is an oligomeric NTPase, is localized to viroplasms, possesses helix-destabilizing activity, and is possibly involved in RNA encapsidation and virulence (Taraporewala *et al.*, 1999). NSP3 is a sequence-specific RNA-binding protein that requires only four nucleotides at the 3' end for RNA recognition (Poncet *et al.*, 1994). NSP3 functions similarly to the cellular poly (A)-binding protein (PABP). It binds to eIF4G and thereby inhibits cellular RNA translation and protein synthesis, while enhancing the translation of viral mRNA (Vende *et al.*, 2000). NSP5 is an *O*-glycosylated phosphoprotein. It self-assembles into dimers, has autokinase activity and exists in several phosphorylated isomers in infected cells (Afrikanova *et al.*, 1996). NSP5 interacts with NSP2 (Gonzalez *et al.*, 1998), and when co-expressed, NSP2 induces hyperphosphorylation of NSP5 (Poncet *et al.*, 1997). NSP6 is encoded in an alternative ORF on gene segment 11 of most rotaviruses and is known to interact with NSP5 and accumulate in viroplasms (Torres-Vega *et al.*, 2000). NSP2, NSP5, and NSP6 are associated with viroplasms and co-expression of NSP2 and NSP5 is sufficient for the formation of viroplasms (Fabbretti *et al.*, 1999). These proteins may therefore function to recruit other viral proteins into the viroplasms, or enhance RNA encapsidation or movement of nascent viral particles from the viroplasms to the ER membrane. In contrast, NSP1 and NSP3 show a diffuse cytoplasmic staining in cells (Mattion *et al.*, 1992). NSP4 is the only nonstructural protein that does not bind to RNA. The nonstructural protein NSP4 encoded by gene 10 is 175 amino acids in length and has been shown to play a key role in viral morphogenesis (Estes, 2001). The primary product of gene 10 is a 20kD protein that is cotranslationally glycosylated to a 29kD species. Oligosaccharide processing then eventually yields the mature 28kD protein (Kabcenell and Atkinson,

1985). NSP4 is an ER glycoprotein that has been proposed as a receptor for double-layered particles (lacking the outer capsid proteins VP4 and VP7) and is crucial to translocate these immature viral particles across the endoplasmic reticulum membrane (Au *et al.*, 1989). During the process of budding, the subviral particles, assembled in the viroplasm adjacent to the ER, acquire a transient membrane envelope, which is lost when the two outer capsid proteins VP4 and VP7 are assembled. The receptor role for NSP4 is further confirmed by the observation that double-layered particles bind to ER membranes containing only NSP4 (Estes, 2001). Glycosylation of NSP4 is required for removal of the transient envelope from the budding particles (Petrie *et al.*, 1983). The topography of NSP4 in the ER membrane indicates that the hydrophilic carboxy terminus is projected into the cytoplasm and that the amino acid terminus, containing three hydrophobic domains, is responsible for maintenance in the ER membrane (Tian *et al.*, 1996). The carboxy terminus (amino acids 161-175) was found to be responsible for binding to VP6 present on nascent particles (Au *et al.*, 1993). NSP4 contains a VP4 binding domain between amino acids 112 and 146 (Au *et al.*, 1993) and forms heterooligomeric complexes with VP4 and VP7 (Poruchynsky and Atkinson, 1991). This association however is transient and NSP4 is not incorporated in the mature virion (Maass and Atkinson, 1990). The amphipathic a-helix region is predicted to adopt an a-helical coiled-coil structure, and this region is thought to mediate oligomerization of the virus-binding domains into a homotetramer (Taylor *et al.*, 1996). Glycosylation of NSP4 is required for interaction with calnexin, but is not required for its binding activity to double-layered particles or for oligomerization (Mirazimi *et al.*, 1998). Increasing lines of evidence show that the NSP4 protein is involved in pathogenicity of rotaviral infection and that it acts as a viral enterotoxin and can induce age-dependent diarrhoea (see section mechanisms of rotavirus pathogenesis) (Mori *et al.*, 2002). Reassortment analyses showed that NSP4 might function as a virulence factor (Hoshino *et al.*, 1995). Mutations in NSP4 have been associated with avirulence, and sequence changes correlated with an altered protein function (Zhang *et al.*, 1998). Not all attenuated (avirulent) viruses however have been shown to contain mutations in NSP4 (Chang *et al.*, 1999). This is most probably caused by the fact that there are many mechanisms of virus attenuation, and other viral proteins (NSP1, NSP2, VP3, VP4, VP7) are also virulence factors (Burke and Desselberger, 1996).

2.4 BINDING AND ENTRY

Rotavirus predominantly infects the mature enterocytes in the mid and upper part of the villi of the small intestine, which ultimately leads to diarrhoea. Rotavirus binding and entry of host cells is thought to be a multifactorial process (Lopez and Arias, 2006). Some animal rotavirus strains require sialic acid (SA, N-acetylneuraminic acid) residues on the surface of target cells for initial attachment (Delorme, 2001). The infectivity of these rotavirus strains is greatly diminished by the treatment of cells with neuraminidase, and consequently, these strains are neuraminidase sensitive. In contrast, many animal strains and most strains isolated from humans are neuraminidase-resistant (Ciarlet and Estes 1999). This does not mean that these strains do not use sialic acid for cell attachment because sialic acid moieties that are internal in oligosaccharide structures are either less sensitive or not sensitive at all to neuraminidase (Delorme, 2001). In their review, López and Arias proposed a model describing rotavirus-host cell interactions (Lopez and Arias 2006). For neuramidase sensitive rotavirus strains, initial binding to the cell surface is through a sialic acid (SA)-containing cell receptor, using the VP8 domain of VP4. This initial cell surface receptors containing sialic acid may be either glycolipids (gangliosides) or glycoproteins (Delorme, 2001). The interaction with sialic acid probably induces a conformational change in VP4. This change allows the virus to subsequently interact with the integrin $\alpha 2\beta 1$. This binding is mediated by the integrin ligand sequence DGE on VP5 (Graham *et al.*, 2003). Neuraminidase-resistant rotavirus strains have been hypothesized to use either a neuraminidase-resistant sialic acid-containing molecule (i.e. GM1) or a non-acidic sugar residue (i.e. galactose) for initial cell attachment or may be able to entirely bypass the binding to sialic acid and directly interact with integrin $\alpha 2\beta 1$ (Graham *et al.*, 2003). After this second interaction, three more interactions are thought to take place; binding of VP5 and the ligand-binding domain of cell-surface heat shock cognate protein hsc70 (Guerrero *et al.*, 2002), binding of VP7 (through its CNP region) and integrin $\alpha v\beta 3$ (Zarate *et al.*, 2004), and binding of VP7 (via its integrin ligand sequence GRP) integrin $\alpha x\beta 2$ (Graham *et al.*, 2003). The order of these events however has not been established. The initial interactions of the virus with the cell surface might trigger conformational changes in VP4 to facilitate subsequent interactions of VP5 with hsc70 and VP7 with integrins. Host cell binding ultimately leads to penetration and uncoating of the virus

(removal of VP4 and VP7 proteins from the virus to yield the transcriptionally active double-layered particles). There are several hypotheses on how rotavirus enters host cells. Virus internalization was previously thought to happen via direct entry or fusion (Kaljot *et al.*, 1988) or through Ca2+-dependent endocytosis (Chemello *et al.*, 2002). In the latter process, the virus is internalized within clathrin-coated endocytic vesicles. Within these vesicles a decrease in the calcium concentration, solubilizes the surface proteins from the virus particle. The solubilized outer layer proteins then permeabilize the vesicle's membrane to release the double-layered particle into the cytosol (Chemello *et al.*, 2002). A recent study however, showed that specific drugs that are known to impair clathrin- and caveolae-mediated endocytosis, did not affect rotavirus cell infection (Sanchez-San Martin *et al.*, 2004). Instead cholesterol and dynamin seemed to play a role in the entry of rotaviruses. The entry process was recently proposed to be mediated by lipid rafts (Lopez and Arias, 2006). The pathway described as raft-dependent endocytosis is clathrin and caveolin independent (Lopez and Arias, 2006). Cellular lipid rafts are membrane lipid microdomains enriched in glycosphingolipids (gangliosides among others), cholesterol and a specific set of associated proteins. Rafts have been implicated in a variety of cellular functions such as apical cell-sorting of proteins, signal transduction, and endocytosis (Lopez and Arias, 2006). In the rotavirus entry process, the rafts are also thought to serve as platforms to facilitate the efficient interaction of cell receptors with the virus particle (Lopez and Arias, 2006). It however still remains ambiguous how virus particles penetrate the plasma membrane and how they loose the outer layer.

2.5 GENOME ANALYSIS FOR VIRUS DETECTION

Rotaviruses are the only mammalian agents known to contain 11 segments of dsRNA. In most cases, the genome of the group A viruses is composed of four high-molecular weight dsRNA segments (segments 1 to 4), five middle-sized segments (segments 5 to 9) including a distinctive triplet of segments (segments 7 to 9), and two smaller segments (segments 10 and 11). Analysis of the genome pattern of virus specimens is relatively easy and rapid; therefore, this technique has become a useful and popular procedure for virus detection and for molecular epidemiology studies (Estes and Kapikian, 2007).

2.6 GENE-CODING ASSIGNMENTS

The gene-coding assignments and known properties of the proteins encoded in each of the 11 genome segments are now well established, although new protein functions continue to be identified. These assignments have been determined by *in-vitro* translation studies with mRNA or denatured dsRNA and by analyses of reassortant viruses. The information on simian 11 (SAl1) serves as the basis for comparative studies with other rotavirus strains. Such comparisons have shown that the absolute order of migration of a gene coding for a particular protein (cognate gene) may differ for different virus strains, and so gene assignments cannot be based on RNA patterns alone. Instead, identification of cognate genes must be based on hybridization with gene-specific probes, analysis of reassortants, or protein identification based on biochemical or immunologic identification of the protein translated in a cell-free system programmed with mRNA specific to the gene. The ability to directly obtain sequence information from dsRNAs or ssRNAs and the accumulating nucleic acid sequence data bases also make it possible to identify cognate genes solely on the basis of sequence homology following reverse transcription-polymerase chain reaction (RT-PCR) amplification (Estes and Kapikian, 2007).

The rotavirus genome segments code for structural proteins found in virus particles and for nonstructural proteins found in infected cells but not in mature particles. The consensus is that the protein products (VP1 to VP4, VP5*, VP6, VP7, VP8*) of six of the genome segments are structural proteins found in virus particles and that the other five genome segments code for nonstructural proteins. It remains unknown whether the additional open reading frames found in genome segment 11 codes for other proteins. Early studies often contained seemingly conflicting conclusions concerning the numbers and locations of the rotavirus proteins. Many of these were resolved, as reviewed elsewhere (Mattion *et al.*, 1994), when it was recognized that posttranslational modifications (glycosylation, trimming of carbohydrate residues, and proteolytic cleavages) occur following polypeptide synthesis. In addition, strain variations (such as the presence of more than one glycosylation site on VP7 in some bovine and human rotavirus strains) have been clearly shown, and these provide explanations for other discrepancies.

2.7 EPIDEMIOLOGY

2.7.1 Distribution

Rotaviral enteritis has been described in poults in the United Kingdom (Horrox, 1980), United States (Castro *et al.*, 1992), and France (Andral and Toquin, 1984). Rotavirus isolation from, or detection in, chicken faeces has been documented in Argentina (Bellinzoni *et al.*, 1987), Belgium (Meulemans *et al.*, 1985a), Brazil (Alfieri *et al.*, 1989), China (Wang *et al*., 1995), Cuba (Fraga *et al.*, 1985), the United Kingdom (McNulty *et al.*, 1981), the United States (Yason and Schat 1985), and the former Soviet Union (Bakulin *et al.*, 1991). Rotaviral antibody has been reported in chickens in Japan (Takase *et al.*, 1990), ducks in the United Kingdom (McNulty *et al.*, 1979a), and pigeons in Belgium (Vindevogel *et al.*, 1981). Rotaviruses have been found in faeces of diseased pheasant chicks in Italy (Foni *et al.*, 1989), the United Kingdom (Gough *et al.*, 1985), and the United States (Yason and Schat 1985). Rotaviruses were isolated from, or detected in, faeces of clinically nor mal ducks in Japan (Takase *et al.*, 1986) and the United Kingdom (Varley *et al.*, 1993), apparently normal feral pigeons in Japan (Minamoto, 1988a), diseased racing pigeons in the United Kingdom (Gough *et al.*, 1992), diseased partridges in Italy (Pascucci and Lavazza, 1994) and the United Kingdom (Gough *et al.*, 1990), diseased partridges and Japanese quail in Italy (Pascucci and Lavazza, 1994), a wild bird (*Melanitta fusca)* in Japan (Theil, 1987), and diseased ratites in South Africa (Els and Josling, 1998) and the United States (Hines *et al.*, 1995). A chicken embryo lethal rotavirus was isolated from the liver and small intestine of a diseased lovebird in England (Gough *et al.*, 1988). This evidence indicates that rotaviruses have a worldwide distribution in a wide variety of avian species.

2.7.2 Natural and Experimental Avian Hosts

As discussed above, turkeys, chickens, pheasants, partridges, ducks, guinea fowl, pigeons, and lovebirds naturally are infected with rotaviruses, and some have been experimentally infected. Most naturally occurring infections in turkeys, chickens, pheasants, partridges, and ducks involve birds 6 weeks old. Paradoxically, older chickens (56—119 days) and turkeys (112 days) were more susceptible to experimental infection than birds in the first few weeks of life (Yason and Schat, 1987). This observation is interesting; however, its relevance to the field situation is questionable because available evidence indicates that

most turkeys and chickens will have been infected, and presumably will have developed some immunity, well before this age. Lack of age resistance to infection, however, is illustrated by an outbreak of diarrhoea associated with rotavirus infection in commercial laying hens between 32 and 92 weeks of age (Jones *et al.*, 1979). Longitudinal surveys have shown that flocks of broilers and turkeys frequently experience simultaneous or sequential infections with different rotavirus electropherogroups (Theil and Saif, 1987).

2.7.3 Transmission, Carriers, and Vectors in Avian species

Rotaviruses are excreted in avian faeces in very large numbers (Yason and Schat, 1987). No information is available concerning survival of avian rotaviruses in faeces, but, by extrapolation from mammalian rotaviruses, environmental contamination is likely to be persistent. Horizontal transmission occurs readily between birds in direct and indirect contact.

Evidence demonstrates that larvae of the darkling beetle act as a mechanical vector for turkey rotaviruses (Despins *et al.*, 1994). Egg transmission of rotaviruses has not been demonstrated, but rotavirus detection in 3-day-old turkey poults prompted speculation that transmission occurs either in or on the egg (Theil and Saif, 1987). No evidence exists for a carrier state in birds.

2.7.4 Incubation Period, Clinical Signs, Morbidity, and Mortality in Poultry

Both the incubation period and the course of the disease are short. In experimentally infected turkeys, watery-to soft droppings were passed 2-5 days post infection. Orange-tinged mucus was observed in soft faeces. Rotavirus infection caused significant impairment of D-xylose absorption from the intestinal tract at 2 and 4 days post infection. Inoculated turkeys were depressed with loss of appetite between 1 and 5 days post infection. Pasting of the vents of experimentally infected poults has also been described (Hayhow and Saif, 1993). In the majority of studies, no mortality occurred in experimentally infected turkeys or chickens, but in one series of experiments, 3 of 23 poults inoculated at 2 days of age died (Shawky *et al.*, 1993). Mild (McNulty *et al.*, 1983) or no clinical signs (Meulemans *et al.*, 1985b; Yason and Schat, 1986a) were observed following experimental infection of chickens. When signs occurred, their onset coincided with peak virus excretion about 3 days postinfection. Chicks had mild diarrhoea (McNulty *et al.*, 1981) or passed increased quantities of cecal droppings (McNulty *et al.*, 1983).

Laying hens experimentally infected with rotavirus showed a drop in egg production 4-9 days postinfection (Yason and Schat, 1987). Rotavirus was detected in faeces of experimentally infected chickens and turkeys from 24 hours postinfection, and in some birds, excretion continued for more than 16 days (McNulty *et al.*, 1983; Yason and Schat, 1986a; Yason and Schat, 1986b; Yason and Schat, 1987). Under field conditions, clinical signs associated with rotavirus infection in broilers have varied from subclinical infections to outbreaks of diarrhoea severe enough to warrant attention to the litter, with associated dehydration, poor weight gains, and increased mortality (Alfieri *et al.*, 1989; Bellinzoni *et al.*, 1987). In poults, variations in severity of clinical signs have also been observed, including a very mild scour in the first week of life, which caused mortality only if vent pecking occurred (Horrox, 1980); a more severe disease in 12-21 day-old poults characterised by restlessness, litter eating, and watery droppings with mortality between about 4 and 7% (Bergeland *et al.*, 1977); and profuse scouring in 2-5 week-old poults(McNulty *et al.*, 1978), with affected birds huddling together, mortalities from suffocation, and stunting of survivors. In other outbreaks, predominant signs have been diarrhoea and wet litter. Pheasant chicks 2-3 weeks old in the United States had diarrhoea and increased mortality associated with rotavirus infection (Reynolds *et al.*, 1987a). In the United Kingdom, rotavirus infection was associated with stunting and increased mortality in pheasant chicks in the first week of life (Gough *et al.*, 1985; Gough *et al.*, 1986). Six of twenty 2-day-old pheasants inoculated with intestinal contents containing rotaviruses from naturally occurring cases died 5-6 days postinfection (Gough *et al.*, 1986); a high mortality rate was also observed in pheasant chicks inoculated with a group D rotavirus (Haynes *et al.*, 1994). In Italy, infected pheasants between 6 and 40 days of age showed depression, drooping wings, yellowish watery diarrhoea, and dehydration; mortality was 20-30% (Foni *et al.*, 1989). Diarrhoea, lethargy, and loss of appetite were associated with rotavirus infection in 3-4 month old racing pigeons in the United Kingdom (Gough *et al.*, 1992). Variations in severity of clinical signs associated with rotavirus infections could be due to genuine differences in virulence of avian rotavirus strains, as has been shown for mammalian rotaviruses (Estes, 2001), and interaction of rotavirus with additional factors such as other infectious agents (Hayhow and Saif, 1993) or

environmental stress. Morbidity is high. Most faecal specimens taken randomly from birds in affected flocks will contain rotaviruses.

2.8 PATHOLOGY

2.8.1 Gross

The most common finding at necropsy is the presence of abnormal amounts of fluid and gas in the intestinal tract and ceca. Pallor of the intestinal tract accompanied by loss of tonicity may be evident. Secondary findings may include dehydration, stunting of growth, pasted vents, inflamed vents, anemia due to vent pecking, litter in the gizzard, and inflammation and encrustation with droppings of plantar surfaces of the feet (Haynes *et al.*, 1994). Hemorrhages were observed in the cecal walls of some experimentally infected pheasant chicks (Gough *et al.*, 1986), and discrete, multifocal, superficial brownish-red erosions were found in the duodenum and jejunum of turkeys experimentally infected at 84 and 112 days of age (Yason *et al.*, 1987).

2.8.2 Microscopic

Immunofluorescence (IF) studies using chickens and turkeys experimentally infected with rotavirus have demonstrated the principal site of virus replication to be the cytoplasm of mature villus absorptive epithelial cells in the small intestine. Infected cells were most numerous in the distal third of villi. Small numbers of infected cells were also detected in colon epithelium, ceca, cecal tonsils, and lamina propria of some villi. No IF was observed in proventriculus, gizzard, spleen, liver, or kidney (Yason and Schat, 1987). Within the small intestine, different rotavirus strains may show preference for specific areas. A group A rotavirus grew best in the duodenum of experimentally infected chickens, and a group D rotavirus favored the jejunum and ileum (McNulty *et al.*, 1983). In general, experimental infections using chickens and turkeys of differing ages showed that increasing amounts of viral antigen were detected in birds of increasing age (Yason and Schat, 1987). Microscopic lesions in the small intestines of turkeys experimentally infected with group A rotaviruses consisted of basal vacuolation of enterocytes, separation of enterocytes from the lamina propria with subsequent desquamation, villus atrophy accompanied by widening of the lamina propria, scalloping of the villus surface, fusion of villi, and leukocytic infiltration of the lamina propria (Shawky *et al.*, 1994). In general, mean villus lengths were decreased and crypt depths were increased following experimental infection,

resulting in decreased villus to crypt ratios; morphometric changes were more pronounced in the duodenum and jejunum than in the ileum (Shawky *et al.*, 1993). There was infiltration of polymorphonuclear and mononuclear cells into the lamina propria of the cecum and colon in some birds (Yason and Schat, 1987). Scanning electron microscopy demonstrated roughened villus surfaces, irregularly shaped and sized villi (Yason and Schat, 1987), and loss of microvilli in enterocytes located at the tips of villi (Yason and Schat, 1987). In experimentally infected chickens, only minimal leukocytic infiltration of the lamina propria, with minimal loss of microvilli on cells at villus tips, was found in one study (Yason and Schat, 1987). Moderate villus atrophy, mainly in the ileum, however, has also been described by other workers in experimentally infected chickens (Meulemans *et al.*, 1985b).

Similar lesions to those found in turkeys were reported in pheasant chicks experimentally infected with a group D rotavirus. However, when microscopic lesions were most severe, at 7 days PI, viral antigen was detected along the entire length of villi and occasionally in the crypts. Lesions were most severe in the duodenum and jejunum (Haynes *et al.*, 1994). No histopathologic changes were observed in poults with naturally acquired rotavirus infection (Horrox, 1980). Degeneration and inflammation of villi of the duodenum and jejunum have also been reported, however, in poults with rotaviral enteritis (Bergeland *et al.*, 1977). Lesions were not found in ileum, cecum, colon, cloaca, or other organs. Neither gross nor microscopic lesions are pathognomonic for rotavirus infection.

2.9 PATHOGENESIS OF THE INFECTIOUS PROCESS

Little information about the pathogenesis of avian rotavirus infections exists, but inferences can be drawn from what is known about mammalian rotaviruses. With both avian and mammalian rotaviruses, the target cells are mature columnar absorptive cells that are located in the villus epithelium. Under normal circumstances, these cells have a relatively short life in mammals and birds and they desquamate from the tips of the villi. They are replaced by a process of cell division in the crypts, followed by migration of cells up the sides of the villi to the tips. Crypt cells are immature and poorly differentiated. As they migrate up the villi, they differentiate and start to produce disaccharidases, alkaline phosphatase, and mechanisms to transport sodium. In rotavirus-infected piglets, infection and destruction of mature villus epithelial cells results in increased division and

accelerated migration of crypt cells, so that the villi are clothed with immature, poorly differentiated cells, deficient in disaccharidases, alkaline phosphatase, and (Na-K) ATPase. Glucose-stimulated sodium transport and the net absorption of sodium, potassium, chloride, and water is also decreased, producing a rapid-onset, severe, watery diarrhoea with loss of electrolytes in the faeces (Hamilton and Gall, 1982). It is assumed that broadly similar mechanisms operate in rotavirus-infected birds. Decreased absorption of D-xylose from the intestinal tract has been observed in experimentally infected turkeys (Hayhow and Saif, 1993). However, shortening of the villi in experimentally infected turkeys (Hayhow and Saif, 1993) and chickens (Yason *et al.*, 1987) was less severe than in infected calves and piglets. It has been suggested that the differences in lesions between infected birds and mammals and the age-related susceptibility of turkeys and chickens may result from differences in the development of intestinal villi between mammals and birds (Yason *et al.*, 1987). It has been postulated that the frothy fluids found in the ceca of infected birds may result from impaired digestion and absorption of carbohydrates and sugars that, in turn, lead to their fermentation by cecal bacteria, producing metabolites that draw water into the ceca by osmosis (Yason and Schat, 1986b).

2.10 DIAGNOSIS

The clinical manifestations of rotavirus illnesses are not sufficiently distinctive to permit diagnosis on this basis alone. The diagnosis therefore requires detection of virus or viral antigen, demonstration of a serologic response, or both. The epidemiologic pattern of rotavirus disease at any one time may suggest this diagnosis, but laboratory confirmation is required.

Many assays have been developed for detection of rotavirus in faeces (Yolken and Wilde, 1994) and are as follows:

- Electron microscopy
- Antigen detection
- Enzyme immunoassay (EIA)
- Latex agglutination and lateral-f low immunoassays
- Virus isolation
- Nucleic acid detection (PAGE) and nucleic acid amplification (RT-PCR)

Most of these methods are relatively efficient at detecting rotaviruses, at least in part because of the large amount of intact rotavirus present in stool specimens of children with gastroenteritis.

2.10.1 Electron microscopy

Electron microscopy is highly specific for detection of rotavirus and is as sensitive as some EIAs. However, the method is too labor intensive for routine detection of rotavirus in large numbers of stool specimens. In addition, EM requires an expensive instrument and highly trained personnel and cannot distinguish between rotaviruses of different groups.

2.10.2 Antigen detection

The most widely used methods for rotavirus diagnosis are based on detection of protein antigens on rotavirus particles in stool specimens. The most appropriate antigen detection format for large-scale surveillance studies is an EIA that uses rotavirus specific antibodies to capture antigen onto wells of plastic plates. The antigen is then detected in a colorimetric reaction using a second rotavirus-specific antibody coupled to a detector enzyme. The EIA format is highly sensitive and specific and is adaptable to large sample volumes in the 96-well plate format. The optical density (OD) results can be easily recorded with a standard plate reader, permitting analysis of results with standard computer programs. Latex agglutination, utilizing latex particles coated with anti-rotavirus antibodies can be used as an alternative to EIA and rapid near patient tests using immunochromatographic methods are being used widely in consulting rooms.

Because of the importance of rotaviruses in clinical settings, many antigen detection methods have been commercialized, and data are available on their sensitivity and specificity.

2.10.3 Virus isolation

The first report of rotavirus isolation in cell culture was made by Mebus *et al.* (1971), who was also the first to describe the virus (Mebus *et al.*, 1969). From then on, several other attempts to isolate the virus in cell culture have been reported, however delays in the adaptation period and loss of infectivity after serial passages were observed (Banatvala *et al.*, 1975). The use of MA104 cells, associated to trypsin in the pre-treatment of the inoculums and maintenance medium by Sato *et al.* (1981b), increased rotaviruses isolation from several animal species. After that, different concentrations of trypsin and treatments

were described by Ferrari *et al.* (1986). The limitation in the understanding of the mechanisms by what means trypsin improved the rotavirus infectivity lasted until Arias *et al*. (1996) demonstrated they specifically cleaved VP4 to VP5 and VP8 in rotavirus strain SA11. However, cultivation of rotaviruses from faeces is not always possible for reasons that are not fully understood (Arnold *et al.*, 2009).

2.10.4 Nucleic acid detection

Due to the large quantities of rotavirus present in stool samples from children with gastroenteritis, the viral nucleic acid segments can be visualized directly after extraction from virus particles, by electrophoresis on acrylamide gels, and staining with ethidium bromide or silver nitrate. After electrophoresis, human rotavirus Groups A, B, and C have distinct patterns of gene-segment distribution, designated electropherotypes. The results of electropherotyping correlate with the presence of viruses of a specific group as shown by using other methods. Thus, the presence of distinct electropherotype patterns has long been considered diagnostic for the presence of individual rotaviruses of Groups A, B, and C (Steele *et al.*, 2004). For Group A rotaviruses, most samples that are positive for rotavirus by EIA will be positive for the characteristic pattern of rotavirus RNA segments after electrophoresis and silver staining. In some cases, silver nitrate staining of viral nucleic acid has roughly the same sensitivity as EIA methods (Herring *et al.*, 1982). Consequently, the PAGE method has sometimes been used to diagnose Group A rotavirus infections for surveillance studies. However, this method is very labor intensive and time consuming.

A variety of sensitive conventional or real-time reverse-transcription polymerase chain reaction (RT-PCR) methods have been developed based on primers specific for several different rotavirus genes (Gouvea *et al.*, 1991; Wilde *et al.*, 1991; Kang *et al.*, 2004). These methods have been particularly useful in detecting rotavirus in extra-intestinal tissues, in studies of the duration of viral shedding in stool and the correlation between disease severity and virus load (Wilde *et al.*, 1991; Richardson *et al.*, 1998).

2.11.5 Rotavirus Characterisation

2.11.5.1 Serotyping and subgrouping with monoclonal antibodies

Enzyme immunoassays described by groups in Australia, Japan, and the United States allow determination of rotavirus VP6 subgroup and VP7 serotype using serotype specific

monoclonal antibodies (Taniguchi *et al.,* 1987; Greenberg *et al.*, 1983; Boom *et al.*, 1990). The five most common rotavirus G serotypes (G1, G2, G3, G4, G9) can be assigned a serotype directly from faecal material using several ELISA formats incorporating monoclonal antibodies (Mabs) that bind in a serotype-specific manner to the VP7 protein. Similarly, VP6 subgroupings I, II, I & II and non-I, II can be assigned using binding specificity of VP6 Mabs. Three types of combined subgroup (VP6) and serotype (VP7) ELISA's are commonly used in rotavirus research. One uses a polyclonal antibody to coat the solid phase, which will bind the serotype- or subgroup-specific Mabs to capture the rotaviruses in the faecal samples. The second method uses the serotype or subgroup-specific Mabs bound directly to the solid phase to capture the virus. The third uses a polyclonal anti-rotavirus VP6 cross-reactive antibody bound to the solid phase to capture the virus, which will then be incubated with the different serotype- or subgroup-specific Mabs.

Studies using serotyping Mabs have typically typed 60%-70% of strains circulating in the community. The method is rapid and inexpensive. It provides G serotype information and, with suitable reagents, might provide information on antigenic differences between rotavirus strains of the same serotype.

2.11.5.2 VP7 serotyping

In the early to mid 1980s, Mabs against the VP7 (G) serotype antigen of the four most common rotaviruses were isolated and shown to bind in a serotype specific manner to intact virus particles. Subsequent development of serotype-specific EIA methods with these Mabs permitted the direct serotyping of rotaviruses in faecal specimens (Coulson *et al.*, 1987; Taniguchi *et al.,* 1987). Large-scale serotyping studies of rotaviruses in stool samples showed that these serotypes (now called G1 to G4) were globally common causes of gastroenteritis in children (Koshimura *et al.*, 2000). The antibodies are now used for direct serotyping studies. The Mab technique provides an antigenic measure of strain serotype rather than the indirect result provided by RT-PCR genotyping. Mabs specific for different variants of common serotypes have also been isolated, and their use in Mab-serotyping EIAs allows the detection of these antigenic variants (designated monotypes) in circulating rotaviruses (Coulson *et al.*, 1999). Other serotype-specific Mabs have proven valuable for detection of less common serotypes such as G5, G6, G8 and G10. Several

Mabs to serotype G9 have been important in the detection of this serotype, subsequently determined to be common worldwide (Coulson *et al.*, 1999; Kirkwood *et al.*, 2003).

A disadvantage of Mab serotyping is that a substantial fraction of the rotaviruses in faecal specimens cannot be serotyped. Reasons include insufficient numbers of intact virus particles, antigenic variation in common serotypes that renders them non-reactive with Mabs, and stool inhibitors that alter the binding of virus to antibody. The problem can be partially overcome by using larger panels of Mabs containing antibodies to different antigenic variants of the various serotypes. However, for some collections of rotavirus specimens, a large percentage of samples will need to be analyzed by RT-PCR to determine the genotypes of the strains not typeable with Mabs (Gouvea *et al.*, 1991). Another drawback of Mab serotyping is the need for continual supplies of Mabs and rotavirus hyperimmune antisera, both of which must be produced in animals. These processes require considerable resources in the context of the declining use of animals in research.

2.11.5.3 VP4 serotyping

Serotyping assays based on Mabs specific for the three most common human rotavirus P (VP4) serotypes/subtypes, P1A[8], P1B[4], and P2A[6], have also been developed (Coulson, 1992; Padilla-Noriega *et al.*, 1993). These assays have proven valuable in defining antigenic variation in serotypes such as the globally common P1A[8] (Padilla-Noriega *et al.*, 1998).

2.11.5.4 VP6 subgrouping

Polyclonal antibodies to the most abundant virion protein, VP6, are cross-reactive among all human and animal rotaviruses and largely define the group reactivity of rotaviruses (Kapikian *et al.*, 2001). In contrast, some Mabs to the VP6 protein react specifically with different rotavirus strains. For example, one group of antibodies reacts with the VP6 protein of typical serotype G2 strains with short electropherotypes, but do not react with most serotype G1, G3, G4, and G9 strains with long electropherotypes. Another group of VP6-specific antibodies has reciprocal reactivity (i.e., react with typical long electropherotype strains but not with short strains). These reactivity patterns referred to as VP6 subgroup I (SGI) and subgroup II (SGII) for Mabs that react with short and long electropherotype strains, respectively, have been important tools in rotavirus epidemiology

(Greenberg *et al.*, 1983). Less commonly, typical human rotaviruses might react with both SGI and SG II Mabs or with neither type of Mab. Animal strains and some uncommon human rotaviruses (e.g., G6, G8, and G10) have phenotype SGI specificity and a long electropherotype. When analyzed, these rare human strains usually show strong genetic homology with animal strains and might have derived from reassortment between human and animal rotaviruses. Subgrouping studies have also been useful in detecting human rotavirus strains with relationships to animal rotaviruses. A growing body of evidence suggests that reassortment among animal and human rotaviruses represents an important source for generating genetic diversity in human rotaviruses (Desselberger *et al.*, 2001b; Nakagomi and Nakagomi, 2002; Santos and Hoshino, 2005).

2.11.6 Polyacrylamide gel electrophoresis (PAGE)

Rotavirus dsRNA can be detected in clinical specimens by extraction of the viral RNA and analysis by electrophoresis on a polyacrylamide gel followed by silver staining. Rotavirus dsRNA has 11 segments. During electrophoresis through the gel, these negatively charged macromolecules separate according to size. The patterns of dsRNA can be visualized in the gel by staining with silver nitrate. Silver staining is a sensitive procedure to detect small amounts of nucleic acid in polyacrylamide gels. Silver ions form a stable complex with nucleic acids. After staining, the gels can be dried and stored. The dsRNA extracted from Group A rotaviruses can be split into four size classes: four large segments, two medium-sized segments, three small segments, and the two smallest segments. Group A human and animal rotaviruses also display two electropherotypes: “long” and “short.” Short electrophoretic patterns exhibit a larger segment 11 (encoding NSP5) that migrates more slowly and is located between gene segments 9 and 10 (Matsui *et al.*, 1990). Although most Group A rotaviruses have either a short or a long pattern, super-short electropherotypes have been documented. These correlations between RNA patterns and serotypes have been maintained and have become a useful epidemiologic tool. Detailed descriptions of the correlations between electropherotype and viral antigenic and genetic properties have been published (Gentsch *et al.*, 1996; Matsui *et al.*, 1990; Desselberger *et al.*, 2001a; Nakagomi and Nakagomi, 2002; Santos and Hoshino, 2005). Although this relatively time-consuming method requires a trained technologist, the main advantage of PAGE is the lack of ambiguity in the results. The genome pattern obtained from a Group

A rotavirus can be readily distinguished from, for example, a Group C rotavirus genome pattern. The sample is positive for Group A rotavirus if 11 segment of dsRNA are visible and the pattern is similar to Group A rotavirus control RNA. Uncommon patterns can be tested against Group B and Group C rotavirus controls if necessary

2.11.7 RNA extraction methods

Several methods have been described for the release and/or extraction of rotavirus RNA from clinical specimens. The purpose of these methods is to:

- Disrupt infected cells and microorganisms, resulting in the release of nucleic acids
- Protect the nucleic acids from degradation
- Remove inhibitors of amplification
- Concentrate the target nucleic acid
- Recover the nucleic acid in an environment suitable for its use in the PCR

The complexity of the method chosen will depend on:

- Type and volume of specimen
- Concentration of target nucleic acid/volume of specimen
- Presence of PCR inhibitors that might interact with the target nucleic acid and/or the polymerase enzyme

The method should consist of the fewest steps possible to reduce the chance of contamination with exogenous DNA or RNA, or, in the case of RNA extraction, exogenous ribonucleases. Also, use of protocols with complex and multiple procedures increases the potential for loss of target nucleic acid. Methods involving crude lysis of microorganisms in specimens containing a high concentration of the target nucleic acid might be suitable for PCR, but additional nucleic acid extraction procedures are typically required to remove inhibitors and concentrate the target nucleic acid. Crude lysis might involve physical methods, such as freeze-thawing or sonication, or chemical methods using detergents, enzymes, or chaotropic agents. Some nucleic acid extraction methods rely on the differential solubilities of nucleic acids and proteins in phenol and water, as in phenol-chloroform extraction methods, or the ability of nucleic acids to bind to silica, as in silica/guanidinium isothiocyanate extraction methods. Concentration of extracted nucleic acid is accomplished by precipitation with ethanol. The pathogenesis and cellular tropisms of microorganisms determine the most appropriate sample and potential number of target

molecules of nucleic acid available. Therefore, the specimen and volume required, the methods of transport and storage, and the presence or absence of high concentrations of endogenous nucleic acids and/or PCR inhibitors should be determined for each specimen type to maximise the quantity and quality of target nucleic acid available for use in the PCR.

The original methods for extracting rotavirus dsRNA from faecal specimens were based on standard phenol-chloroform extraction and ethanol precipitation (Coulson and Kirkwood, 1991). However, when RT-PCR techniques were developed for rotavirus detection and genotyping, RNA prepared by phenol-chloroform extraction could not always be amplified by RT-PCR, even when a large amount of RNA could be identified by PAGE and silver staining (Gouvea *et al.*, 1990). This failure was attributed to inhibitors of the RT-PCR enzymes that were not removed by the extraction procedure.

Subsequently, a variety of methods were developed to reduce the amount of stool inhibitors in RNA extracts. These methods incorporated a step in which the extracted RNA was mixed with a substrate known to bind nucleic acid, such as CF11 cellulose, hydroxyapatite, or finely ground silica beads. Several variations of the silica method in which extracted RNA is bound to the glass beads in the presence of guanidinium isothiocyanate (GTC) of high molarity and subsequently eluted have become the most widely used manual method and the method of choice when the RNA will be analyzed by RT-PCR subsequently. The GTC/silica method not only reduces stool inhibitors of RT-PCR but also irreversibly inactivates the RNAse present in stool, likely resulting in more stable RNA preparations. In addition, the extracted RNA can be used in PAGE and staining techniques for direct visualization of rotavirus genome segments.

Because the manual method is so labor intensive, automated instruments are available for GTC/silica extraction. Several commercial kits based on a modified phenol- extraction procedure (acid-phenol method) followed by alcohol precipitation of RNA have also been shown to reduce the concentration of stool inhibitors, as indicated by efficient detection or genotyping of viral RNA.

2.11.8 RT-PCR

Rotaviruses in clinical specimens can be detected and 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 the genes encoding the VP7 (G-type) or VP4 (P-type). The objective is to obtain genotype-specific PCR products for analysis on an agarose gel or sequencing gel. RT-PCR of rotavirus dsRNA has three steps: 1) denaturation of dsRNA, 2) reverse transcription of dsRNA, and 3) amplification of cDNA. PCR consists of these steps: 1) heating the DNA to be amplified to separate the two template strands, 2) adding two primers that are complimentary to the region
to be amplified, 3) adding a heat-stable DNA polymerase enzyme that catalyses the extension of the primers using the DNA strand as template, and 4) repeating the cycle, with the newly synthesised cDNA heat-denatured and the enzymes extending the primers attached to the liberated single DNA strands.

2.11.8.1 Genotyping

Rotavirus genotyping methods are based on semi-nested RT-PCR, in which viral RNA extracted from faecal specimens is reverse-transcribed and amplified by PCR in the presence of consensus primers for the rotavirus genes specifying G (gene 9) or P (gene 4) serotype (Gouvea *et al.*, 1990; Das *et al.*, 1994; Gentsch *et al.*, 1992; Gunasena *et al.*, 1993; Iturriza-Gomara *et al.*, 2000b). The primers are selected to be homologous to strains from different serotypes, so that one primer pair can be used to amplify most rotavirus strains. The DNAs from the first amplification cycle are used as a template in a second PCR in the presence of one of the original consensus primers and a mixture of genotype-specific primers of opposite polarity from the consensus primer, each designed to yield a product of different size. The genotypes are then determined based on the size of the product after analysis by agarose gel electrophoresis.

In an alternative procedure, complementary DNA (cDNA) is made in the presence of random hexamers and then as a template for semi-nested PCR with the consensus and genotype-specific primers described above (Iturriza-Gomara *et al.*, 1999). Results from this method are comparable to those from procedures using specific priming during cDNA synthesis. In addition, because random hexamers prime from any RNA template in the faecal specimen, the cDNA and DNA generated can subsequently be amplified with specific primers for other enteric viruses, bacteria, and parasites.

As described in the protocols for G and P genotyping by RT-PCR, several sets of primers have been developed and used successfully. These include the original set for G genotypes

1-4, 8, and 9, a subsequent set for G types 1-4 and 9, the original P genotyping set for P[4], P[6], P[8], P[9], and P[10], and a second set for P[4], P[6], P[8], and P[9]. Each set has been shown to genotype large collections of rotavirus strains and has been used successfully with isolates from many regions of the world.

2.11.8.2 Issues in strain genotyping

Although rotavirus genotyping primers have been documented to work well and provide generally accurate results, several issues must be considered regarding their use.

2.11.8.3 Non-typeable strains resulting from genetic variation in common strains

Regardless of the primers used, a fraction of strains (i.e., a few percent to >50%) cannot be typed for P or G genotype or for both P and G genotypes. In several recent studies, genetic variations in the VP4 and VP7 genes of globally common rotavirus strains (e.g., P[8] and G1) have precluded amplification of these isolates with the original genotyping primers that are homologous to rotavirus strains isolated before 1990 (Iturriza-Gomara *et al.*, 2000b; Cunliffe *et al.*, 2001). This inability to genotype has been manifested by the detection of strains that produce a high yield of the consensus PCR product in the first-round PCR but do not yield a genotyping PCR product. Sequencing of the VP4 and/or VP7 genes of some of these non-typeable (NT) strains showed 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. New primers based on the variant sequence of the NT strains were designed at the same genome position as the original primer and subsequently used to genotype the remaining strains, suggesting that the variant strains belonged to the same P[8] or G1 genotype. These studies show the importance of considering genetic variation when using RT-PCR for strain genotyping. Investigators setting up genotyping methods using the originally published genotyping primers are likely to encounter NT strains and will need to develop a strategy (e.g., use of alternative primers) to reduce the number of NT strains detected. If the available primers do not work well for given specimen collections, genotyping primers might also need to be tailored to strains circulating in certain countries or regions. Investigators might need to apply molecular techniques such as nucleotide sequencing to characterise NT strains and redesign genotyping primers. Local laboratories might consider collaborating with

Regional laboratories to obtain sequence information on circulating rotavirus strains to facilitate modification of primers. All redesigned primers need to be tested against a variety of field isolates and standard strains bearing common genotypes to detect cross-reactivity. In addition, results obtained with new primer sets will need to be selectively confirmed. Finally, because new rotavirus variants might cocirculate with parental strains, the design of degenerate primers capable of binding to both strains should be considered.

2.11.8.4 Non-typeable strains resulting from the presence of novel strains

Although NT strains are often genetic variants of common genotypes that no longer bind to the original genotype-specific primer, further characterisation of NT strains, often by nucleotide sequencing, has demonstrated the presence of novel rotaviruses. Examples include the detection of types G5, G6, G10, G12, P[11], and P[14] among NT strains. The availability of sequence data permitted the design of a specific primer or primer pair for the novel strains that subsequently could be used in monoplex or multiplex PCR to genotype related strains. Although these novel strains have usually been detected at very low frequency, examples of high-incidence detection of such strains have also been reported (Desselberger *et al.*, 2001b; Santos and Hoshino, 2005).

2.11.8.5 Other reasons for an inability to type strains

Samples positive for rotavirus antigen might fail to yield any PCR products after amplification with G and P consensus primers and genotyping primers. Untypeable samples might be the result of a false-positive EIA, insufficient or degraded RNA, the presence of residual stool inhibitors in the RNA extract, the presence of novel strains, or technical problems with the assay itself. If untypeable samples represent a significant percentage of the analyzed strains, it is important to design a strategy to identify them. A possible first step might be to confirm the presence of rotavirus particles by EM or rotavirus antigen and RNA by one of several methods, including a repeat of the antigen EIA and subsequent PAGE analysis, or the use of a detection RT-PCR with consensus primers (Iturriza Go´mara *et al.*, 2002). If rotavirus is detected or RNA is present by PAGE, then a repetition of the RNA extract might be considered, followed by a repeat of the typing procedure. If RNA is absent by PAGE and/or RT-PCR, then the samples should be categorized as “RNA not detected” rather than NT. If these additional steps fail to

identify a sample with intact RNA, then characterisation of such strains might require testing a variety of primers to obtain products for sequencing or using advanced methods.

2.11.8.6 Confirmation of results

Although genotyping methods have been shown to be >90% accurate, misidentification by RT-PCR methods does occur (Gouvea *et al.*, 1990; Masendycz *et al.*, 1997). To ensure the accuracy of results, selective confirmation of genotype assignments should be carried out. Although several confirmation methods have been described (e.g., Southern hybridization with cDNA and oligonucleotide probes or serotyping methods), sequence analysis has become the standard for both confirmation and identification of NT strains. For confirmation, sequencing can be performed either on the genotype specific products or on a fragment of the VP7 or VP4 gene after amplification. The advantage of sequencing the genotype-specific PCR products is the ability to confirm infections by purifying and sequencing different sized products isolated from an agarose gel. For the VP7 gene, a variety of consensus primer-pairs have been described including beg9/end9 and VP7-F/VP7-R and degenerate versions, 9con1/9con2, and 9con1-L/VP7-R deg. Consensus primers for VP4 gene fragments include con2/ con3, HumCom5/HumCom3 and VP4-F/VP4-R (Gunasena *et al.*, 1993). After sequencing, the strain genotype can be determined by comparing the genes of strains with known VP4 or VP7 types from the GenBank database (Green *et al.*, 1988).

In some cases, the PCR products might need to be cloned before sequence analysis. An advantage of cloning is that only a small amount of PCR product is required, thereby enabling sequencing of strains even when the concentration of product is too low for direct sequencing.

2.11.9 Other characterisation methods

Some strains might require additional characterisation techniques. An unusual level of stool inhibitors or a low level of intact virus in some samples might make it difficult to identify strain genotypes by RT-PCR or sequencing, and the samples might not be typeable by EIA. The presence of novel strains might also preclude characterisation by routine methods.

Additional characterisation techniques include cultivation in cell culture to amplify the amount of virus present and dilute out stool inhibitors, followed by repetition of routine

methods or sequencing. If the sequences of the VP4 and/or VP7 genes suggest a novel serotype, it might be necessary to prepare hyperimmune sera to the strains and conduct cross-neutralization tests to determine if the strains are antigenically distinct from known rotavirus serotypes. Such studies have been used in the past to define new rotavirus serotypes (Hoshino and Kapikian, 2000). If the genotype of the strain is novel or shows relationships to animal strains, additional studies can be carried out to define the potential origin of the strain. These studies might include sequencing additional genes, with comparison to human and animal rotaviruses, or conducting whole-genome hybridization studies to define the relationships to common rotaviruses of animals and humans. These types of studies have helped demonstrate that some rare human rotaviruses arose through interspecies transmission of an animal rotavirus to humans. Such studies also suggest that some strains, both common and uncommon, probably arose through reassortment between human and animal rotaviruses (Nakagomi and Nakagomi, 2002; Palombo, 2003). Thus, the analysis of untypeable rotavirus strains from surveillance studies has been important in defining the genetic diversity and possible origin of many rotaviruses.

2.12 MANAGEMENT PROCEDURES

In humans, besides oral rehydration solutions (ORS), which are highly efficacious in the treatment of rotavirus disease, encephalinase inhibitors, for example, racecadotril, or serotonin receptor antagonists, such as Granisetron, are showing benefit for treatment (Estes and Desselberger, 2012). At present in poultry, no specific treatment or means of control exists. The effect of diarrhoea on the litter can be minimized by increasing ventilation rate and temperature and by adding fresh litter. Where litter is reused several times, infection will build up, and problems are likely to be more severe than in situations in which houses are cleaned and fumigated and fresh litter is used for each batch of birds. If severe problems arise, remove litter and thoroughly clean the house and equipment and fumigate with formaldehyde before restocking with a new flock.

2.12.1 Vaccination

The clinical significance of rotavirus disease, resulting in significant morbidity in the US in the pre-vaccination era, and in almost half a million deaths worldwide, mainly in subSaharan Africa and SE Asia, motivated efforts to develop a human vaccine. Vaccine development faced multiple hurdles. The first live, attenuated vaccine (Rota- ShieldTM,

quadrivalent), licensed in the USA in 1998, was epidemiologically linked with gut intussusception (IS) resulting in discontinuation of vaccine production. In 2006, two other live, attenuated RV vaccines, the monovalent Rotarix™ vaccine (called RV1) and the pentavalent human-bovine reassortant RotaTeq™ (termed RV5), were found to be efficacious in large phase III clinical trials with no apparent risk of gut IS (Ruiz-Palacios *et al.*, 2006; Vesikari *et al.*, 2006). Both vaccines provide considerable protection from heterotypic infection associated with disease. As a consequence, the isolation of rotaviruses and admission to hospital for rotavirus associated acute gastroenteritis has significantly decreased in developed countries, and in some settings rotavirus vaccination has also decreased the mortality from rotavirus disease.

In poultry commercially available vaccines have not yet been developed. Given the extent of antigenic diversity that exists in avian rotaviruses and the difficulty in growing non-group A rotaviruses in cell culture, obvious problems exist in vaccine development. Preliminary work on experimental group A rotavirus vaccines in turkeys (Shawky *et al.*, 1994) and pheasants (Gough *et al.*, 1999) indicates that inactivated vaccines administered to the breeders are unlikely to protect the progeny against challenge for more than the first week of life, unless much higher titers of anti-rotavirus antibody can be produced.

CHAPTER THREE

3.0 PRIMER DESIGN FOR DETECTION OF AVIAN ROTAVIRUS

3.1 INTRODUCTION

A primer is a strand of nucleic acid that serves as a starting point for DNA synthesis. It is required for DNA replication because the enzymes that catalyze this process, DNA polymerases, can only add new nucleotides to an existing strand of DNA. The polymerase starts replication at the 3'-end of the primer and copies the opposite strand (http://en.m.wikipedia.org/wiki/primer_(molecular_biology) (2014). The use of molecular methods for rotavirus characterisation provides increased sensitivity for typing, and allows the identification of putative reassortant strains. However, due to the constant accumulation of point mutations through genetic drift; and to the emergence of novel genotypes; and possibly zoonotic transmission and subsequent reassortment, the reagents and methods used for genotyping require close monitoring and observation.

Primer design is aimed at obtaining a balance between two goals: specificity and efficiency of amplification. *Specificity* is defined as the frequency with which a mispriming event occurs. Primers with mediocre to poor specificity tend to produce PCR products with extra unrelated and undesirable amplicons as visualized on an ethidium bromide-stained agarose gel. *Efficiency* is defined as how close a primer pair is able to amplify a product to the theoretical optimum of a two fold increase of product for each PCR cycle. Given a target DNA sequence, analysis software attempts to strike a balance between these two goals by using preselected default values for each of the primer design variables (Dieffenbach *et al.*, 1993). The specificity is generally controlled by the length of the primer and the annealing temperature of the PCR reaction. Oligonucleotides between 18 and 24 bases tend to be very sequence specific if the annealing temperature of the PCR reaction is set within a few degrees of the primer melting temperature (Tm). These types of oligonucleotides work very well for standard PCR of defined targets that do not have any sequence variation. The longer the primer, the smaller the fraction of primed templates there will be in the annealing step of the amplification. In exponential

amplification, even a small inefficiency at each annealing step will propagate to produce a significant decrease in amplified product. Short oligonucleotides of 15 bases or less are useful only for a limited amount of PCR protocols such as the use of arbitrary or random short primers in mapping simple genomes (Williams, 1990; Liang and Pardee, 1992). Depending on the genome size of the organism, there is a minimum primer length. In general, it is best to build in a margin of specificity for safety. For each additional nucleotide, a primer becomes four times more specific; thus, the minimum primer length used in most applications is 18 nucleotides. Clearly, if purified cDNA is being used, or genomic DNA is not present, the length could be reduced because the risk of nonspecific primer/template interactions will be greatly reduced. Yet, it is generally a good idea to design primers such that the synthesized oligonucleotides can be used in a variety of experimental conditions, and the small marginal cost of oligonucleotides with four to five additional bases makes it worth the expense. The upper limit on primer length is somewhat less critical and has more to do with reaction efficiency. For entropic reasons, the shorter the primer, the more quickly it will anneal to target DNA and form a stable double-stranded template to which DNA polymerase can bind. In general, oligonucleotide primers 28-35 bases long are necessary when amplifying sequences where a degree of heterogeneity is expected. This has proved to be generally useful in two types of applications: (1) Amplifying sequences encoding closely related molecules, such as isoforms of a protein or family of proteins within a species, as well as in the cloning of the homologous gene from a different species, (Dveksler *et al.*, 1993) and (2) in amplifying the sequences of viruses such as HIV-1, where the possibility of having a set of primers with perfect complementary to all the templates is not expected (Mack and Sninsky, 1988; Ou *et al.*, 1988). In both cases, one first uses primer design software to compare all available related sequences and, in such a manner, determines the DNA region with the least amount of sequence variability. These regions serve as starting places for selecting the primers. With the amino acid sequence information and the help of codon usage tables for different species, both primers, or at least one of them, could be designed around the "conserved sequence." The placement of the 3' end of the primer is critical for a successful PCR reaction. If a conserved amino acid can be defined, the first 2 bases of the codon, or 3 bases in the case of an amino acid encoded by a single codon (methionine and tryptophan),

can serve as the 3' end. Perfect base-pairing between the 3' end of the primer and the template is optimal for obtaining good results; minimal mismatch should exist within the last 5 to 6 nucleotides at the 3' end of the primer. Attempts to compensate for the mismatches between the 3' end of the primer and the template by lowering the annealing temperature of the reactions do not improve the results, and failure of the reaction is almost guaranteed.

With this concept in mind, one should evaluate all possible strategies in the design of primers when the nucleotide sequence of the template to be amplified is not known with certainty. Cases like the one described above are routinely encountered when the researcher wishes to amplify a cDNA using information from a partial protein sequence (Dveksler *et al.*, 1993). Several approaches that include the use of degenerate oligonucleotide primers covering all possible combinations for the bases at the 3' end of the primer in the pool, as well as the use of inosine to replace the base corresponding to the third or variable position of certain amino acid condons, have been successful for cDNA cloning and for detection of sequences with possible variations (Lin and Brown, 1992). For primers shorter than 20 bases, an estimate of Tm can be calculated as T m = 4 (G + C) + 2 (A + T), (Suggs *et al.*, 1981) whereas for longer primers the Tm requires the nearest neighbor calculation, which takes into account thermodynamic parameters and is employed by most of the available computer programs for the design of PCR primers (Breslauer *et al.*, 1986; Freier *et al.*, 1986).

Polymerase chain reaction primers should maintain a reasonable GC content. Oligonucleotides 20 bases long with a 50% G + C content generally have Tm values in the range of 56-62. This provides a sufficient thermal window for efficient annealing. Within a primer pair, the GC content and Tm should be well matched. Poorly matched primer pairs can be less efficient and specific because loss of specificity arises with a lower Tm and the primer with the higher Tm has a greater chance of mispriming under these conditions. If too high a temperature is used, the primer of the pair with the lower Tm may not function at all. This matching of GC content and Tm is critical when selecting a new pair of primers from a list of already synthesized oligonucleotides within a sequence of interest for a new application. The ideal primer generally has the following characteristics:

i. Melting temperature (Tm) between 55 and 65°C (usually corresponds to 45-55% G+C for a 20-mer).

ii. Absence of dimerization capability.

iii. Absence of significant hairpin formation (usually >3 bp).

iv. Lack of secondary priming sites in the template.

v. Low specific binding at the 3' end, to avoid mispriming.

Care also has to be taken that the primers are not complementary to each other, particularly at their 3' ends. Complementarity between primers leads to the undesirable primer-dimer phenomena in which the PCR product obtained is the result of the amplification of the primers themselves. This sets up a competitive PCR situation between the primer-dimer product and the native template and is detrimental to the success of the amplification. In cases when multiple primer pairs are added in the same reaction (multiplex PCR), it is very important to check for possible complementarity of all the primers added in the reaction. (Chou *et al.*, 1992). Bearing this in mind, primers were designed to detect avian rotavirus in faeces.

3.1.1 JUSTIFICATION

The two outer layer surface proteins of rotavirus, VP4 and VP7, are used to classify the virus into P and G types, respectively. Both proteins are targets of neutralising antibody responses, and protection from infection is thought to be at least in part type specific. Genotyping of rotavirus by RT-PCR has revealed significant strain diversity and provided information on virus strains circulating throughout the world (Simmonds *et al.*, 2008). Successful surveillance of rotaviruses requires that genotyping methods be updated regularly in order to detect the emergence of novel G and/or P types through zoonotic introduction of “unusual types”, for which typing reagents may not be available, and because of genetic drift, which can cause nucleotide changes at the primer binding sites that result in typing failures (Iturriza-G´omara *et al.*, 2003). High incidences of infection with unusual types which may represent zoonotic introductions has often been reported in different parts of the world: G5 in Brazil, G8 and P[6] in Africa, G10 and P[11] in India (Adah *et al.*, 2001; Cunliffe *et al.*, 1999; Das *et al.*, 1993, 1994; Esona *et al.*, 2004; Gouvea *et al.*, 1994; Iturriza-G´omara *et al.*, 2004b; Santos *et al.*, 1998). During infection of a single cell with two different virus strains, reassortment of genes can occur, as the

genes encoding VP4 and VP7 segregate independently (Iturriza-G´omara *et al.*, 2001). Mixed infections are observed frequently and give rise to a variety of G- and P-type combinations (Iturriza-G´omara *et al.*, 2001; Fischer *et al.*, 2005). Due to the variability and reassortment nature of rotaviruses, the present study was thus carried out to design new pairs of oligonucleotide primers, in order to increase the chances of detecting avian rotavirus in faeces and also to increase the specificity of detection. This would aid in the elimination of false negative results in the detection of different segments of group A avian rotavirus.

3.1.2 AIM

To design and evaluate new oligonucleotide primer pairs that provide increased sensitivity and specificity for detecting avian rotavirus.

3.1.3 OBJECTIVES

3.1.3.1 General objective

i. To design primers to detect the VP4, VP6 and VP7 segments of avian rotavirus.

3.2 MATERIALS AND METHODS

3.2.1 Primer design

A new VP4, VP6 and VP7 gene-specific primer pairs were designed by analyzing different rotavirus strains isolated during the last decade by downloading them from the GenBank. The alignments were generated using clustal analysis from the BioEdit program (Hall, 1999). Overlapping sequences were selected in other to be able to sequence the complete genome of each segment. Degenerate nucleotides were included because of the reassortant nature of rotavirus. The consensus sequences were aligned using the BioEdit program and then treated with the Fast PCR software (Kalendar *et al.*, 2009) to derive the primers. The derived primer sequences were submitted for a BLAST search in order to ensure alignment was exclusive to the desired target genes which include the VP4, VP6 and VP7. The binding position, sequences and product size of the forward and reverse primers are as shown in table 3.

3.2.1.1 Forward primer

A region of interest consisting of ~18 to 25 nucleotides (nt) with the following characteristics was located on nucleotide sequence of the segment to be detected.

- 5' : rich in GC (for primer annealing stabilisation)
- 3' : more AT (increase specificity, if primer sequence is not perfectly identical, less chance that this region will bind to a template and the polymerase will not amplify something else).

Different primers were tested by deleting some nt at the 5' end or some nt at the 3' end. A "Primer list test" was then carried out with the different primers combinations. The best primer was then identified with the following:

- GC% : between 40-60%
- Tm : between 55-60°C
- No dimer
- No repeated motif (example GGGGGTTAAAA)
- No woobled nt

3.2.1.2 Reverse Primer

Based on the sequence, a 5' region rich in AT and 3' region rich in GC was chosen (this will give a 5' GC rich region and 3' AT rich region when reverse transcripted as shown in figure 3.

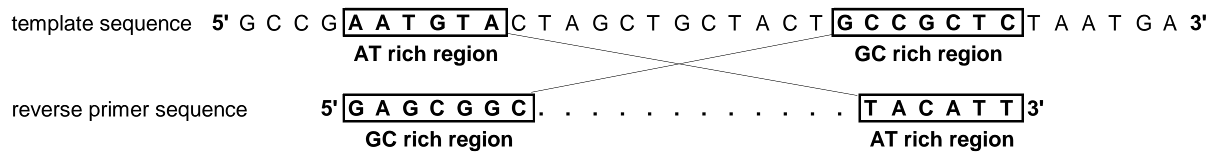


Figure 3: Representation of reverse transcription of reverse primer.

The primer sequence was then entered in fast PCR software for further analysis.

Table 3: Primers used for the detection of segments 4 (VP4), 6 (VP6), 7 (VP7) and 10 (NSP4) of Avian group A rotaviruses.

Name	Gene	Binding position	Sequence	Product size
[a]Rota-seg4-s	VP4	1-21	5'-GGCTATAAAATGGCTTCTCTC-3'	
[a]Rotaseg4-686R	VP4	666-686	5'-AATGGTCTRCCTCCAATWCA-3'	685bp
[c]Seg4-540f	VP4	539-557	5'-GAACAGTGCCAAATGTRCAA-3'	
[c]seg4-1309r	VP4	1291-1308	5'-CGAATGATGGCTCACCAA-3'	769bp
[c]Seg4-1215f	VP4	1212-1231	5'-CTCCGACGCTGTAACATTAT-3'	
[c]Seg4-1786r	VP4	1763-1786	5'-TGTTGCCGTATCTATTAAGTCATT-3'	574bp
[c]Seg4-1704f	VP4	1704-1722	5'-CAGACGCAGCTACTTCAAT-3'	
[c]Seg4-2215r	VP4	2196-2214	5'-CGCTTGCTCTCCAGTTATT-3'	510bp
[c]vp6-begf	VP6	11-29	5'-CGAAGTCTTCATCATGGAT-3'	
[c]vp6-470r	VP6	450-470	5'-RCCTGTRCGTTGYCTTCTGTT-3'	441bp
[a]Rota-Seg6-s	VP6	317–338	5′-GTAATGGAATWGCDCCNCAATC-3′	
[a]Rota-Seg6-as	VP6	1072–1094	5′-ATACCTGSWGGAAAWACTGGTCC-3′	777bp
[c]seg6-953f	VP6	953-974	5'-GCAGCACCATTTCCWAATCAT-3'	
[c]seg6end	VP6	1330-1349	5'-GGTCACATCCTCTCACTATA-3'	375bp
[c]RotaVP7-135F	VP7	135-154	5'-GAATGGCTAGCTCATATGTT-3'	
[c]RotaVP7-647R	VP7	642-664	5'-TATGCCCAYTAAATACTCAGACA-3'	529bp
[c]RotaVP7-587F	VP7	587-606	5'-CAGACYTCAGAGGCTAATAA-3'	
[c]RotaVP7-1003R	VP7	985-1003	5'-TATGTCTAGAAGATCGCGC-3'	416bp
[b]NSP4 F30	NSP4	30-49	5'-GTGCGGAAAGATGGAGAAC-3'	
[b]NSP4 R660	NSP4	640-660	5'-GTTGGGGTACCAGGGATTAA-3'	630bp

[a]Schuman *et al.*, 2009, [b]Pantin-Jackwood *et al.*, 2007, [c]Own design

D, R, S, W, Y= Degenerate nucleotides

3.3 RESULTS

3.3.1 NSP4 PRIMERS

Figure 4 shows the agarose gel pictures for the detection of avian rotavirus using the NSP4 primers. From these figure, it is seen that unspecific bands were prominent, in most cases only the positive control was specific. Figure 5 represents purified PCR products.

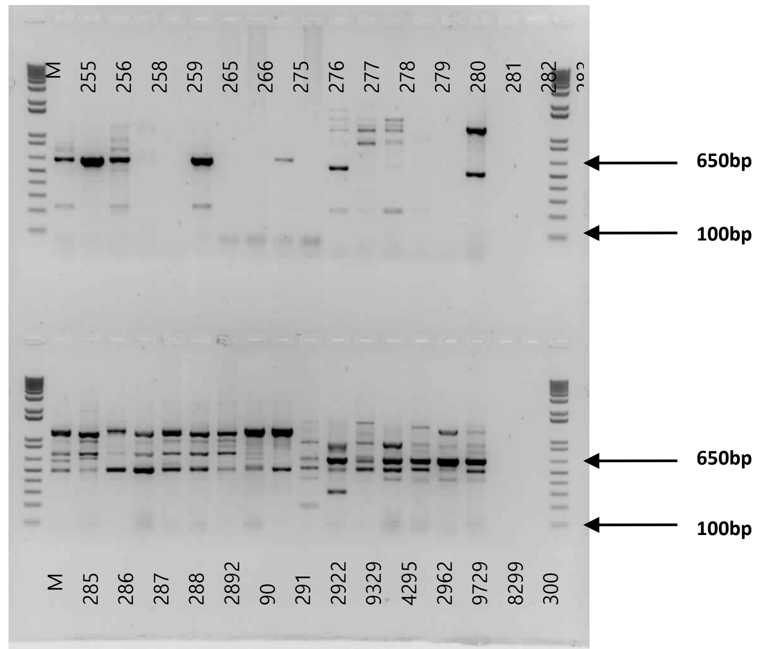


Figure 4: Detection of genome segment encoding NSP4 of chicken rotavirus (samples 255-300).

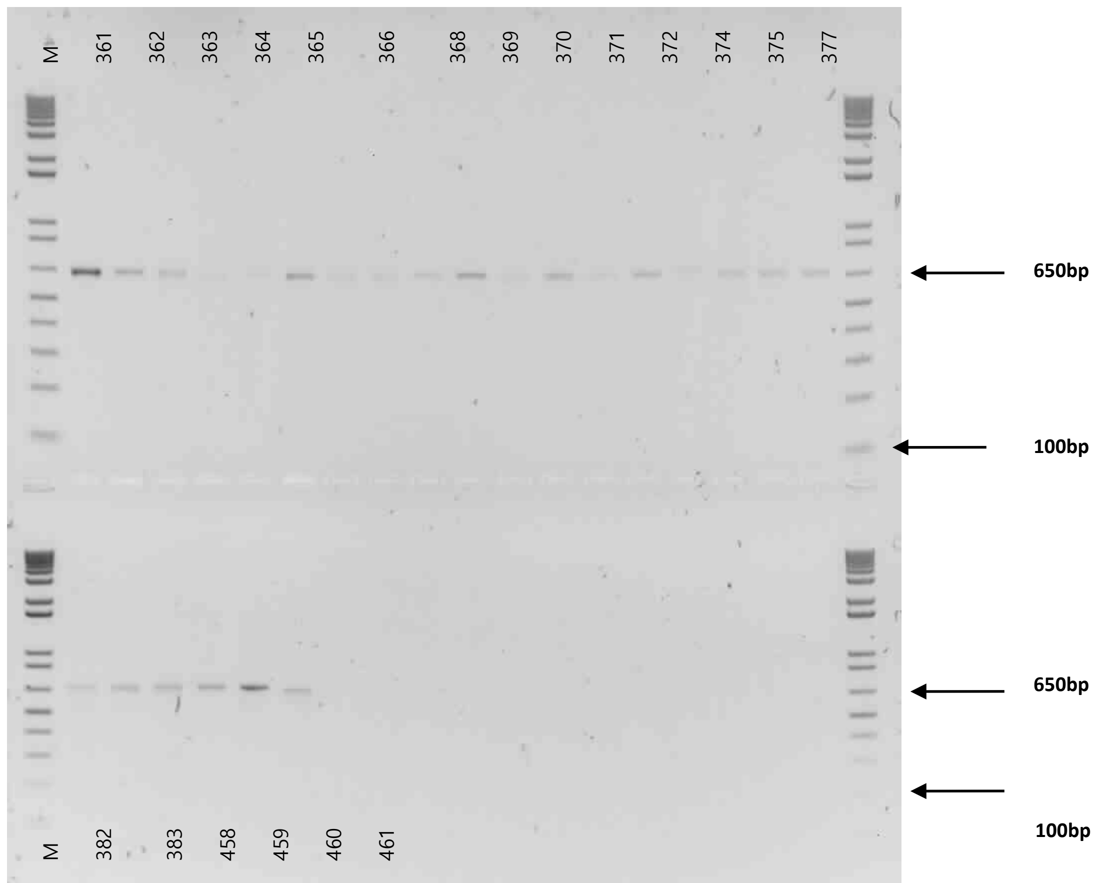


Figure 5: Agarose gel electrophoresis for confirmation of DNA purity by gel extraction method

3.3.2 VP4 PRIMERS

Figures 6 show the agarose gel pictures for the detection of avian rotavirus using the VP4 primers. Specific bands at the appropriate base pairs for all the positive controls used in this study are as shown on figure 6. However, for the detection of the virus in field samples (figure 7) none specific (Seg4s + Seg4-686r; Seg4-540f + Seg4-1309r) bands were detected using our designed primers. Some of the primers (Seg4-1704f + Seg4-2215r, Seg4-1215f + Seg4-1786r) were also incapable of detecting some part of the segments.

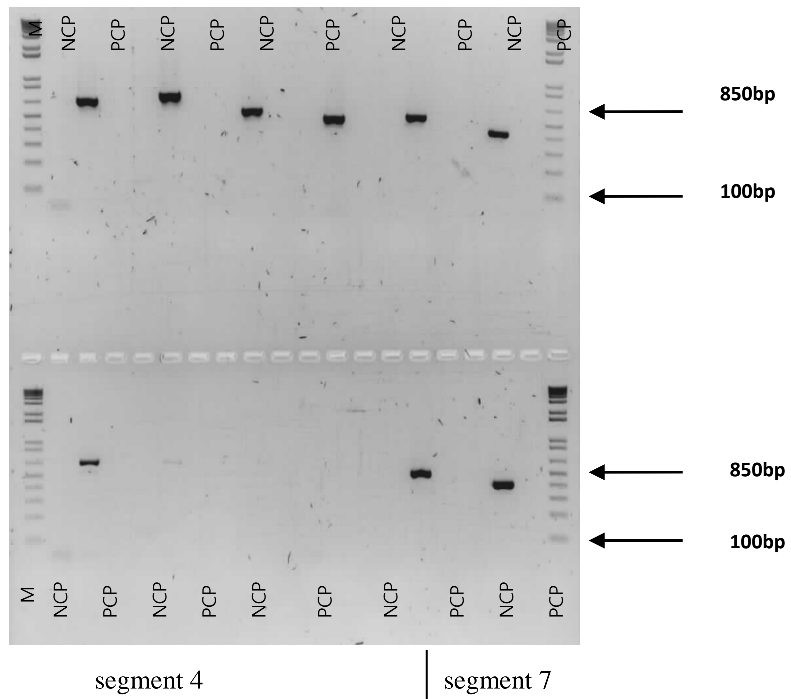


Figure 6: Rotavirus A (upper wells) and D (lower wells) detection test for segment 4 and segment 7

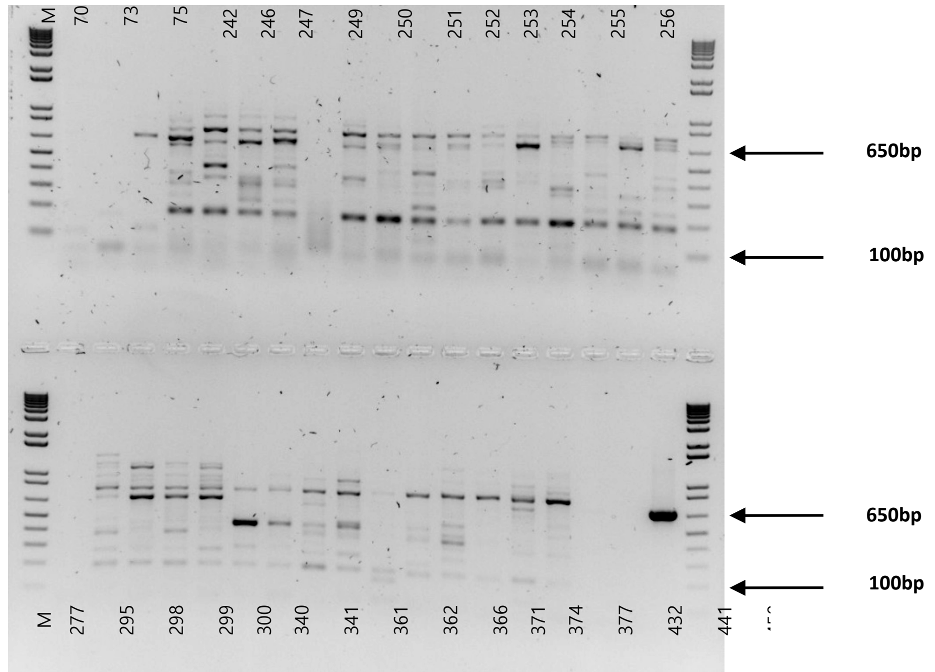


Figure 7: Detection of genome segment encoding VP4 of chicken rotavirus (samples 70-450) using Seg4s+seg4-686r primers at 686bp. PCR products were separated on Sybr safe stained 1.5% agarose gels.

3.3.3 VP6 PRIMERS

Figure 8 shows specific bands for the positive controls using the primers (Rota-Seg6-s + Rota-Seg6-as) designed by Schumann *et al.*, 2009 at two different temperature gradients. However, bands seen on figure 9 which represents field samples were not specific using these primers. So two primer pairs were designed for VP6 segment in this study, the VP6-begf + VP6-470r and VP6-953f + VP6-endr. The two pairs were more sensitive and specific in detecting the VP6 segment with prominent bands at 459bp and 416bp respectively. Some samples which were also negative with the primers designed by Schumann *et al.* (2009) were seen to be positive with our designed primers (figure 10).

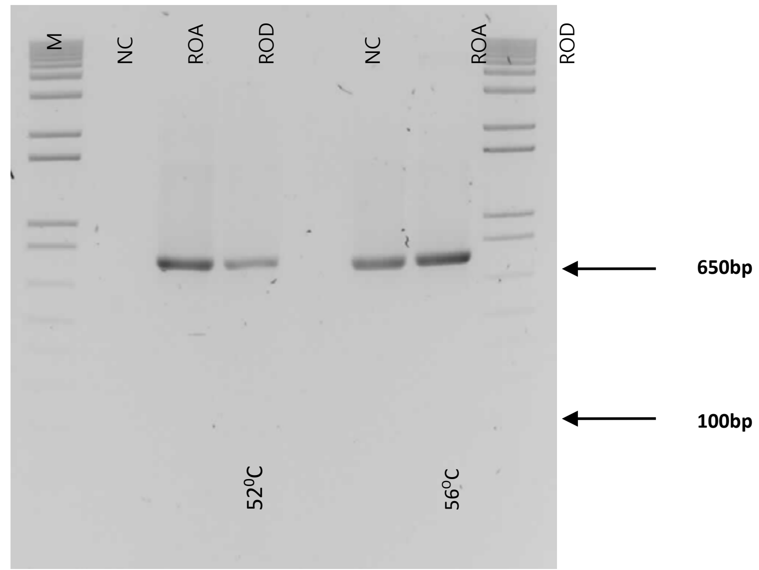


Figure 8: Detection of genome segment encoding VP6 of chicken rotavirus using positive controls for Rotavirus A and D at 2 different temperature gradients (52°C and 56°C). PCR products were separated on Sybr safe stained 1.5% agarose gels.

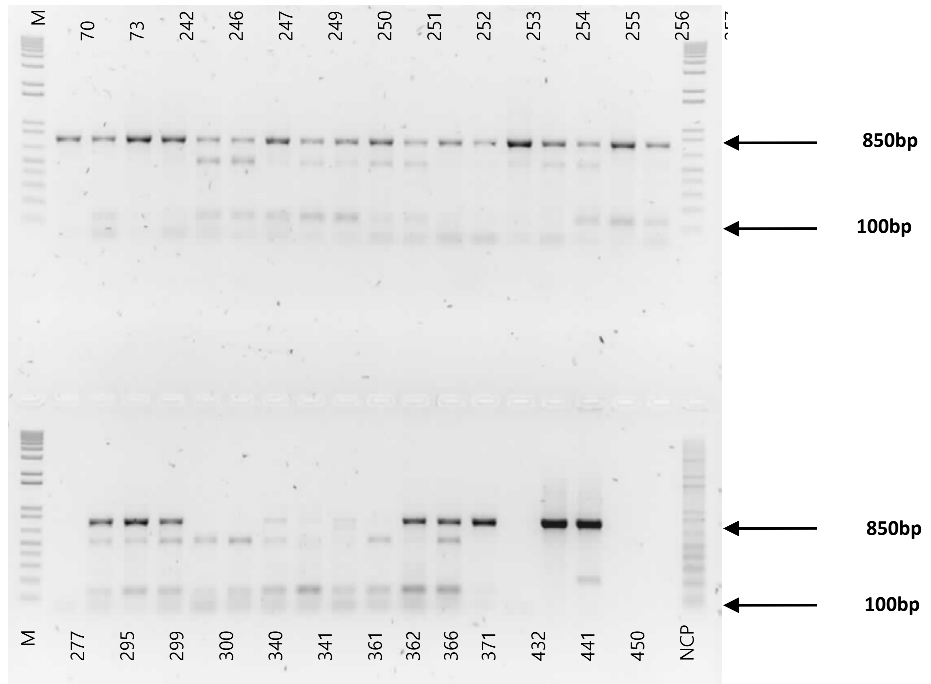


Figure 9: Detection of genome segment encoding VP6 of chicken rotavirus (samples 70-450) using Rota-Seg6-s + Rota-Seg6-as at 777bp.

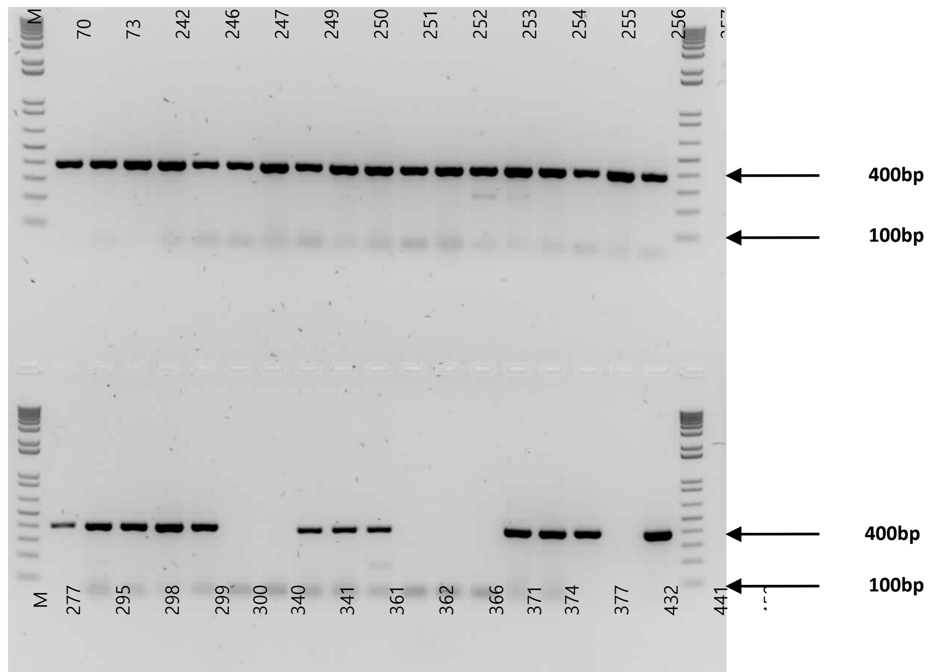


Figure 10: Detection of genome segment encoding VP6 of chicken rotavirus (samples 70-450) using VP6-953f+VP6-endR primers (own design) at 416bp. PCR products were separated on Sybr safe stained 1.5% agarose gels.

3.3.4 VP7 PRIMERS

Figures 11 and 12 show the agarose gel pictures for the detection of avian rotavirus using our own designed VP7 primers. The two primer pairs (VP7-135f + VP7-647r and VP7-587f + VP7-1003r) were sensitive and specific for the detection of the VP7 segment with prominent bands at 512bp and 416bp (figures 11 and 12) respectively. The primers also detected rotavirus samples which were previously presumed to be negative using the VP6 primers designed by Schumann *et al.* (2009). The new primer pairs allowed typing of rotavirus strains and provided increased sensitivity.

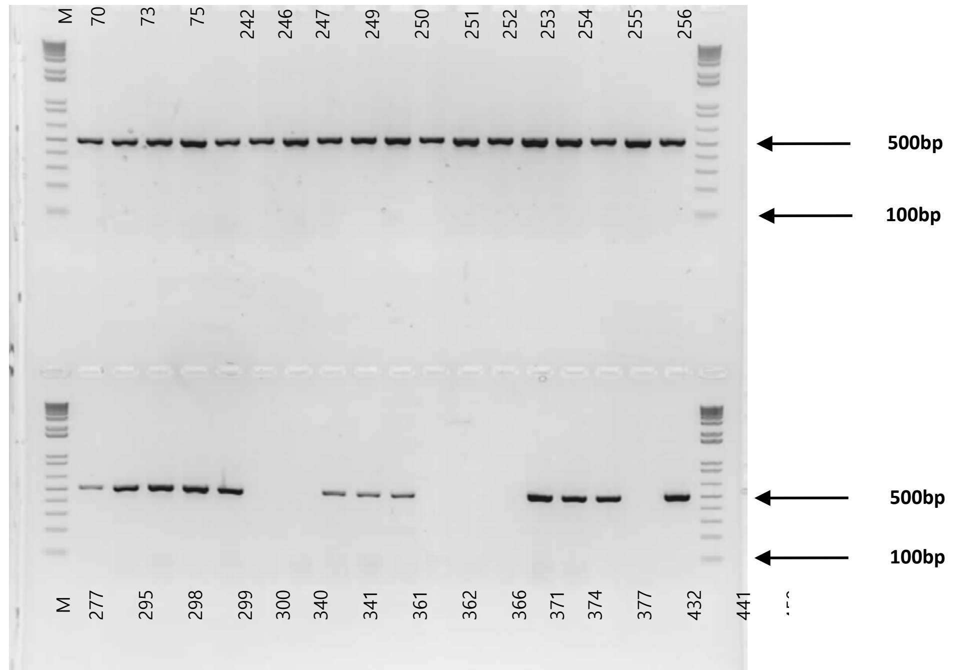


Figure 11: Detection of genome segment encoding VP7 of chicken rotavirus (samples 70-450) using VP7-135f+VP7-647r primers at 512bp. PCR products were separated on Sybr safe stained 1.5% agarose gels.

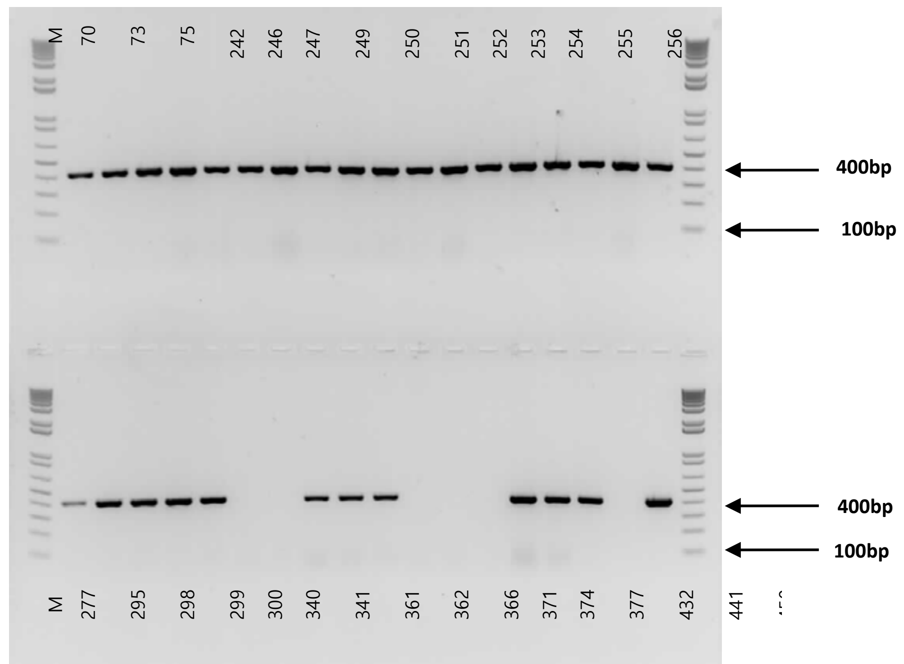


Figure 12: Detection of genome segment encoding VP7 of chicken rotavirus (samples 70-450) using VP7-587f+VP7-1003r primers at 416bp. PCR products were separated on Sybr safe stained 1.5% agarose gels.

3.4 DISCUSSION

In this study, the initial detection of rotavirus in faeces was carried out with NSP4 primers designed by Pantin-Jackwood *et al.*, 2007. Results from the use of these primers showed bands which were non-specific. Just a few bands were specific using the NSP4 primers. Also, in some breed of birds, rotavirus was not detected when primers NSP4 and VP6 designed by Pantin-Jackwood *et al.* (2007) and Schumann *et al.* (2009) respectively were used. These samples turned out to be positive when rescreened with primers designed for the present study. The reason for this is probably due to the ability of rotaviruses to undergo reassortment, thereby bringing about changes in some nucleotides in its genome sequence. Rotaviruses evolve mainly through two mechanisms: (a) genetic shift, which can lead to the acquisition of novel or unusual gene combinations; and (b) genetic drift, which leads to the accumulation of point mutations, that can result in antigenic drift. Drift has often been associated with genotyping and serotyping failures (Adah *et al.*, 1997; Iturriza-G´omara *et al.*, 2000a, 2004a; Banyai *et al.*, 2003; Cunliffe *et al.*, 2001; Santos *et al.*, 2003; Martella *et al.*, 2004;). Successful rotavirus molecular surveillance therefore requires regular revision and updating (Iturriza-G´omara *et al.*, 2003, 2004a). For this reason, primers were designed in other to increase the chances of detecting rotavirus in avian faeces.

The non-specificity could bring about false positive results and also a waste of reagents. Avoidance of such situations contributed to designing primers which would be specific for rotaviruses. Results determined from the use of these primers showed more precise and specific bands. In addition some breeds of birds which tested negative initially were also determined to be positive. Also, some samples which were previously negative were rescreened with the newly designed primers and were seen to be positive. The increased sensitivity of the new primer pairs may be due to complementarity to a wider diversity of rotavirus strains. It is probable that the NSP4 and VP6 primer pairs were designed from sequences derived from a limited number of cell-culture adapted rotavirus strains available in public databases at the time of the study (Gentsch *et al.*, 1992) or viruses detected in the present study are reassortants. The new primer pairs were designed by analysis derived from a wider strain of different types of rotavirus strains isolated in the last decade. However, the difference in sensitivity could also be due to increased efficiency of the PCR

reaction. The use of degenerate nucleotides to represent reassorted nucleotides also increased the sensitivity of the designed primers and thus aided the detection of rotavirus. In a study carried out by Iturriza- G´omara *et al.* (2000a) in the UK, they reported 3% of the strains tested failed to P-type using the widely published methods. However, new VP4 consensus primer pair VP4F/VP4R which they later designed amplified the target sequence from rotavirus isolates that were previously not P-typed using Con2/Con3 consensus primers (Gentsch *et al.*, 1992). This was due to reassortment of the viruses. In addition, the new primer typed 63.7% of previously untypeable strains. A significantly higher percentage of previously untypeable strains in the UK samples were thus successfully P-typed. Similar result was obtained in this present study. It is likely that mutations were responsible for the failure to type the isolates, similar to previously reported findings (Adah *et al.*, 1997; Iturriza-G´omara *et al.*, 2000c, 2004a; Martella *et al.*, 2004). Monitoring of typing failures is therefore important in order to adapt the typing methods as necessary.

This study highlights the importance of a constant reconsideration of primer sequences employed for the molecular typing of rotaviruses. The new primers designed for this study proved successful in identifying currently circulating virus strains some of which could have gone undetected. These primers are now readily available and reliable for further studies. It is also recommended that specific primers be designed for the detection of other groups of avian rotavirus.

CHAPTER FOUR

4.0 GENETIC DETECTION OF ROTAVIRUS IN POULTRY FLOCKS AND SOME OTHER AVIAN SPECIES

4.1 INTRODUCTION

Avian rotaviruses represent a diverse group of intestinal viruses, which are suspected as the cause of several diseases in poultry with symptoms of diarrhoea, growth retardation or runting and stunting syndrome. Viral enteric diseases in poultry, especially in turkeys and chickens, have an important economic impact because of production losses due to poor weight gain. The most important viruses associated with poult enteritis are adenoviruses, astroviruses, turkey coronavirus, entero-virus-like viruses, reovirus, rotavirus (RV) and turkey torovirus (Reynolds *et al.*, 1987b; Fitzgerald, 2008; Jones, 2008; Reynolds and Schultz-Cherry, 2008; Saif, 2008). Rotaviruses have a worldwide distribution in a wide variety of avian species. However, in Nigeria, to the best of our knowledge, no data about avian rotavirus (ARV) and types are available. There are however information on human (Aminu *et al.*, 2008), porcine and bovine (Ojeh *et al.*, 1995, Adah *et al.*, 2001) rotavirus in Nigeria. Numerous surveys of virus incidence have shown that poultry flocks are frequently infected with RV (McNulty *et al.*, 1978, 1979, 1984a,b; Saif *et al.*, 1985; Bellinzoni *et al.*, 1987; Reynolds *et al.*, 1987b,c; Minamoto *et al.*, 1988; Alfieri *et al.*, 1989; Takase *et al.*, 1990; Gough *et al.*, 1992; Legrottaglie *et al.*, 1997; Otto *et al.*, 2006; Pantin-Jackwood *et al.*, 2007). Most of the RV strains detected in humans and animals belong to group A. Accordingly, a high percentage of avian RVs belong to group A, but group D strains have also been frequently detected in avian species. Furthermore, ARVs of group F and group G have been occasionally found.

Electron microscopy, PAGE and enzyme- linked immunosorbent assays (ELISA) are commonly used to detect ARVs (McNulty *et al.*, 1979; Theil *et al.*, 1986; Todd and McNulty, 1986; Bellinzoni *et al.*, 1987; Legrottaglie *et al.*, 1997; Elschner *et al.*, 2002; Otto *et al.*, 2006). More recently however, RT-PCR protocols have been employed (Day *et al.*, 2007; Pantin-Jackwood *et al.*, 2007) but until now the examination of poultry samples by the latter has been limited to detection of ARVs of group A, as sequence data of other

ARV groups were lacking. Due to the availability of the genomic sequences of groups A and D ARV strains (Otto *et al.*, 2006; Schumann *et al.*, 2009; Trojnar *et al.*, 2009, 2010), PCR- based methods could be developed for detection of both virus groups. In detecting RVs, PAGE is almost as sensitive as electron microscopy and enables identification of non-ARV-A or–D strains (Otto *et al.*, 2006; Johne *et al.*, 2011). RT-PCR is however, more sensitive than PAGE and EM. Electron microscopy (EM) can be used to simultaneously detect and identify most enteric viral agents from a single stool specimen. However, the method is labor intensive and is relatively insensitive, requiring up to 106 intact viral particles per ml stool (Richards *et al.*, 2003). Commercially available antigen detection kits, such as enzyme immunoassay (EIA) and latex agglutination (LA) tests, are often the method of choice for the diagnosis of adenovirus and rotavirus infection in stool samples. These kits are easy to use and highly specific; however, due to the levels of strain diversity among circulating gastrointestinal viruses, the estimated sensitivity for many antigen detection kits is low. Various molecular techniques have been exploited for the development of highly sensitive and rapid assays for the detection of causative agents of viral gastroenteritis (O'Neill *et al.*, 2002). RT-PCR has reportedly increased the detection rate of rotavirus A by up to 48% compared to EIA or electron microscopy (Gunson *et al.*, 2003). The screening of faecal samples for rotavirus in this study using highly sensitive molecular methods would provide an indication of the true prevalence of rotavirus on some farms in south western Nigeria.

4.1.1 JUSTIFICATION

Rotaviruses have been identified as one of the main etiological agents of diarrhoea and enteritis in mammals, including humans, and in avian species. In poultry, both in layer hens and broilers, rotavirus has already been established as the etiological agent of enteritis, originated from viral replication in intestinal epithelium, resulting in diarrhoea and nutrient malabsorption which causes an increase in feed conversion ratio and large economic losses to poultry industry (Pantin-Jackwood *et al.*, 2008). This virus has been isolated from a wide variety of avian species around the world, including turkeys, chickens, and pheasants (Estes and Desselberger, 2012). Enteric viruses are the primary cause of injuries to the gut, allowing other agents, especially bacteria, to attach, to penetrate, and to replicate in the enteric tissue, leading to further damage.

There are surprisingly no epidemiological and molecular data on avian rotavirus in Nigeria. Enteric diseases cause substantial economic losses to the poultry industry because they cause decreased weight gain, increased morbidity, increased mortality, and increased production cost. There are however no data to facilitate the determination of the extent of such losses in the Nigerian poultry. This study was thus carried out to detect rotavirus in the faeces of poultry birds by using molecular techniques and provide information on the prevalence of the virus in some poultry birds.

4.1.2 AIM

To investigate the presence and prevalence of rotavirus in poultry flocks and some avian species in south western Nigeria

4.1.3 OBJECTIVES

4.1.3.1 General objective

i. To detect rotavirus in avian species.

4.1.3.2 Specific objectives

i. To develop specific and sensitive PCR protocol for detecting avian rotavirus.

ii To determine the prevalence of rotavirus in some poultry farms in south western Nigeria.

iii. To detect rotavirus in faeces of some other avian species using molecular techniques.

4.2 MATERIALS AND METHODS

4.2.1 Study locations:

This study investigated the presence of rotavirus in poultry birds on some farms in Lagos, Ogun and Oyo State, all in Southwestern Nigeria. Samples were collected from commercial poultry farms, backyard poultry, live bird market and poultry slaughter slabs using the simple random technique.

4.2.2 Samples and sampling procedure:

Faecal samples were collected from different age group of birds ranging from 9 days to 60 weeks. One hundred and nine flocks from 50 farms comprising chickens, crowned cranes, ducks, eagles, geese, guinea fowls, parrots, pigeons and turkeys were sampled between June 2009 and August 2012. Faecal samples in the form of cloacal swabs were collected from birds. Approximately 3g of fresh faecal samples were collected from the litter and placed in 500µl of viral transport medium (VTM) containing Hank's balanced salt solution (Appendix 1). The cloacal swabs were similarly placed in VTM. Clinical signs for each flock were noted. All samples were directly placed on ice, transported to the laboratory on ice pack and stored at -80°C.

4.2.3 Laboratory analysis:

4.2.3.1 RNA extraction:

Two methods were employed in extracting RNA from faecal samples and are as follows:

4.2.3.1.1 Extraction using QIAamp Viral RNA Mini Kit:

Isolation of Rotavirus RNA from samples and its purification was done using the QIAamp Viral RNA minikit.

4.2.3.1.1.1 Procedure:

Faeces were resuspended in 500µl of Medium 199 (Lonza, Belgium) (Appendix I). Samples were clarified at 800 rpm for 10 min and 140µl of supernatant was used for RNA extraction using QIAamp Viral RNA Mini Kit (Qiagen, Venlo, Netherlands), according to the manufacturer's description as follows:

Buffer AVL was incubated at 80°C until precipitate was dissolved, 1ml of buffer AVL was then added to lyophilized carrier RNA to dissolve and transferred into buffer AVL bottle and cooled down to room temperature. Twenty five milliliters of ethanol was added to wash buffer AW1 while 30ml of ethanol was added to buffer AW2.

Samples and all reagents were equilibrated to room temperature. Five hundred and sixty microliters of AVL buffer was pipette into a labeled 1.5 ml eppendorf tube and 140µl of faecal suspension added, then mixed by pulse vortexing for 15 seconds. This was then incubated at room temperature for 10 min, briefly centrifuged to remove drops from the lid and 560µl of ethanol added. This was mixed by pulse vortexing for 15 sec, briefly centrifuged and 630µl of the solution was transferred to a labeled RNA extraction column in a 2 ml collection tube. The collection tube was centrifuged at 8000 rpm for 1 min., spin column was then placed in a new 2 ml collection tube and the old one containing the filtrate was discarded. The steps from addition of 630µl of the solution up to centrifugation were repeated. Washing was performed by addition 500µl of buffer AW1 to the column and centrifuged for 1 min at 8000 rpm. The column was placed in a new 2 ml collection tube and the old one containing the filtrate discarded. Washing was repeated by addition of 500 µl of buffer AW2 and centrifuged for 3 min at full speed. Column was then placed in labelled 1.5ml eppendorf tube and collection tube discarded. Elution was carried out by addition of 60µl of buffer AVE into the spin column and incubated at room temperature for 1 min. After incubation, the spin column was centrifuged at 8000 rpm for 1 min. Eluted RNA was then stored at – 80°C until used.

4.2.3.1.2 Extraction using laboratory prepared reagents:

4.2.3.1.2.1 Reagents:

Lysis buffer, silica suspension, wash buffer, elution buffer, proteinase k, proteinase k digestion buffer, 70% ethanol, 100% acetone, guanidine m-isothiocyanate, 0.1M Tris-Hcl, triton X-100, 0.2M EDTA solution, NaCl, 2% SDS, pH meter, Heating block.

4.2.3.1.2.2 Preparation of laboratory reagents for extraction (Appendix I):

4.2.3.1.2.3 Preparation of silica suspension:

Sixty grams of silica (Sigma, S-5631) was added to dimineralized water in a total volume of 500ml in a glass cylinder. Silica was allowed to sediment under gravity for 24hours at room temperature (RT) in the dark. After 24hours, 430ml of supernatant was removed and dimineralized water was added up to 500ml and vortexed. This was allowed to sediment for 5hours at RT. Four hundred and forty ml of supernatant was extracted and 600µl Hcl was added to adjust pH to 2. Silica was then sterilized by autoclaving and stored at RT in the dark.

4.2.3.1.2.4 Lysis buffer preparation

One hundred and twenty grams of Guanidine isothiocyanate (GTC, Fluka, 50990) was dissolved in 100ml 0.1M Tris-HCL pH 6.4 in a glass beaker followed by addition of 2.6g triton X-100 and 22ml of 0.2M EDTA solution was done (adjusted to pH 8.0 with NaOH) and stored in the dark at RT.

4.2.3.1.2.5 Washing buffer preparation

One hundred and twenty (120) grams of guanidine isothiocyanate was dissolved in 100ml 0.1M Tris-Hcl pH 6.4 in a glass beaker and stored in the dark at RT.

4.2.3.1.2.6 RNA Elution

RNAse free distilled water (Gibco) was used to elute RNA.

4.2.3.1.3 Procedure

4.2.3.1.3.1 Rotavirus cell lysis

Ten to fifty milligrams (10-50mg) of faeces was homogenised in a homogenizer to produce a smooth uniform homogenate. Similarly, cloacal swab in eppendorf tube was vortexed to produce smooth uniform paste. Five hundred µl of PBS was added to homogenate or paste, vortexed and transferred to 1.5ml tube. One hundred and fifty µl was transferred into new sterile eppendorf tube and 150µl proteinase K digestion buffer was added followed by addition of 4µl proteinase K and incubated at 37°C for 30minutes. This was then centrifuged at 8000rpm for 2minutes.

4.2.3.1.3.2 RNA Isolation

One milliliter of lysis buffer was added into the solution. Silica suspension was added at volume of 50µl. Incubated at room temperature for 10minutes and vortexed every 2minutes. Spinned and supernatant decanted away. One milliliter of wash buffer was added and vortexed. Wash was repeated again with wash buffer, 1ml of 70% ethanol and 100% acetone was used to wash 2 times and once respectively. Acetone was then aspirated, dried in heating block at 56°C for 10minutes with cap opened and 50µl elution buffer was then added and vortexed. This was then incubated at 56°C for 10minutes and spinned for 2minutes at 8000rpm. Supernatant (pure RNA) was then transferred into new 1.5ml eppendorf tube and stored at -20°C until used.

4.2.4 Reference Rotavirus strain

The Avian Rotavirus Groups A and D strains were kindly provided by Dr P. Otto, Friedrich-Loeffler Institute, Germany and served as positive controls for all experiments.

4.2.5 Rotavirus detection

4.2.5.1 Detection of rotavirus A and D positive controls

Preliminary detection of Rotavirus groups A and D positive control was carried out at annealing temperature of 50°C. This was carried out using NSP4 primers as designed by Pantin-Jackwood *et al.*, 2007.

4.2.5.2 PCR optimisation using real-time PCR

RNA was denaturated at 95°C for 2 min followed by cooling on ice. Rotavirus detection RT-PCR was performed using the QIAGEN one-step RT-PCR kit (Qiagen) and previously published primers targeting NSP4 (Pantin-Jackwood *et al.*, 2007). Amplification was performed as following: 50°C for 30 min, 94°C for 15 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 50°C, 53°C and 56°C for 30 sec, extension at 72°C for 60 sec. The RT-PCR was carried out in a total volume of 25µl.

Four different mixes (Table 4) with varying components in a total volume of 25.0 µl (Table 5) were used for the detection PCR in order to standardize the detection of rotavirus in faeces.

Table 4: Mix preparations for PCR optimization

Component	Mix 1(µl)	Mix 2 (µl)	Mix 3 (µl)	Mix 4 (µl)
H_2O	12.85	13.65	14.15	13.35
5x qiagen RT-PCR buffer	5	5	5	5
$MgCl_2$ (50 mM)	0.5	0.5	0	0
Sybergreen	0.25	0.25	0.25	0.25
dNTP (Qiagen kit)	0.8	0.8	0.8	0.8
Primer F (25uM)	1	0.6	0.6	1
Primer R (25uM)	1	0.6	0.6	1
RNAse out	0.1	0.1	0.1	0.1
Qiagen 1 step RT-PCR enzyme	1	1	1	1
RNA	2.5	2.5	2.5	2.5
Total	25	25	25	25

Table 5: Reaction components for one-step RT-PCR

Component Master mix	Volume/reaction	Final concentration
RNase-free water	Variable	–
Sybr green	0.25µl	
5x QIAGEN OneStep RT-PCR Buffer*	5.0 µl	1x
dNTP Mix (containing 10 mM of each dNTP)	1.0 µl	400 µM of each dNTP
Primer A	Variable	**0.6 µM**
Primer B	Variable	**0.6 µM**
QIAGEN OneStep RT-PCR Enzyme Mix	1.0 µl	–
RNase inhibitor	Variable	5–10 units/reaction
Template RNA		
Template RNA, added at step 4	Variable	1 pg – 2 µg/reaction
Total volume	**25.0 µl**	**–**

4.2.5.3 Annealing temperatures

The annealing temperatures used for each mix above were 50°C, 53°C and 56°C in other to determine the optimum temperature for annealing the nucleic acid.

4.2.5.4 One step RT-PCR

The reaction components for one-step RT-PCR was the same for the real-time PCR except for none inclusion of sybr green.

4.2.5.5 Two step RT-PCR

Reverse transcription was done to generate cDNA from the RNA by mixing 5µl of extracted RNA with 8µl of mix 1 which consisted of 2µl of distilled H_20, 1µl of 10mM dNTPs, and 5µl of 0.03µg/µl random primer and was incubated at 72°C for 10min. Seven (7)µl of mix 2 which contained 1µl of DTT, 1µl of RNAase inhibitor, 4µl of 5x first strand Buffer and 1 µl of superscript III was added and then incubated at 50°C for 1 hour 20 minutes and 70°C for 15 minutes.

The polymerase chain reaction for Rotavirus was carried out by adding 2.5µl of cDNA to 22.5µl of PCR mix containing 17.2µl of distilled water, 2.5µl of 10X PCR buffer, 2.0µl of $MgCl_2$ (50mM), 0.5µl of dNTP (10mM), 0.1µl of forward primer NSP4-F30, 0.1µl of reverse primer NSP4-R660 (Pantin-Jackwood *et al.*, 2007) and 0.1µl of taq polymerase (5µ/µl).

The PCR reactions was carried out using the following cycling conditions; initial denaturation at 94°C for 5min, 35 cycles of amplification at 94°C for 30 sec, 50°C for 30 sec and 72°C for 1min and final extension at 72°C for 10min.

4.2.5.6 Electrophoresis of DNA

Loading dye (1µl) was mixed with 5µl of PCR products and visualized by UV illumination in 2% agarose gel stained with ethidium bromide or sybr safe and compared to the 1kb+ DNA marker (Invitrogen®) for sample DNA size determination. The positive specimens were detected with band at appropriate base pair (bp) size depending on the expected bp size for each primer pair.

4.2.5.7 PCR product purification using JetQuick purification kit:

Binding buffer H1 was reconstituted with isopropanol and wash buffer H2 with absolute ethanol (96-100%) according to the instructions from the manufacturer.

4.2.5.7.1 Purifying PCR Products using Centrifugation

1. **Loading:** 4 volumes of Binding Buffer (H1) was added to 1 volume of a PCR product. Mixed well and placed in a JetQuick® Spin Column in a 2-mL Receiver Tube. PCR product was the centrifuged at >12,000 × *g* for 1 minute.
2. **Washing:** The column was re-inserted into the empty Receiver Tube and 500 µL of Wash Buffer (H2) was added with ethanol. The column was then centrifuged at >12,000 × *g* for 1 minute. The flow-through was discarded and the column placed in the same 2-mL Receiver Tube. The column was again centrifuged at >12,000 × *g* for 1 minute.
3. **Elution:** The column was placed into a new 1.5-mL microcentrifuge tube. 50 µL of TE Buffer was added to the column and centrifuged at >12,000 × *g* for 2 minutes.

4.2.5.7.2 PCR product purification using Gel Extraction method

Polymerase chain reaction (PCR) product was loaded on a 1% agarose gel. Gel was run in electrophoresis tank until bands were correctly separated. Bands of interest were excised with a scalpel blade under UV light taking precaution by wearing eye protection goggles. The piece of agarose was placed in a 1.5ml Eppendorf tube. This was weighed and values written on the tubes. Three times weight/volume of QG buffer was added and incubated at 50°C for 10minutes. 1 X volume of isopropanol was then added and vortexed. The liquid was then added on the provided column and centrifuged at 13000rpm for 1 minute. Flow through was discarded and 500 µl of QG buffer was added and centrifuged at 13000rpm for 1 minute. Flow through was again discarded and 750 µl of PE buffer added and centrifuged at 13000rpm for 1 minute. Flow through was discarded and centrifuged at 13000rpm for 1 minute. The column was placed in a labeled eppendorf tube. Thirty (30) µl of EB (elution buffer) was added and incubated for 1 minute and then centrifuged at 13000rpm for 1 minute.

4.2.5.7.3 Verification of PCR product purity

A 1.5% agarose gel was prepared and 5 µl of the purified PCR product was mixed with loading dye (6X). Gel was run in electrophoresis tank for 45minutes and efficiency of purification was checked under UV light. Specificity of primers was determined by the presence of specific bands without extraneous bands on the gel. Bands without such extraneous bands indicate pure PCR products.

4.3 RESULTS

4.3.1 PCR OPTIMISATION

4.3.1.1 Detection of rotavirus A and D positive controls

Rotavirus A and D positive controls were both detected with the NSP4 primers, although a much more prominent band was seen with the Rotavirus A sample (Figure 13).

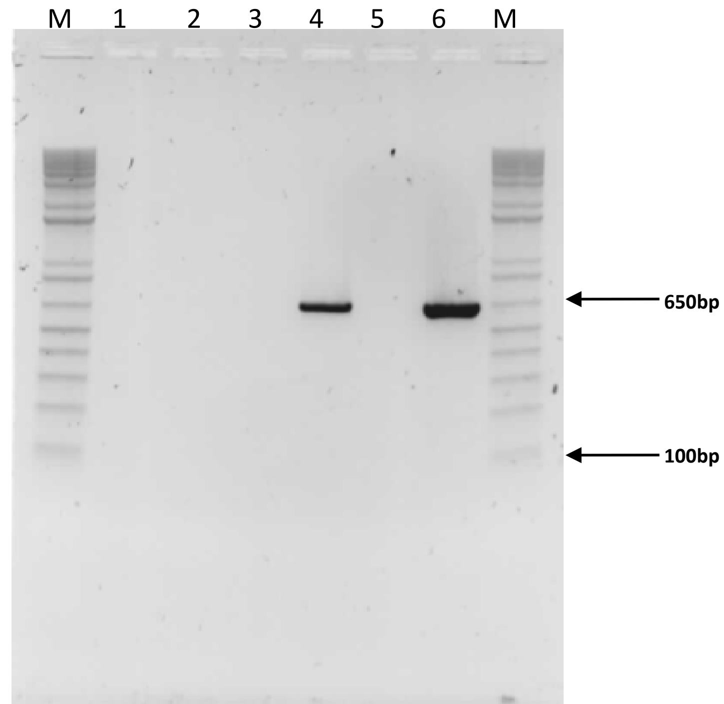


Figure 13: Detection of genome segments encoding NSP4 of chicken rotavirus. PCR products were separated on Sybr safe stained 1.5% agarose gels. Lane 1 is PCR negative control, lanes 2,3 and 5 are extraction negative controls. Lane 4 and 6 are rotavirus D and A positive respectively. M: molecular mass markers.

4.3.1.2 One-step PCR for detection of rotavirus A and D positive controls at different dilutions with varying PCR components

The detection of rotavirus A and D at different dilutions (d1:1, d1:10, d1:100 and d1:1000) with different mixes showed a positive result for all the positive control using the NSP4 primers in the experiment. The bands presented for the different dilutions although specific, varied in intensity at 630bp (Figures 14-19).

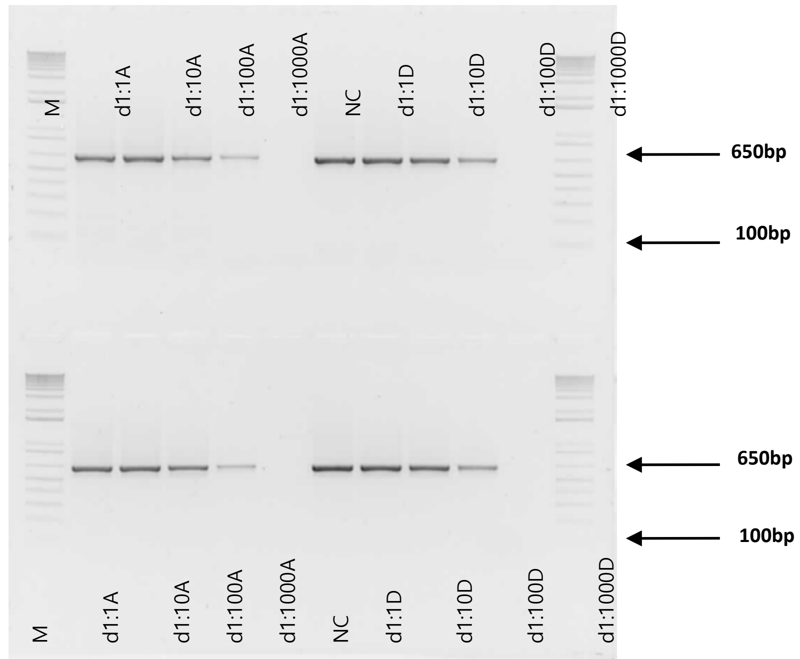


Figure 14: Detection of genome segments encoding NSP4 of Rotavirus groups A and D at different dilutions with annealing temperature at 50°C using mix 1 and 2

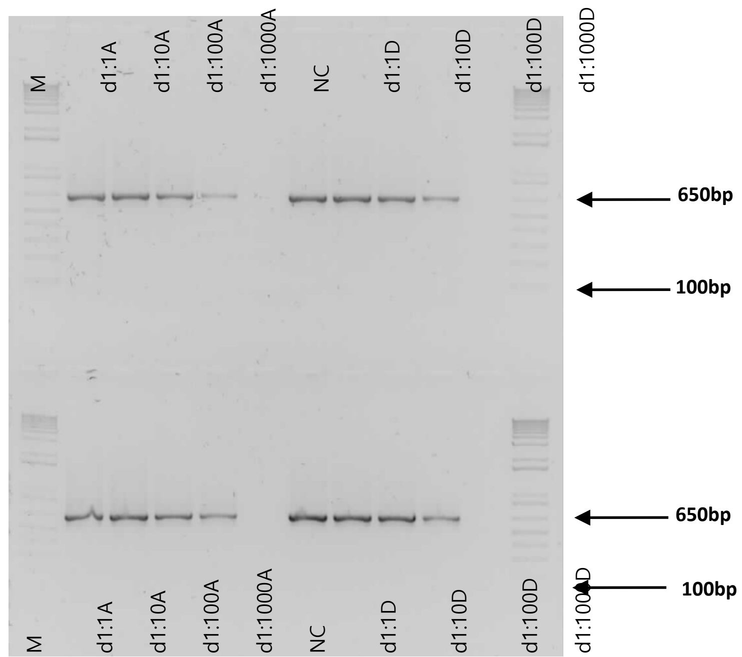


Figure 15: Detection of genome segments encoding NSP4 of Rotavirus groups A and D at different dilutions with annealing temperature at 50°C using mix 3 and 4

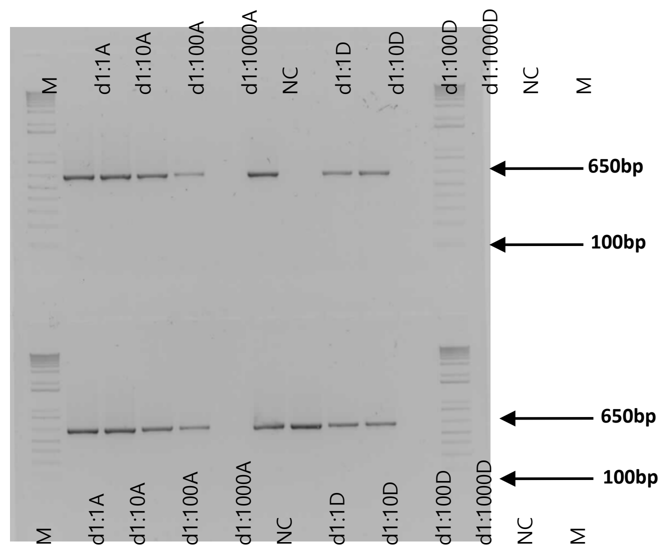


Figure 16: Detection of genome segments encoding NSP4 of Rotavirus groups A and D at different dilutions with annealing temperature at 53°C using mix 1 and 2

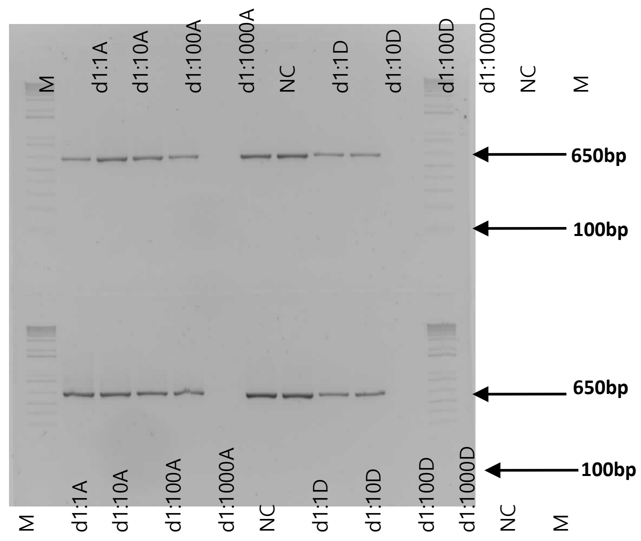


Figure 17: Detection of genome segments encoding NSP4 of Rotavirus groups A and D at different dilutions with annealing temperature at 53°C using mix 3 and 4

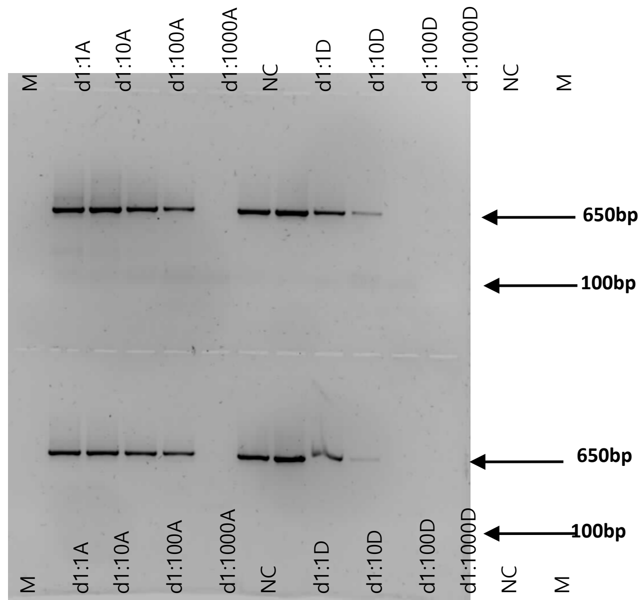


Figure 18: Detection of genome segments encoding NSP4 of Rotavirus groups A and D at different dilutions with annealing temperature at 56°C using mix 1 and 2

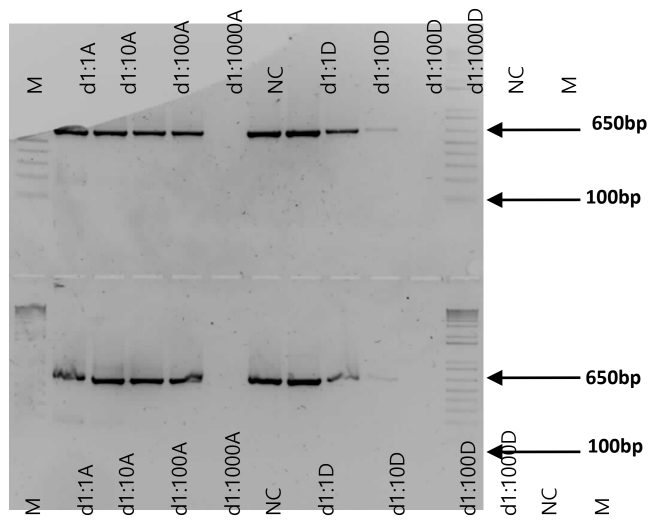


Figure 19: Detection of genome segments encoding NSP4 of Rotavirus groups A and D at different dilutions with annealing temperature at 56°C using mix 3 and 4

4.3.1.3 Real time PCR for detection of rotavirus A positive controls at different dilutions and varying PCR components

The curves (figures 20-31) presented show varying detection levels for the different mixes at different annealing temperature. At 50°C the CT (threshold cycle) values ranged between 19.82 and 33.25 from d1:1 to d1:1000. At 53°C the CT value ranged between 19.90 and 32.60 from d1:1 to d1:1000, and between 21.31 to 30.78 at 56°C. The lowest CT value corresponding to the best detection protocol was achieved at 50°C using mix 4 at d1:1 or d1:10 for rotavirus A (Table 6) and d1:1 for rotavirus D (Table 7). The difference from Pantin-Jackwood *et al.*, 2007, is the volume of primer used for detection. Based on the optimization CT values, mix 4 was adhered to for the detection of rotavirus in the faecal sample in this experiment. All other dilutions at different temperatures have high CT values and not specific for Rotavirus detection.

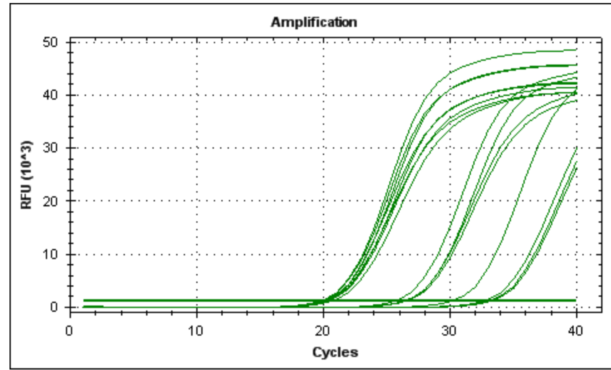


Figure 20: Amplification plots of flourescence intensities (RFU) versus PCR cycle number for detection of Rotavirus A using mix 1,2,3 and 4, at dilutions d1:1, d1:10, d1:100 and d1:1000 at 50°C.

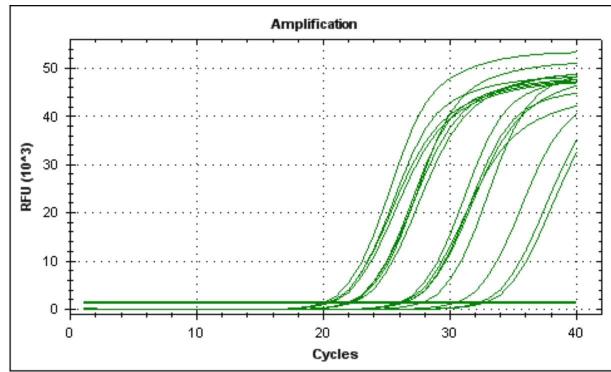


Figure 21: Real time PCR curve for detection of Rotavirus A using mix 1,2,3 and 4, showing CT values for d1:1, d1:10, d1:100 and d1:1000 at 53°C.

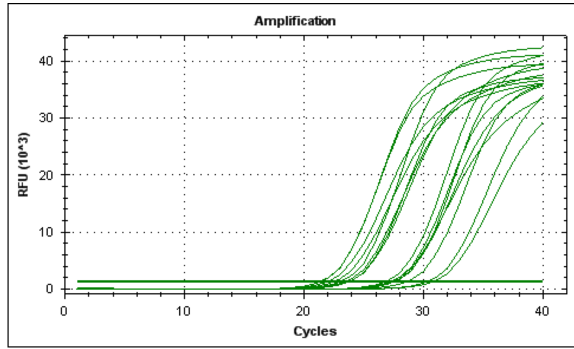


Figure 22: Real time PCR curve for detection of Rotavirus A using mix 1,2,3 and 4, showing CT values for d1:1, d1:10, d1:100 and d1:1000 at 56°C.

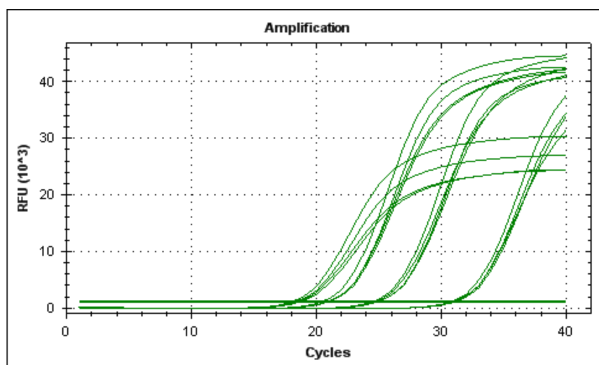


Figure 23: Real time PCR curve for detection of Rotavirus D using mix 1,2,3 and 4, showing CT values for d1:1, d1:10, d1:100 and d1:1000 at 50°C.

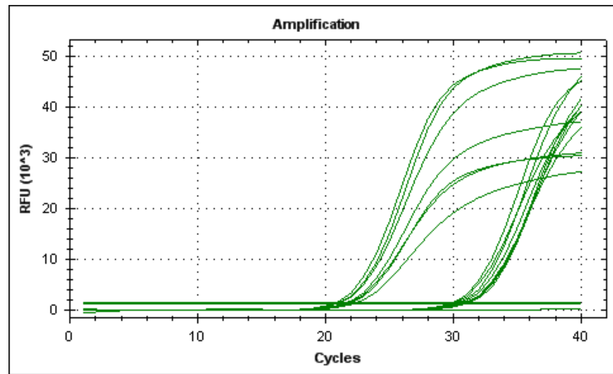


Figure 24: Real time PCR curve for detection of Rotavirus D using mix 1,2,3 and 4, showing CT values for d1:1, d1:10, d1:100 and d1:1000 at 53°C.

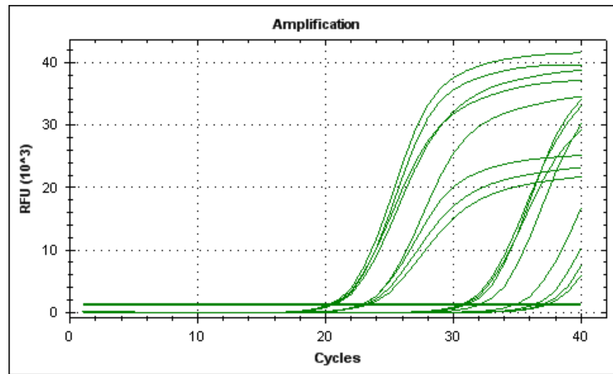


Figure 25: Real time PCR curve for detection of Rotavirus D using mix 1,2,3 and 4, showing CT values for d1:1, d1:10, d1:100 and d1:1000 at 56°C.

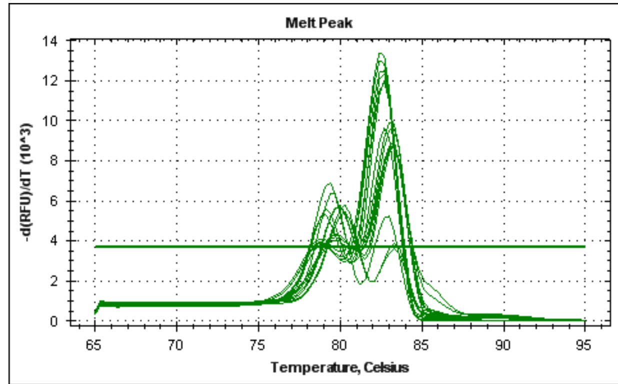


Figure 26: Melt peak for Rotavirus A optimization at 50°C.

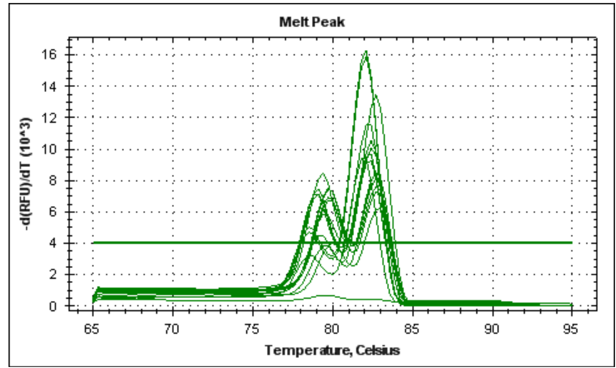


Figure 27: Melt peak for Rotavirus A optimization at 53°C.

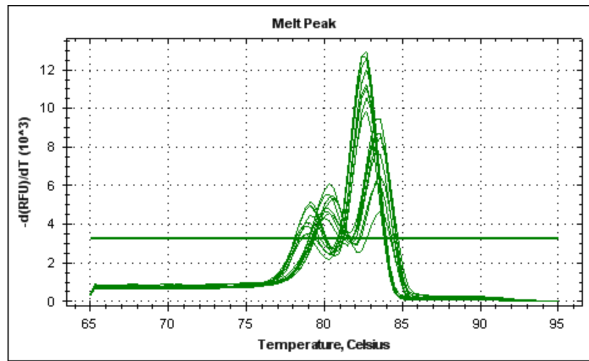


Figure 28: Melt peak for Rotavirus A optimization at 56°C.

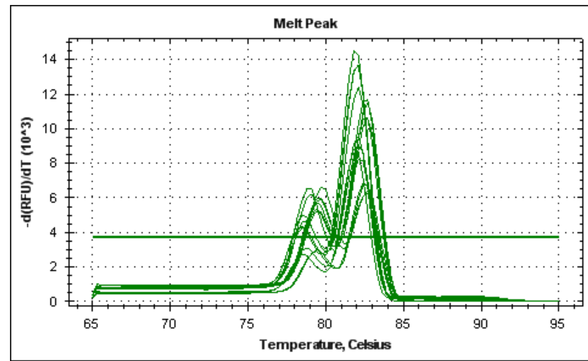


Figure 29: Melt peak for Rotavirus D optimization at 50°C.

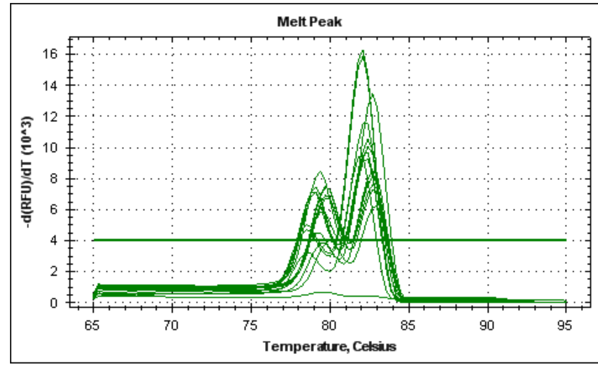


Figure 30: Melt peak for Rotavirus D optimization at 53°C.

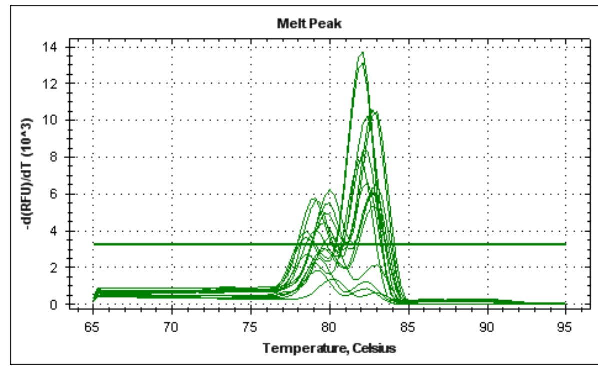


Figure 31: Melt peak for Rotavirus D optimization at 56°C.

Table 6: CT values for Rotavirus A optimization at 50°C, 53°C and 56°C using mix 1,2,3 and 4.

	Dilution	MIX1	MIX2	MIX3	MIX4
50°C	d1:1	20.15	20.64	20.21	19.82
	d1:10	20.03	20.3	20.08	19.82
	d1:100	26.37	26.52	26.38	25.64
	d1:1000	33.25	33.1	32.68	30.16
53°C	d1:1	20.2	20.37	20.21	19.9
	d1:10	21.8	22.12	21.99	21.77
	d1:100	26.01	26.26	26.23	25.8
	d1:1000	32.6	32.14	30.32	27.63
56°C	d1:1	21.98	22.44	21.32	21.31
	d1:10	23.4	23.69	23.05	23.73
	d1:100	27.24	27.41	26.8	27.2
	d1:1000	30.78	30.28	28.57	27.54

Table 7: CT values for Rotavirus D optimization at 50°C, 53°C and 56°C using mix 1,2,3 and 4.

	Dilution	MIX1	MIX2	MIX3	MIX4
50°C	d1:1	18.62	18.86	18.4	18.15
	d1:10	21.02	21.09	21.02	20.5
	d1:100	24.78	25.07	25.05	24.58
	d1:1000	31.08	31.22	31.1	30.9
53°C	d1:1	21.58	22.19	21.77	21.4
	d1:10	N/A	20.97	20.86	20.53
	d1:100	30.74	30.82	31.03	30.17
	d1:1000	29.92	30.36	30.99	29.71
56°C	d1:1	23.08	23.3	22.8	22.89
	d1:10	20.39	20.64	20.25	20.58
	d1:100	30.82	30.6	31.08	32.09
	d1:1000	34.87	37.32	36.31	37.04

4.4 Detection of rotavirus in faecal samples

4.4.1 Detection of rotavirus A complete genome segment 10 using NSP4 primers at 630bp:

In detecting rotavirus with NSP4 primers, the primers were not specific for most of the positive samples, although they were very specific for the positive controls using both the one-step and two-step PCR. However, samples which were screened at the Institute of Immunology, Luxembourg had more prominent bands than those screened at the Department of Veterinary Medcine, University of Ibadan. This could be attributed to the quality of reagents used as power supply in not costant for adequate maintainance of reagents.

Due to the non-specificity, the PCR products of the non-specific samples were purified using the gel method and PCR product subsequently verified to be specific (Figures 32-43).

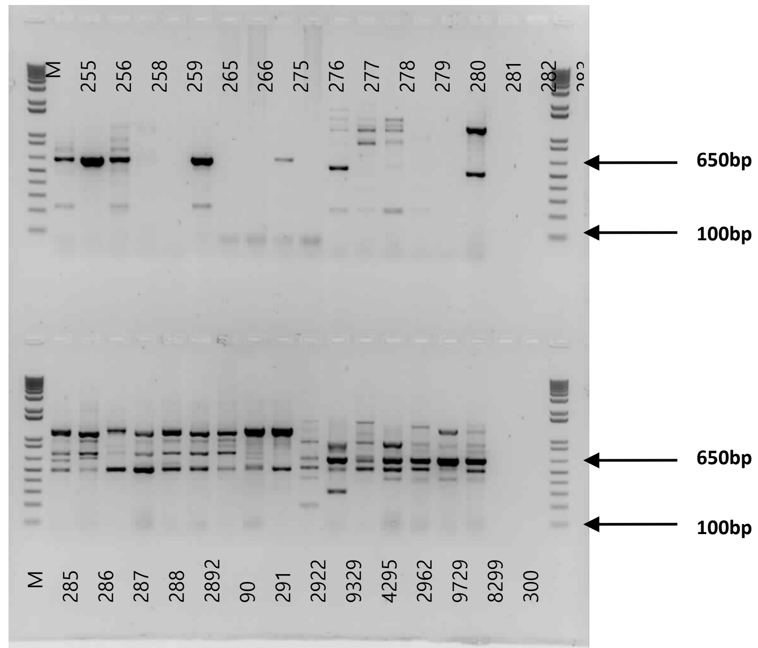


Figure 32: Detection of genome segment encoding NSP4 of chicken rotavirus (samples 255-300). PCR products were separated on Sybr safe stained 1.5% agarose gels.

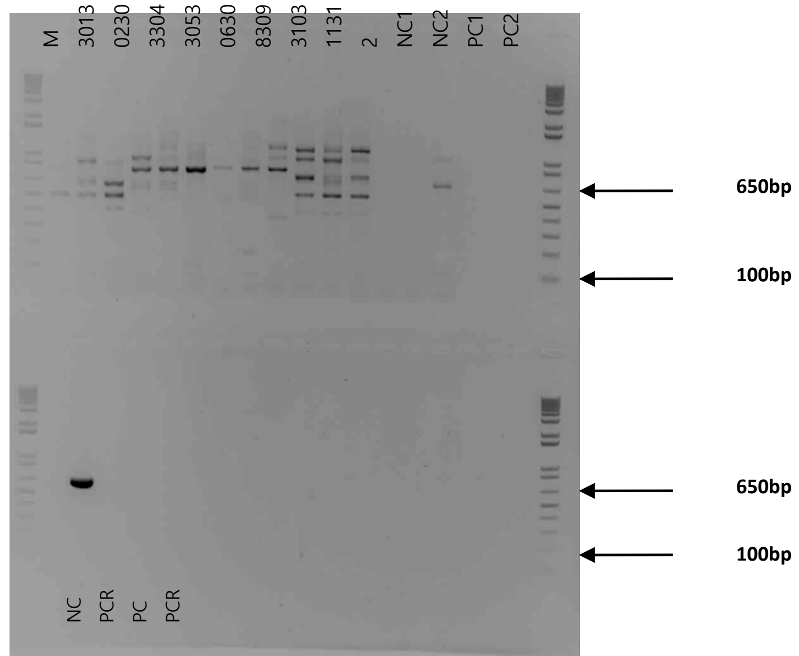


Figure 33: Detection of genome segment encoding NSP4 of chicken rotavirus (samples 301 -312). PCR products were separated on Sybr safe stained 1.5% agarose gels.

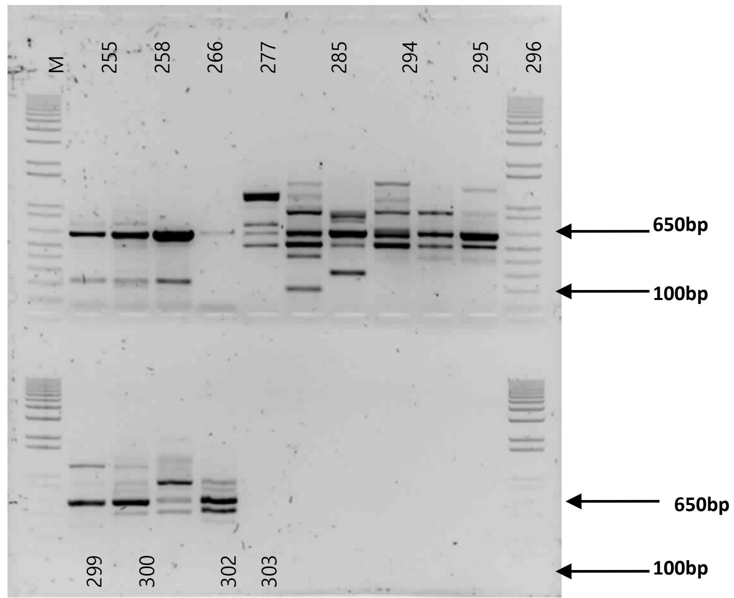


Figure 34: Agarose gel electrophoresis of PCR products (samples 255-303) for purification.

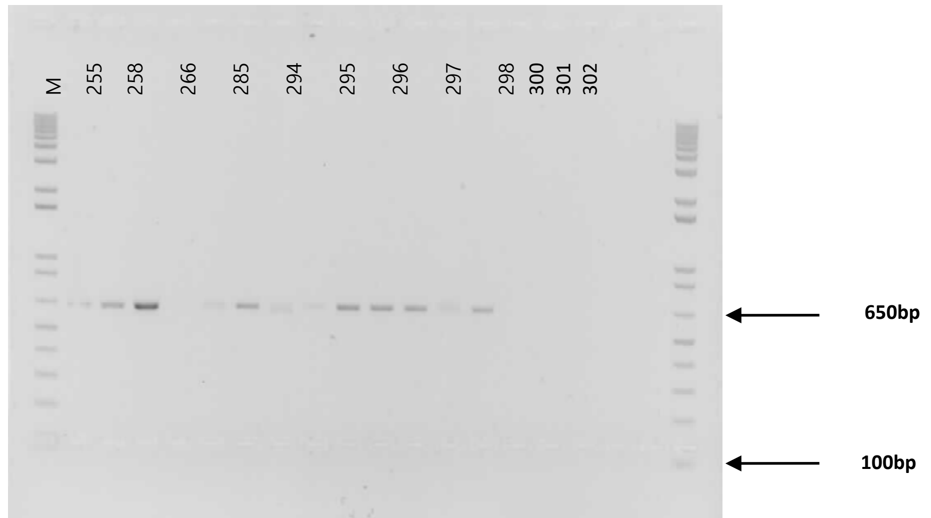


Figure 35: Agarose gel electrophoresis for confirmation of DNA purity by gel extraction method

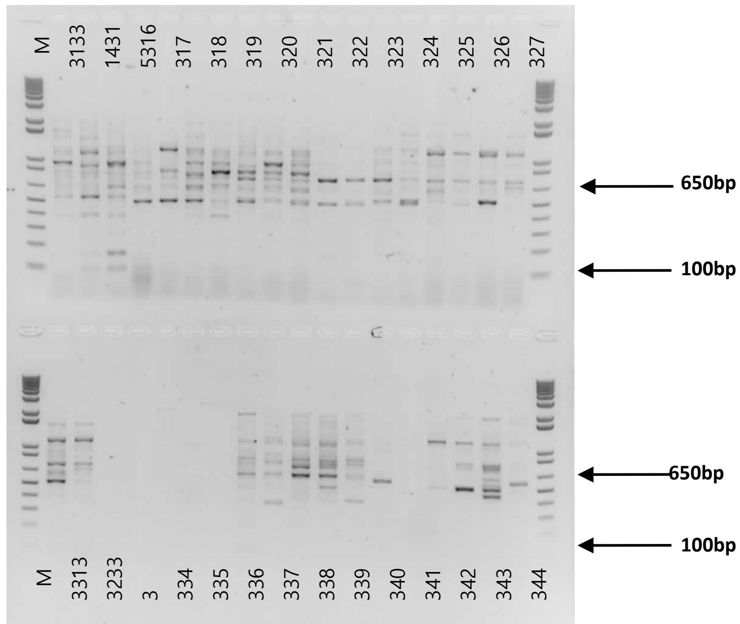


Figure 36: Detection of genome segment encoding NSP4 of chicken rotavirus (samples 313-348). PCR products were separated on Sybr safe stained 1.5% agarose gels.

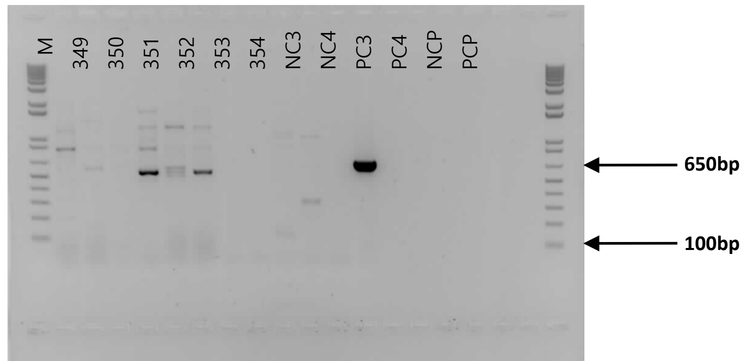


Figure 37: Detection of genome segment encoding NSP4 of chicken rotavirus (samples 349-354). PCR products were separated on Sybr safe stained 1.5% agarose gels.

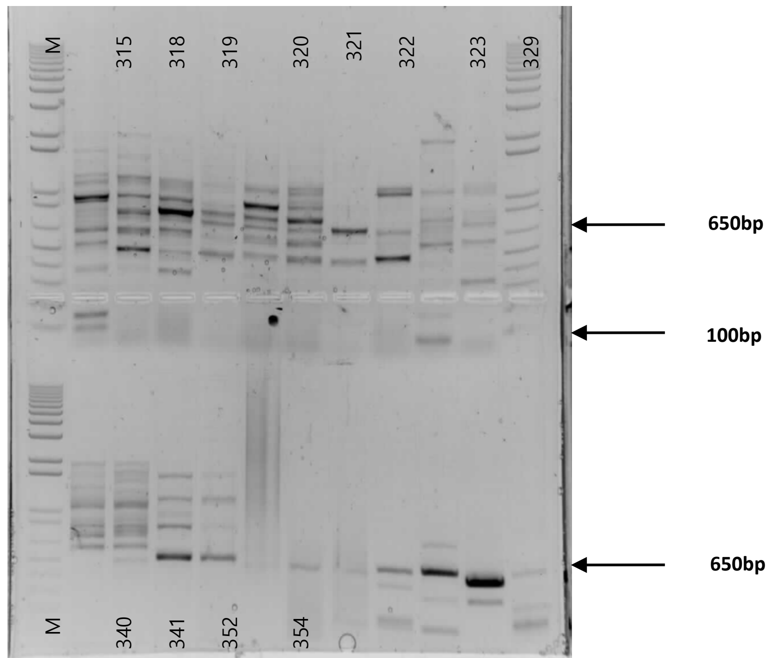


Figure 38: Agarose gel electrophoresis of PCR products (samples 315-354) for purification.

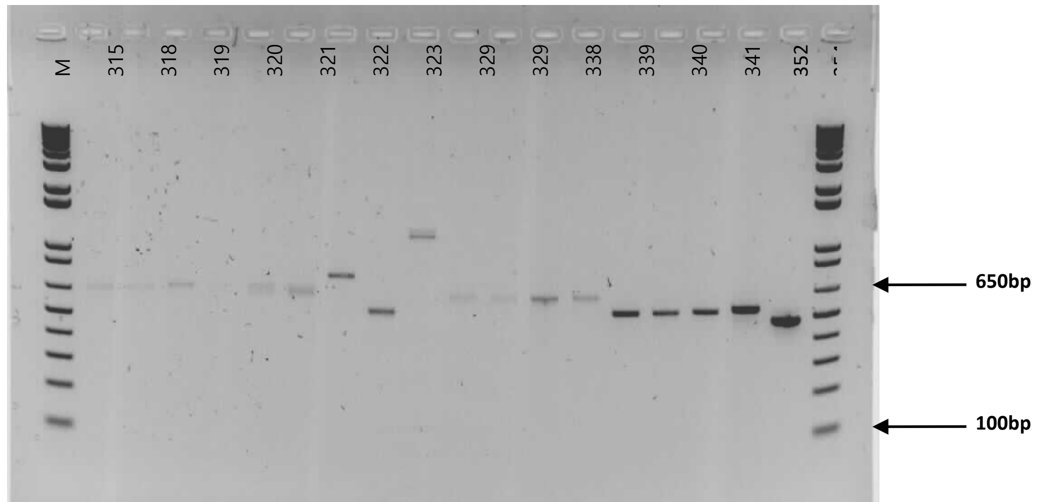


Figure 39: Agarose gel electrophoresis for confirmation of DNA purity by gel extraction method

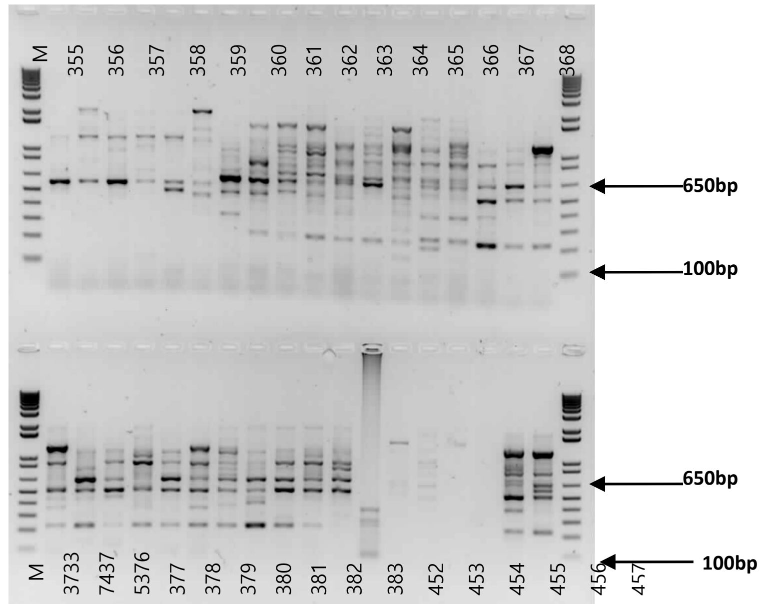


Figure 40: Detection of genome segment encoding NSP4 of chicken rotavirus (samples 355-458). PCR products were separated on Sybr safe stained 1.5% agarose gels.

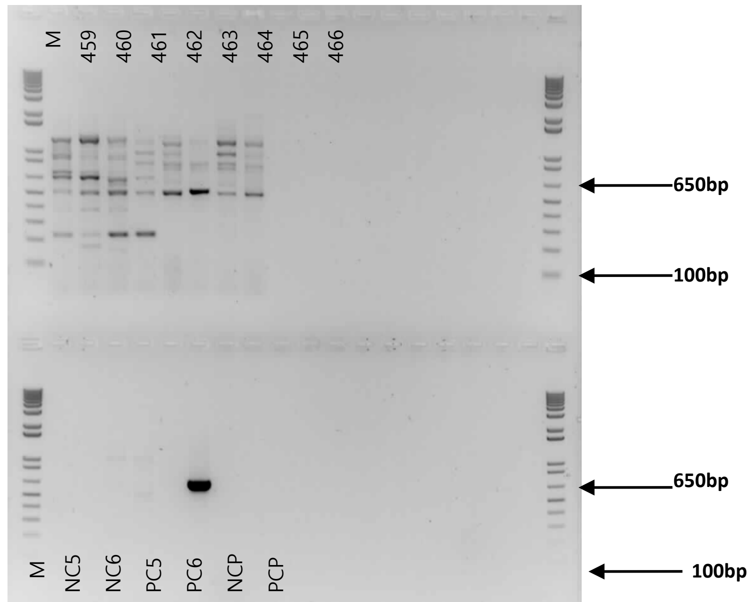


Figure 41: Detection of genome segment encoding NSP4 of chicken rotavirus (samples 459-466). PCR products were separated on Sybr safe stained 1.5% agarose gels.

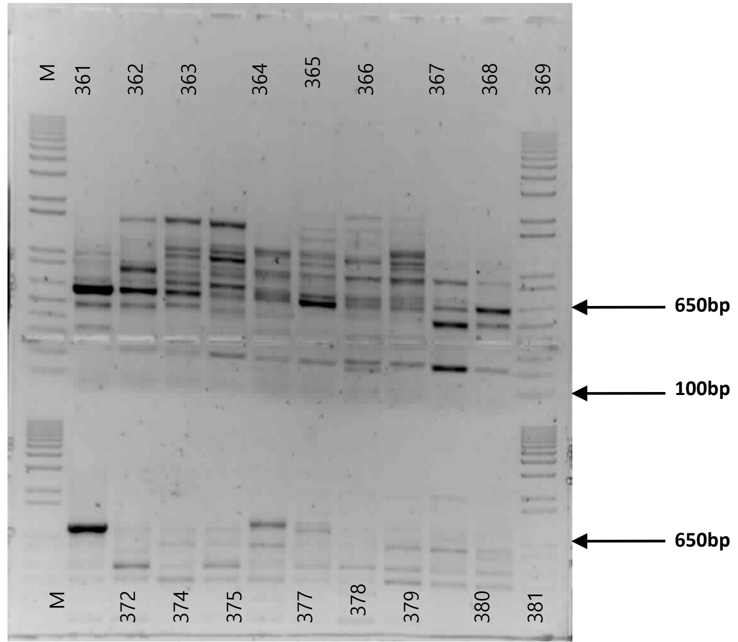


Figure 42: Agarose gel electrophoresis of PCR products (samples 361-383) for purification.

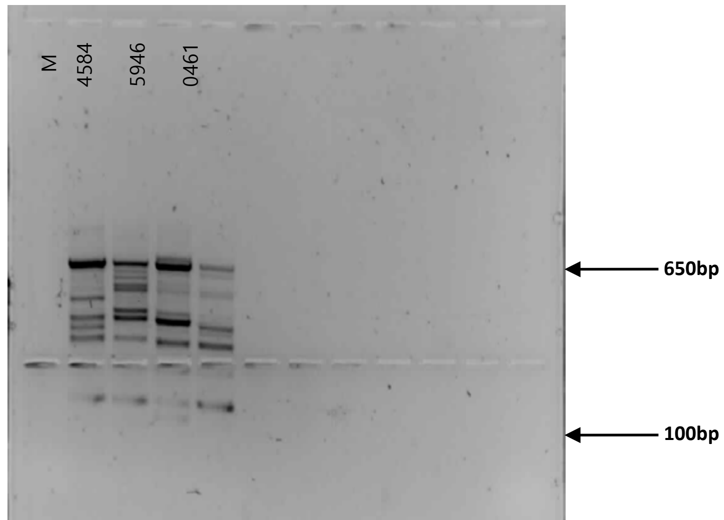


Figure 43: Agarose gel electrophoresis of PCR products (samples 458-461) for purification.

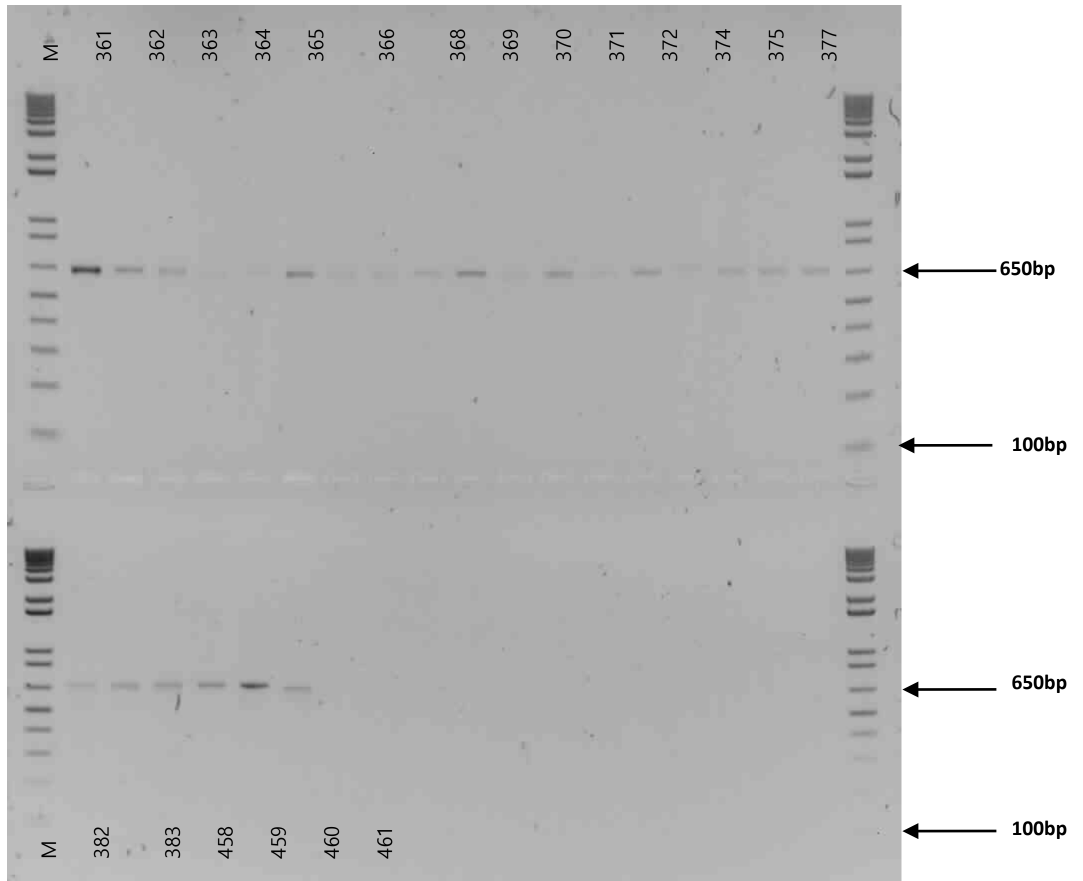


Figure 44: Agarose gel electrophoresis for confirmation of DNA purity by gel extraction method

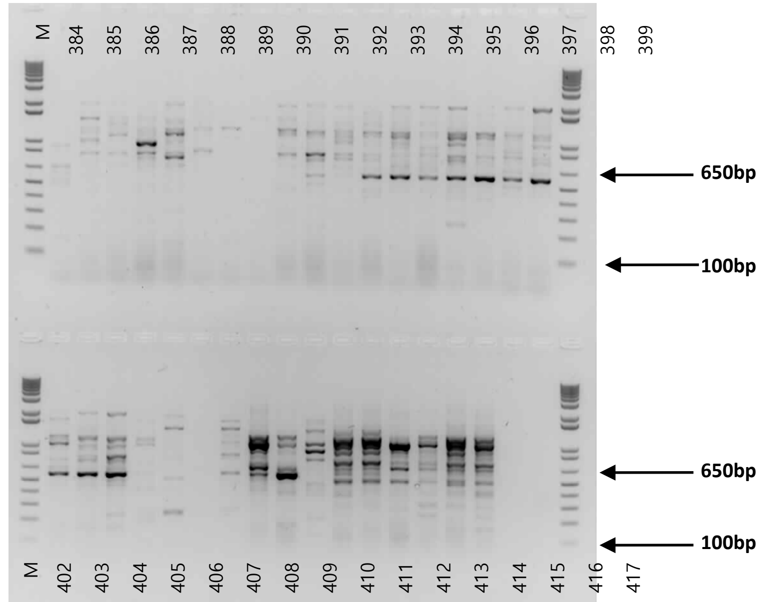


Figure 45: Detection of genome segment encoding NSP4 of chicken rotavirus (samples 384-419). PCR products were separated on Sybr safe stained 1.5% agarose gels.

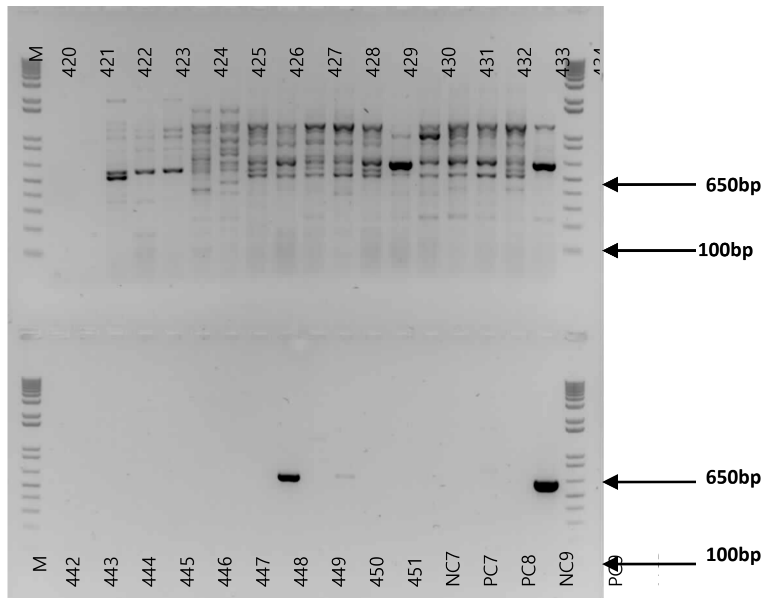


Figure 46: Detection of genome segment encoding NSP4 of chicken rotavirus (samples 420-451). PCR products were separated on Sybr safe stained 1.5% agarose gels.

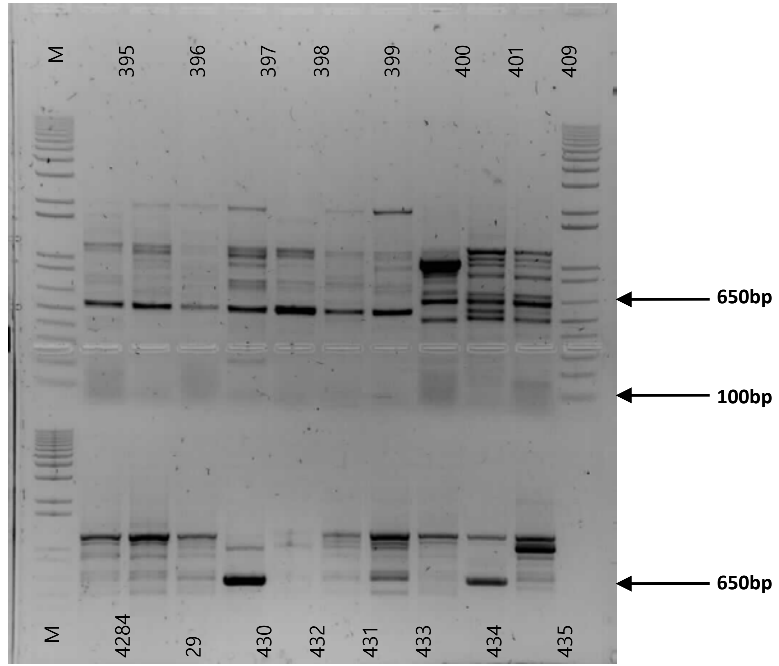


Figure 47: Agarose gel electrophoresis of PCR products (samples 361-441) for purification.

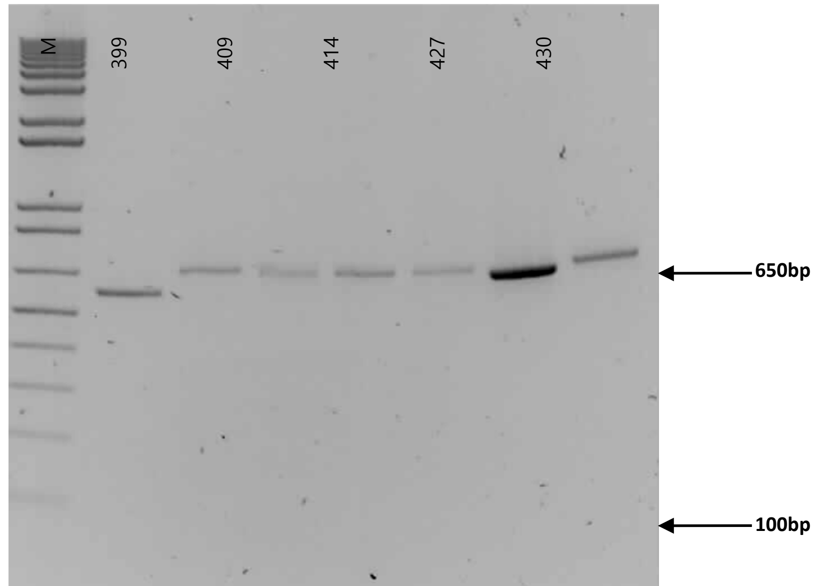


Figure 48: Agarose gel electrophoresis for confirmation of DNA purity by gel extraction method

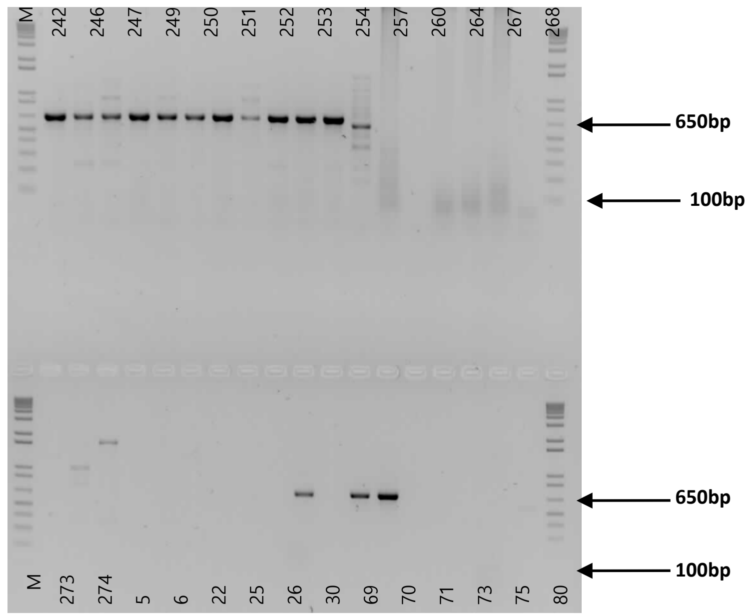


Figure 49: Detection of genome segment encoding NSP4 of chicken rotavirus (samples 5-274). PCR products were separated on Sybr safe stained 1.5% agarose gels.

4.4.2 Detection of rotavirus A and D (positive control) complete gene segment 4 using VP4 primers:

The whole of Rotavirus A positive control gene segment was detected using the designed primer sequence. In detecting rotavirus D positive control, only the initial segment of the gene sequence was detected. This means the designed primers were suitable for detecting only Rotavirus A (Figure 50).

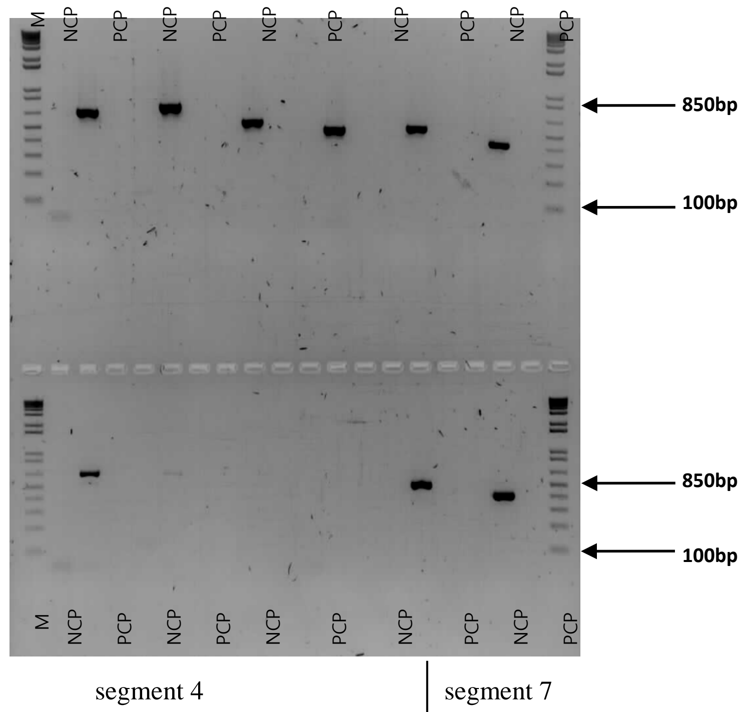


Figure 50: Rotavirus A (upper wells) and D (lower wells) detection test for segment 4 and segment 7

4.4.3 Detection of rotavirus A complete gene segment 4 from field samples using VP4 primers:

Only the initial segment of the Rotavirus A VP4 gene segment was successfully detected. Nucleotide positions 687 to 2215 were not detected. The samples were observed to have the same positive pattern as Rotavirus D for the whole of the segment. The primers were also observed to be non specific for the positive samples but specific for the positive control (Figures 51-56).

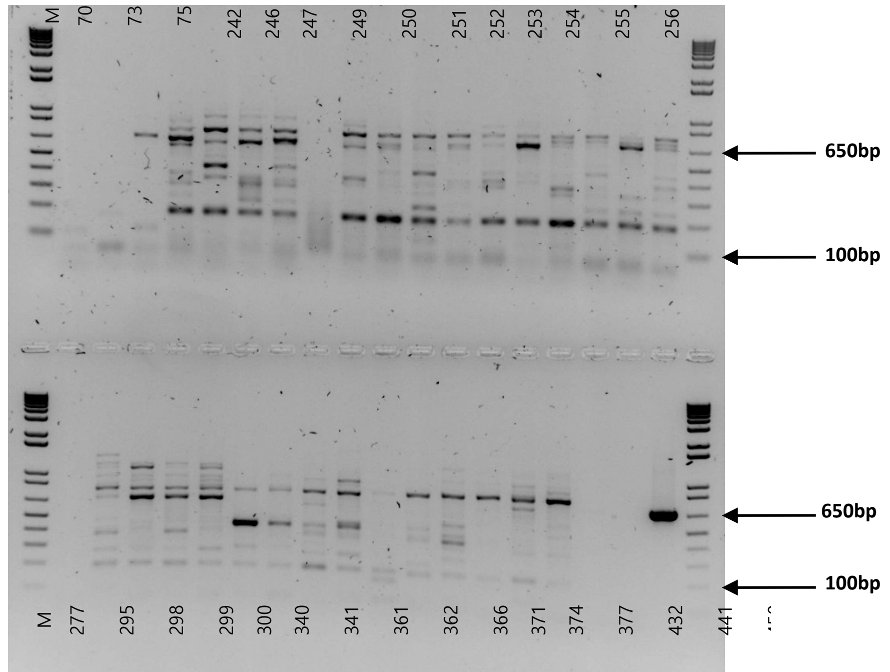


Figure 51: Detection of genome segment encoding VP4 of chicken rotavirus (samples 70-450) using Seg4s+seg4-686r primers at 686bp. PCR products were separated on Sybr safe stained 1.5% agarose gels.

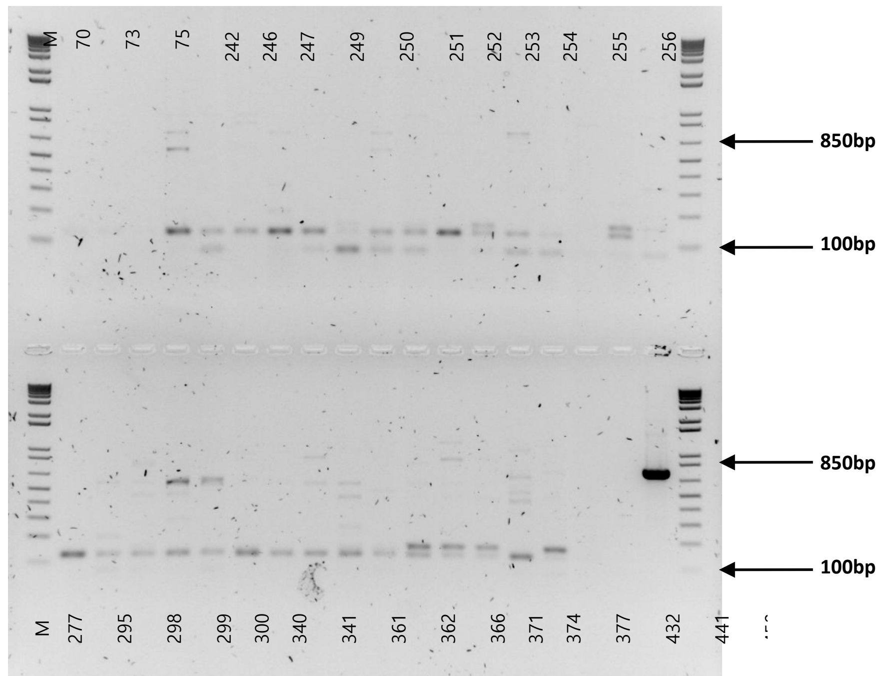


Figure 52: Detection of genome segment encoding VP4 of chicken rotavirus (samples 70-450) using Seg4-540f+seg4-1309r primers at 769bp. PCR products were separated on Sybr safe stained 1.5% agarose gels.

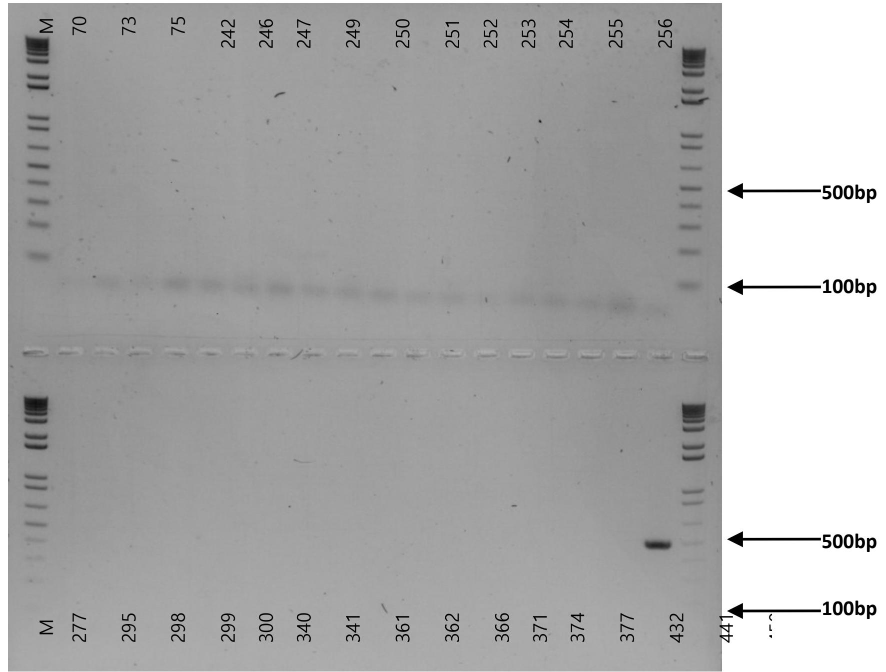


Figure 53: Detection of genome segment encoding VP4 of chicken rotavirus (samples 70-450) using Seg4-1704f+seg4-2215r primers at 511bp. PCR products were separated on Sybr safe stained 1.5% agarose gels.

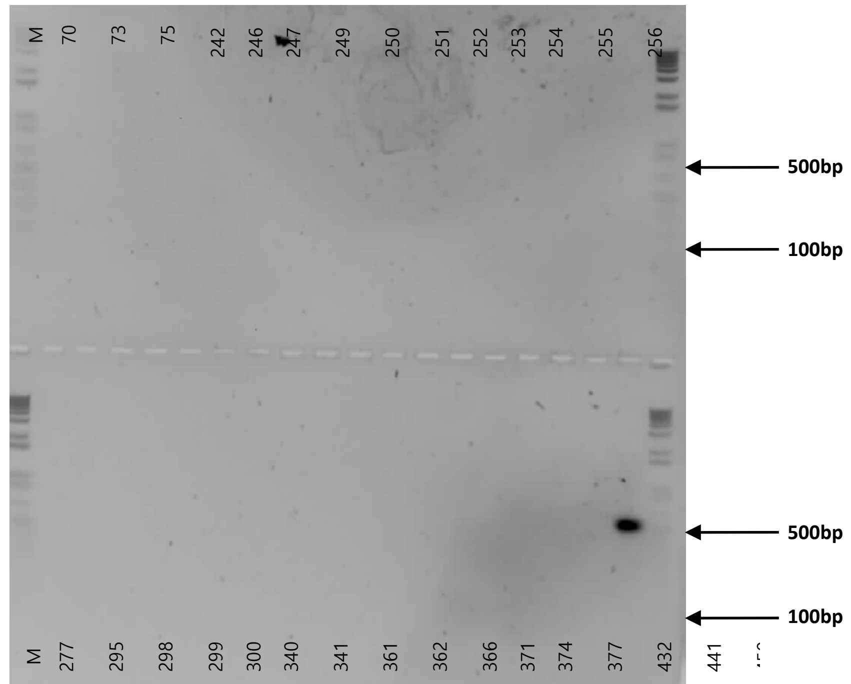


Figure 54: Detection of genome segment encoding VP4 of chicken rotavirus (samples 70-450) using Seg4-1215f+seg4-1786r primers at 571bp. PCR products were separated on Sybr safe stained 1.5% agarose gels.

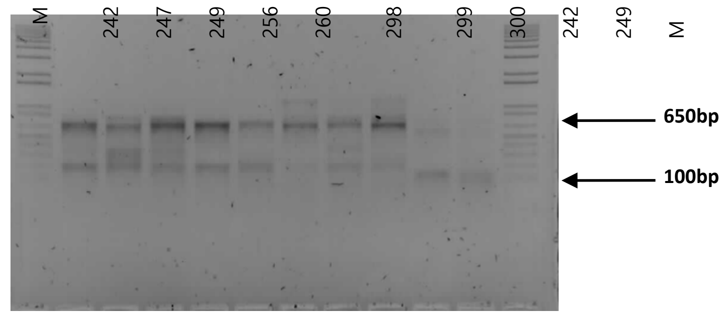


Figure 55: Agarose gel electrophoresis of PCR products (samples 242-300[seg4s+seg4-686r], 242 and 249[seg4-540f+seg4-1309r] for purification .

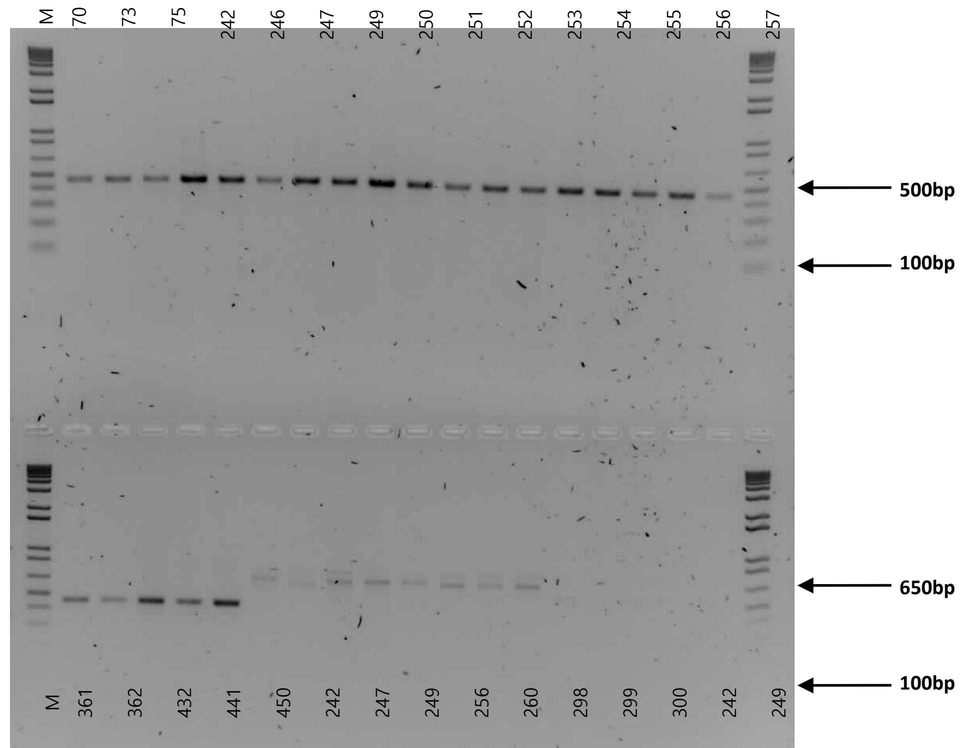


Figure 56: Agarose gel electrophoresis for confirmation of DNA purity (samples 70-450 for VP6 [VP6-begf+VP6-763], 242-300 for VP4 [seg4s+seg4-686r], 242 and 249 for VP4 [seg4-540f+seg4-1309r])

4.4.4 Detection of rotavirus A and D (positive control) gene segment 6 using VP6 primers:

Detection of Rotavirus groups A and D positive control was carried out at annealing temperature of 52°C and 56°C. Rotavirus A and D were both detected with the VP6 primers, although a much more prominent band was seen at 52°C for Rotavirus A and 56°C for Rotavirus D (Figure 57).

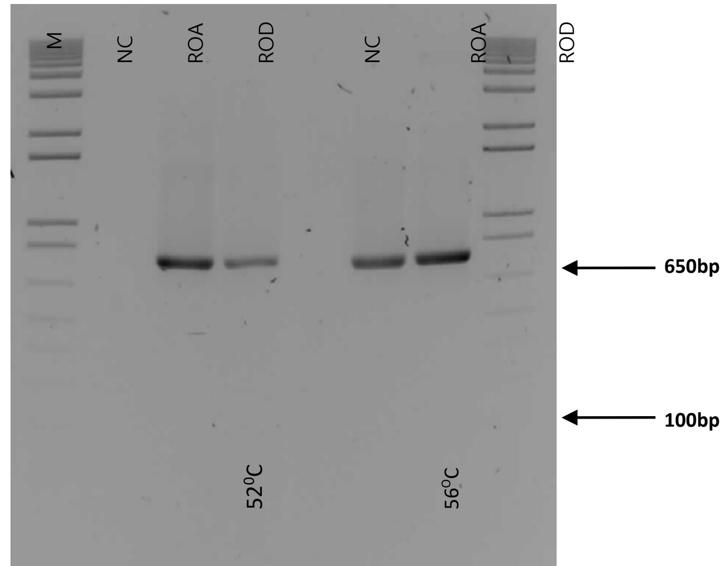


Figure 57: Detection of genome segment encoding VP6 of chicken rotavirus using positive controls for Rotavirus A and D at 2 different temperature gradients (52°C and 56°C). PCR products were separated on Sybr safe stained 1.5% agarose gels.

4.4.5 Detection of rotavirus A complete genome segment 6 using VP6 primers:

Different gene segments for different rotavirus positive samples were detected with the designed primers. The mid portion of the segment was detected with primers designed by Schuman *et al.*, 2009. Prominent and specific bands were detected using the designed primers (figures 58 and 60) than primers from Schuman *et al.*, 2009 (Figure 61).

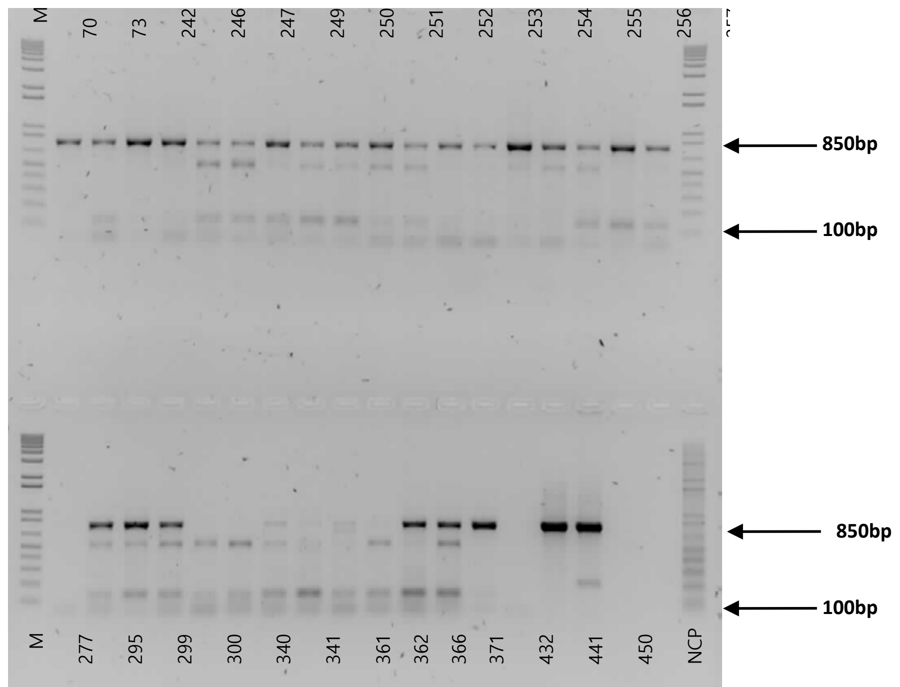


Figure 58: Detection of genome segment encoding VP6 of chicken rotavirus (samples 70-450) using Rota-Seg6-s + Rota-Seg6-as at 777bp.

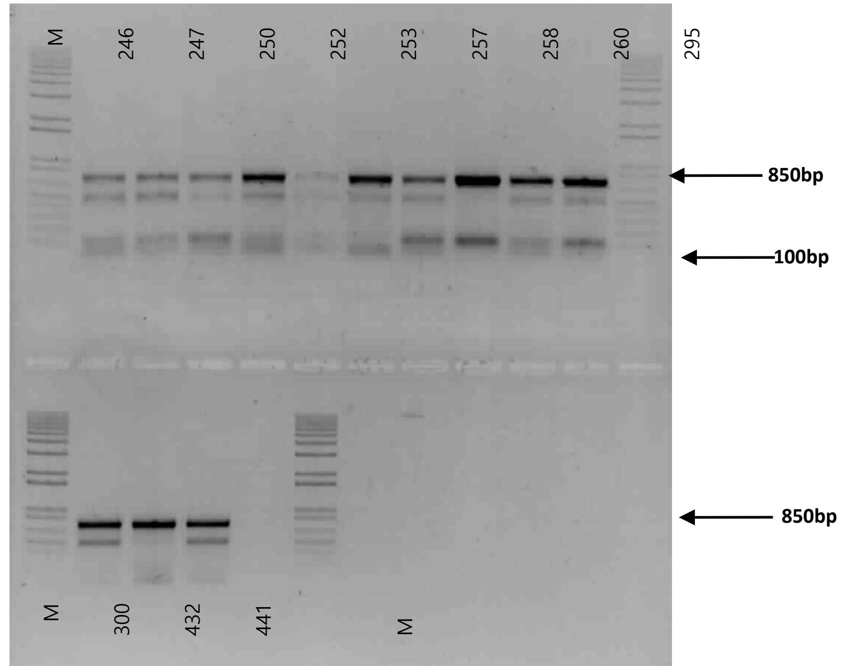


Figure 59: Agarose gel electrophoresis of PCR products (samples 246-441) for purification.

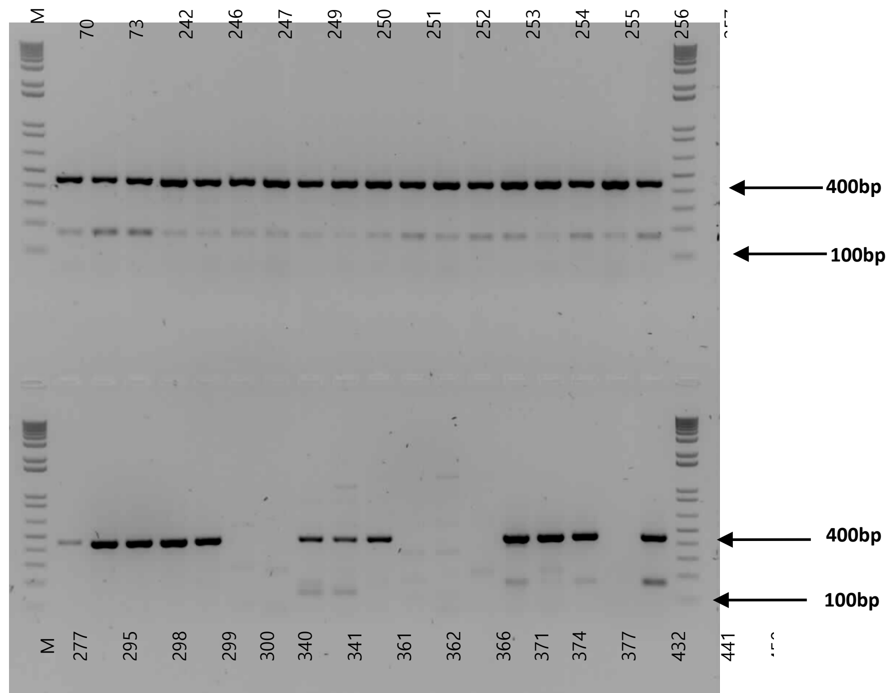


Figure 60: Detection of genome segment encoding VP6 of chicken rotavirus (samples 70-450) using VP6-begf+VP6-470r primers (own design) at 459bp. PCR products were separated on Sybr safe stained 1.5% agarose gels.

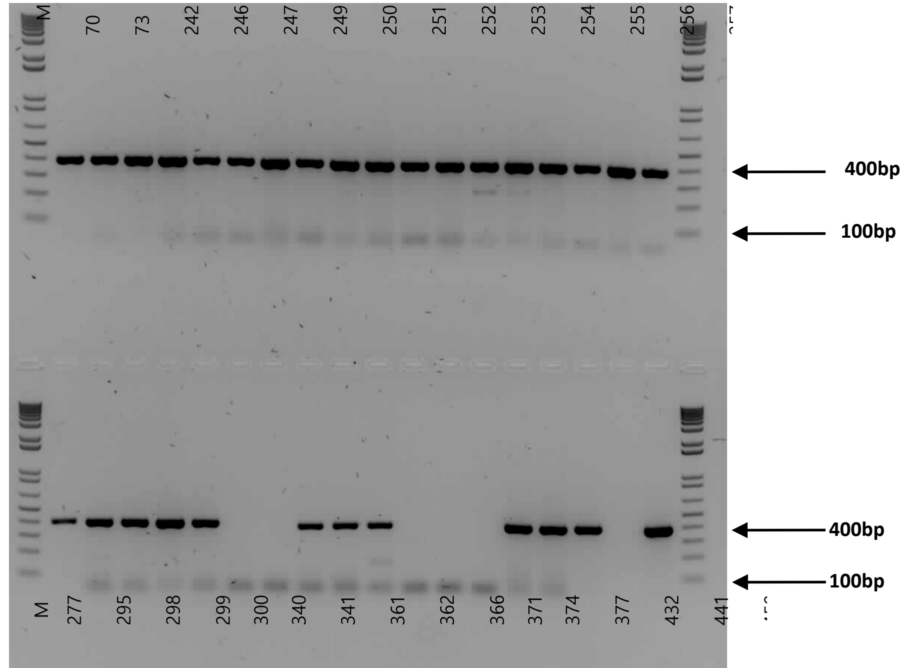


Figure 61: Detection of genome segment encoding VP6 of chicken rotavirus (samples 70-450) using VP6-953f+VP6-endR primers (own design) at 416bp. PCR products were separated on Sybr safe stained 1.5% agarose gels.

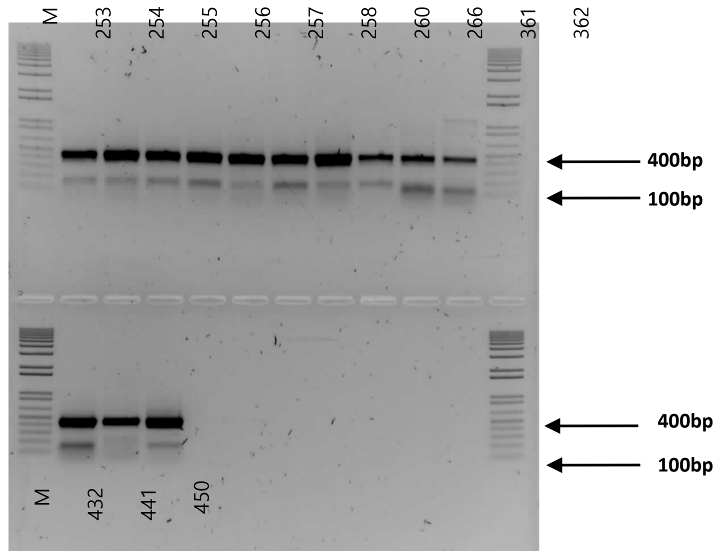


Figure 62: Agarose gel electrophoresis of PCR products of VP6-953f+VP6-end (samples 253-450) for purification.

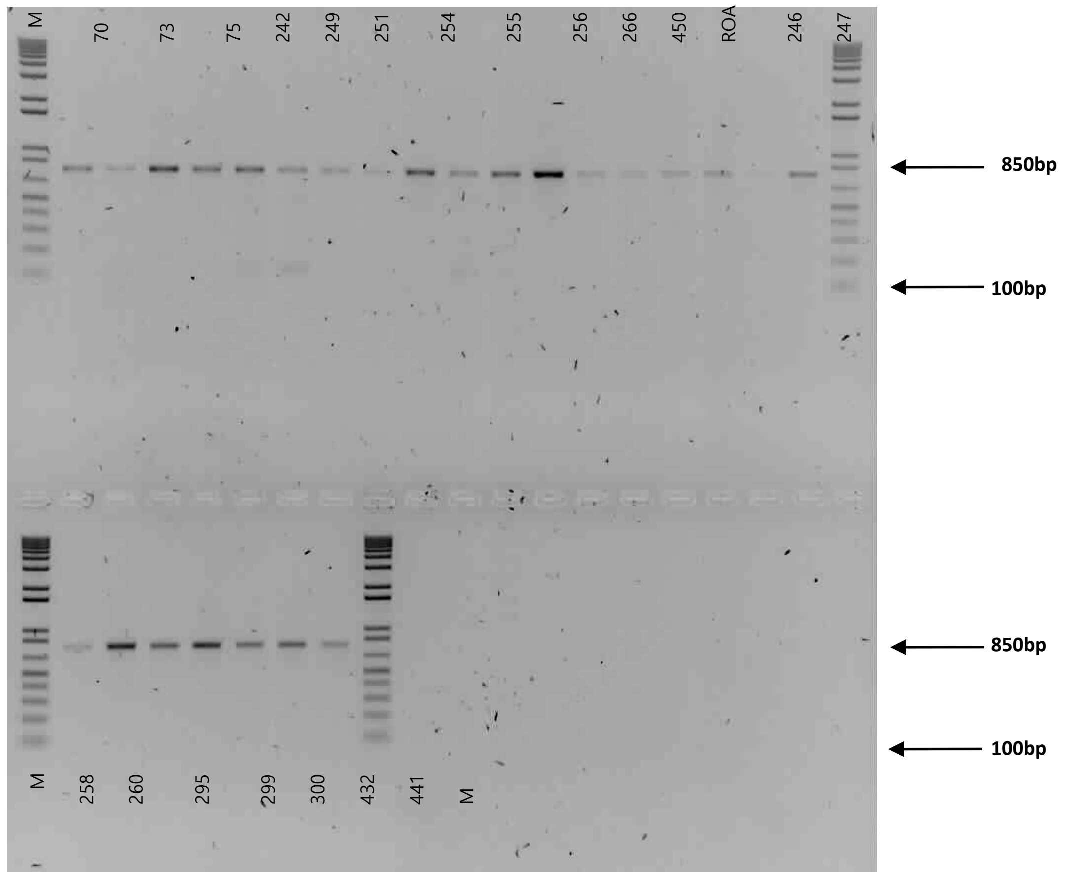


Figure 63: Agarose gel electrophoresis for confirmation of DNA purity by gel extraction and PCR product purification method

4.4.6 Detection of rotavirus A and D (positive control) complete gene segment 7 using VP7 primers:

The whole of Rotavirus A and D positive control gene segment was detected using the designed primer sequence. This means the designed primers were suitable for detecting Rotavirus A and D (Figure 50). The primers were also very specific for the detected gene segment (Figures 64 and 65).

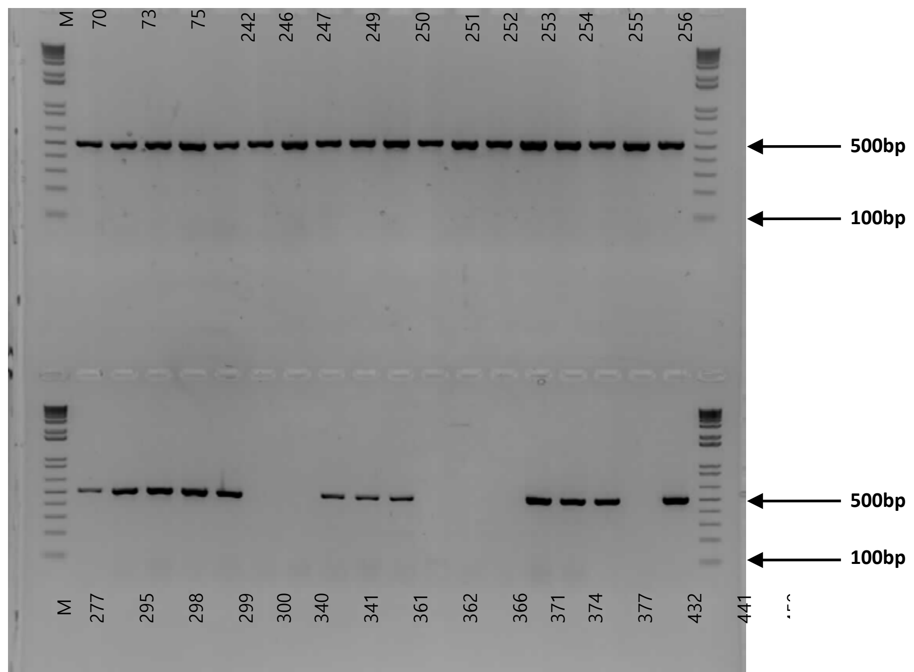


Figure 64: Detection of genome segment encoding VP7 of chicken rotavirus (samples 70-450) using VP7-135f+VP7-647r primers at 512bp. PCR products were separated on Sybr safe stained 1.5% agarose gels.

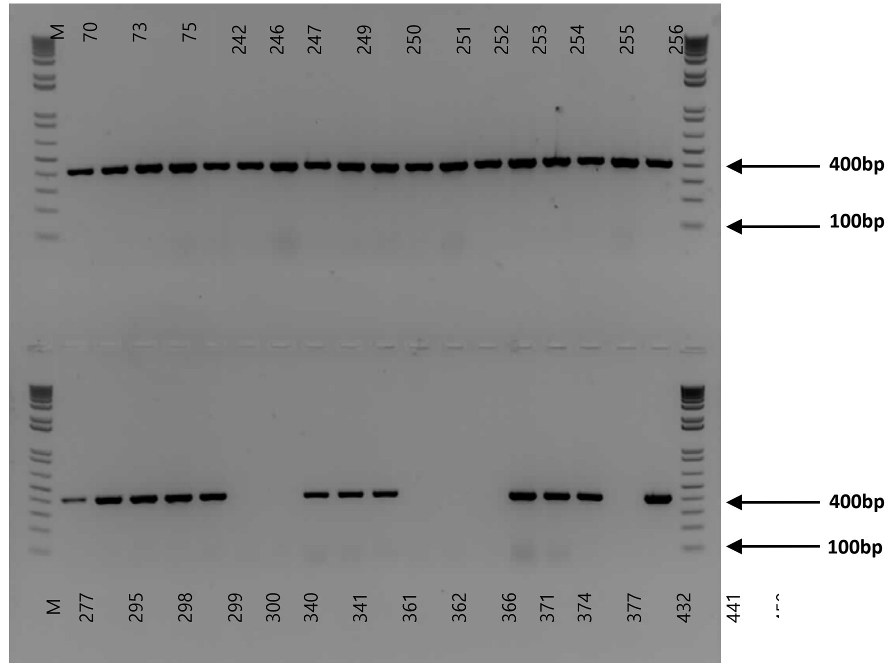


Figure 65: Detection of genome segment encoding VP7 of chicken rotavirus (samples 70-450) using VP7-587f+VP7-1003r primers at 416bp. PCR products were separated on Sybr safe stained 1.5% agarose gels.

4.5 Prevalence of rotavirus in avian species

4.5.1 Age

When grouped according to age, birds younger than 10weeks had a higher percentage (29%) positive for rotavirus than birds older than 10weeks which had 23% of them positive for rotavirus infection. In all 28% of all the samples collected were positive for rotavirus infection (Table 8).

Table 8: Summary of positive samples according to age

Age	Total number of samples	Number Negative	Number positive
<10wks	344	242(70%)	102(30%)
>10wks	120	93(77%)	27(23%)
Total	464	335 (72%)	129 (28%)

In all, 39 (109) flocks from 21 (50) farms were positive for rotavirus infection. A summary of positive samples is as shown in the table above.

4.5.2 Bird type

According to bird type, rotavirus was detected in chickens, crowned crane, eagle, guinea fowl, and parrot (table 9).

Table 9: Distribution of positive samples among birds screened

Bird type	Total num. of samples tested	Number Negative	Number positive (%)
Chicken	360	236	124 (96)
Crowned crane	9	7	2 (1.5)
Duck	10	10	0 (0)
Eagle	2	1	1 (0.8)
Geese	7	7	0 (0)
Guinea fowl	33	32	1 (0.8)
Parrot	3	2	1 (0.8)
Pigeon	27	27	0 (0)
Turkey	13	13	0 (0)
Total	464	335	129

4.5.3 Distribution of rotavirus in flocks screened

From the 109 flocks screened, 39 (36%) were positive for rotavirus infection.

Table 10: Number of flocks screened for each bird type

Bird type	Number of farms screened	Number screened (flock)	Number positive (flock)
Exotic Chicken	26	75	33
Local chicken	5	6	2
Crowned crane	1	4	1
Duck	4	5	0
Eagle	1	2	1
Geese	2	2	0
Guinea fowl	4	5	1
Parrot	1	2	1
Pigeon	4	6	0
Turkey	2	2	0
Total	50	109	39

Table 11: Clinical signs observed in flocks

Clinical signs positive (%)	Number of flock	Number
Apparently healthy	36	8 (22)
Cecal droppings	12	6 (50)
Diarrhea	40	16 (40)
Diarrhea and Mortality	13	7 (53)
Diarrhea, emanciation and Mortality	8	2 (25)
Total	**109**	**39**

4.6 DISCUSSION

The initial detection of rotavirus groups A and D positive controls was carried out with the NSP4 primers (Pantin-Jackwood *et al.*, 2007), the two groups were both detected using these primers, although a much more prominent band was seen with the Rotavirus A sample. In order to have an optimal detection of avian rotaviruses (ARV's) in faecal samples, a PCR optimization experiment was carried out. Results from the real-time PCR optimization experiment indicated mix 4 at an annealing temperature of 50°C had the best detection protocol with a low CT (threshold cycle) value of 19.82 at dilutions d1:1 or d1:10 and CT value of 18.14 at dilution 1:1 for Rotavirus A and D respectively. This reaction protocol was therefore adhered to, for the detection of rotavirus in faeces. It is essential to have an optimized PCR protocol because several studies have missed the diagnosis and typing of rotaviruses due to inadequate PCR protocols.

In determining the presence of ARVs in poultry birds, faecal samples from chickens, crowned crane, duck, eagle, geese, guinea fowl, parrot and turkeys were screened by laying emphasis on the VP4, VP6, VP7 and NSP4 of the genome segments. Despite the optimization experiment which showed good results with the positive controls, the NSP4 primers were not specific for the detected rotavirus in this study. However, detection of rotavirus using the designed primers yielded a better result. Results from the one step RT-PCR were more specific when compared to conventional RT-PCR. The one step RT-PCR has also been reported to be more rapid and sensitive for the detection of rotavirus (Mackay *et al.*, 2002; Kang *et al.*, 2004; Pang *et al.*, 2004). Specific primers were constructed to amplify the RVs-A VP4, VP6 and VP7 protein gene, based on their gene sequences from the GenBank. Due to the diversity and reassortment property of rotaviruses, degenerate nucleotides were included in the designed primers. Some of these primers demonstrated good specificity, as they only amplified the samples that were positive for group A rotaviruses without showing non-specific bands.

Using the VP4 primer pair to detect the positive controls, different regions of the VP4 protein were detected as the primers showed good sensitivity and specificity for the gene segments of rotavirus A and part of rotavirus D. Different regions from beginning to end of the nucleotide sequence were detected for rotavirus A, but not for rotavirus D. However, in detecting rotavirus from samples in this study, the primers showed neither good sensitivity nor specificity for the gene segment. So the gene segments of the VP4

protein were not completely detected. This same pattern of detection occurred for the Rotavirus D positive control also, thus making this segment seem like Rotavirus D VP4. The reason for this inability is because rotaviruses are capable of undergoing reassortment. The Nigerian rotavirus will therefore differ from others based on this reassortment. This segment will thus represent those of the uncommon genotypes of rotavirus. This gene segment consists of 2350nt sequences.

For the detection of the VP6 protein, three different primer pairs were used. Two pairs were designed for the purpose of this study and the third pair was adapted from Schuman *et al.*, (2009). The two pairs were more sensitive in detecting the VP6 segment with prominent bands at 459bp and 416bp respectively. This is because some samples (277, 361, 362 and 366) which were negative for rotavirus with the primers designed by Schumann *et al.* (2009) were seen to be positive with the designed primers. Nucleotides 11-470 were detected with VP6-begf+VP6-470r primers (own design) at 459bp. This primers showed good sensitivity and better specificity than the primer from Schumann *et al.*, 2009. Primer pair Rota-Seg6-s + Rota-Seg6-as at 777bp were used to detect nucleotides 317-1094 (Schuman *et al.*, 2009). The terminal nucleotide sequences were detected with the designed primers seg6-953f + seg6-endR at 375bp and targeting nucleotides 953-1349. These primers were also sensitive and specific although with some primer dimer at less than 100bp. Gene segments for 34 different rotavirus positive samples were detected with the designed primers. Prominent bands were detected using the designed primers than primers from Schuman *et al.*, 2009. Samples which were not specific were purified either by direct PCR product purification or gel purification method. The most sensitive and specific result was achieved using the designed primers for detecting VP7. The VP7 segment consists of 1066nt sequences which was detected with VP7-135f+VP7-647r primers at 512bp and VP7-587f+VP7-1003r primers at 416bp.

A higher prevalence rate (29%) of rotavirus infection in birds younger than 10 weeks of age was seen, with a prevalence rate of 23% in birds older than 10 weeks of age. This is in contrast to the report of Yason and Schat (1986a, 1987) who stated that most naturally occurring infections in turkeys, chickens, pheasants, partridges, and ducks involve birds less than 6 weeks old and older chickens (56-119 days) and turkeys (112 days) were more susceptible to experimental infection than birds in the first few weeks of

life. Birds in this study were naturally infected and not experimentally infected. However, lack of age resistance to infection has been illustrated by an outbreak of diarrhoea associated with rotavirus infection in commercial laying hens between 32 and 92 weeks of age (Jones *et al.*, 1979). Perhaps, diarrhoea occurred in these older birds because of natural infections in the field as seen in this study.

In all, 464 samples were screened using the one and two-step RT-PCR protocols. Of these, 129 samples were positive for rotavirus. All the positive samples were from chicken (exotic and local chicken), crowned crane, eagle, guinea fowl and parrot. The method of management of these birds in Nigeria thus increases the chances of reassortment of rotaviruses between these birds not leaving out a zoonotic transmission to human beings. Birds showing clinical signs related to diarrhea and mortality also had the highest prevalence of rotavirus infection and the least prevalence was in apparently healthy birds and is also as reported by Estes and Kapikian, (2007). None of the other types of birds was positive; this however does not mean they are not susceptible to rotavirus infection. Sequences from the GenBank also indicate these birds could be infected by different strains of the virus. The implication of this is that the virus which is shed in faeces will easily circulate in the environment and probably reassort due to the close proximity at which these birds are reared in some households not leaving out a zoonotic transmission to man. The initial detection of rotavirus in this study was carried out with the NSP4 primers as designed by Pantin-Jackwood *et al.*, 2007. Results from this initial detection indicate the primers were not specific, thus gel and PCR product purifications were carried out using the JetQuick purification kit. The purified products were re-run on agarose gel to confirm they were at the right base pair. This non-specificity of the primers might be due to the presence of contaminants in the tested samples or reassortant viruses.

The infectious diseases of the gastrointestinal tract that affect birds have a worldwide distribution and a variety of etiologies (Yegani and Korve, 2008). Among the viral agents, rotaviruses are the most common viruses in birds and are found both in birds with enteropathic infections and in healthy birds (Tamehiro *et al.*, 2003; Villarreal *et al.*, 2006; Pantin-Jackwood *et al.*, 2008). In this study, some birds were found diarrheic, dehydrated, and anorectic with low body weight and increased mortality in flocks. These

observations are in conformity with the earlier reports of McNulty (2003) and Tamehiro *et al.* (2003) who reported that in field conditions, rotavirus infections in poultry might induce subclinical manifestations, or they might be associated with enteritis, dehydration, anorexia, unrest, litter ingestion, low weight gain and increased mortality. Cumulatively, all these can lead to huge economic losses to poultry production systems (McNulty 2003; Villarreal *et al.*, 2006). Chickhood mortalities in Nigerian poultry have previously been linked to various factors including disease; however, none has been related to rotavirus infection. Ngoveni *et al.* (2012) also stated there are no reports of avian rotavirus on the African continent and in their study in birds, they detected genotypes similar to those commonly detected in humans suggesting human to chicken transmission of rotavirus. Although rotavirus infection is a common cause of diarrhoea in birds, there are surprisingly no epidemiological and molecular data on it in Nigeria to the best of my knowledge. This work provides the first information about the occurrence and prevalence of avian rotavirus in Nigeria.

The potential economic resources of the poultry industry may not be fully utilized until the etiological agent of diseases are recognized and possibly controlled, it is thus important to conduct epidemiological studies to determine prevalence of rotavirus in all regions of Nigerian poultry with subsequent genetic characterisation of the virus. This study has established the presence of rotavirus in some commercial poultry farms, backyard poultry and live bird markets. It is thus important to minimize mixing of different avian species together to prevent cross transmission and reassortment of the virus from different birds. Strict adherence to the hygienic practices on farms may minimize environmental contamination thereby reducing the possibility of human exposure to the pathogen. National surveillance and monitoring programs for rotavirus in humans and animals as obtained in some countries in Africa and developed countries is recommended for Nigeria to facilitate early diagnosis and control. This surveillance is recommended and also carried out by the African Rotavirus Surveillance Network (AFR RSN) which at some of its workshops detected uncommon and mixed genotypes of rotavirus. This network has also identified the human-animal reassortant rotavirus strains circulating in human population (Seheri *et al.*, 2012).

CHAPTER FIVE

5.0 CHARACTERISATION OF AVIAN ROTAVIRUS FROM SOUTHWESTERN NIGERIA

5.1 INTRODUCTION

Viruses of the genus Rotavirus (family Reoviridae) have a genome comprising 11 segments of dsRNA, encoding six structural proteins (VP1-7) and six non-structural proteins (NSP1-5). Classification of rotaviruses in groups is mainly based on antibody reactivity or sequence identity of VP6, which is most conserved among the major structural proteins. Genotyping of viruses within group A is mainly based on sequence identities within VP4 and VP7 leading to P (protease-sensitive) and G (glycoprotein) types, respectively (Gorziglia *et al.*, 1990).

Seven groups (A-G) and two major subgroups (SG I and SG II) are also classified according to VP6 protein antigenicity, which is the middle capsid protein (Estes, 2001). Many epidemiological studies have used subgrouping enzyme immunoassays, and most of the human isolates fall into SG II (Arista *et al.*, 1990; Iturriza-Go´mara *et al.*, 2001) whilst animal isolates fall into SG I (Tang *et al.*, 1997). The non-structural protein NSP4 is encoded by segment 10 and serves as an intracellular receptor on the membrane of the endoplasmic reticulum for double-layered particles (DLPs) and interacts with viral capsid proteins (Taylor and Bellamy, 2003). In addition, NSP4 has been found to have an enterotoxin-like activity, originally mapped between aa 114 and 135. Modifications in the toxigenic activity and virulence of rotavirus have been associated with amino acid changes in this region (Ball *et al.*, 1996; Zhang *et al.*, 1998). NSP4 appears to be well conserved across all genotypes (Lee *et al.*, 2000; Lin and Tian, 2003). The rotavirus A NSP4 gene has been sequenced, and five distinct genetic groups (genotypes), A-E, have been classified. Genotypes A, B and C, or KUN, Wa and AU-1, have been detected in humans, while genotypes D and E, or EW and avian-like, have been detected in animals (Mori *et al.*, 2002; Lin and Tian, 2003). Each NSP4 genotype appears to segregate according to the rotavirus host species (Ciarlet *et al.*, 2000). A novel classification system has been proposed using the nucleotide sequences of all rotavirus genome segments, which enables

a comprehensive characterisation of group A rotavirus strains taking into account possible reassortment events (Matthijnssens *et al.*, 2008a; Matthijnssens *et al.*, 2008b).

Transmission of group A rotaviruses between animals and humans has been described repeatedly. Earlier studies based on RNA–RNA hybridization identified possible reassortment events between human strains (Ward *et al.*, 1990) or between human and animal strains (Nakagomi and Nakagomi, 2002). Recently, genome sequencing led to the detection of an entire animal virus in humans (Matthijnssens *et al.*, 2006) or to the detection of individual genome segments of animal rotaviruses in the backbone of a human virus indicating a reassortment event between both virus types (Khamrin *et al.*, 2006; Mascarenhas *et al.*, 2007; Rahman *et al.*, 2007). From an evolutionary point of view, a common origin of human Wa-like strains with porcine rotaviruses and human DS1-like strains with bovine rotaviruses has been proposed based on a high number of shared genotypes (Matthijnssens *et al.*, 2008a). Interspecies transmission is thought to continuously contribute to genetic variability of human rotaviruses (Müller and Johne, 2007). A variety of rotaviruses of groups A, D, F and G has been detected in several avian species including chicken, turkey and pigeon (McNulty *et al.*, 1980; McNulty, 2003; Estes and Kapikian, 2007); however, only group A rotaviruses have been characterised in more detail. An association of rotaviruses with diseases of poultry including acute diarrhoea and the chronic runting and stunting syndrome has been proposed (McNulty, 2003; Otto *et al.*, 2006). The only avian strain completely sequenced so far is the group A rotavirus strain PO-13, which has been originally isolated from a pigeon (Ito *et al.*, 2001). There have been several reports on the detection of avian rotavirus genotypes similar to those found in humans around the world and in South Africa (Nakagomi and Nakagomi, 1989; Ngoveni *et al.*, 2012) suggesting human to chicken transmission of rotavirus. There are also reports that animal rotaviruses can indeed infect humans and cause disease whenever the chance exists (Nishikawa *et al.*, 1989). This is based on the identification of unusual rotavirus types, with properties of strains more commonly found in animals, which were isolated from various cases of human infection. These unusual human rotavirus types may have arisen either as whole virions or as genetic reassortants between human and animal strains during co-infection of a single cell. This study was thus carried out to provide avian

rotavirus sequences for comparison with those of human and other animals in order to determine their zoonotic potential.

5.1.1 JUSTIFICATION

A binary classification system has been established for rotaviruses, with the viral capsid protein VP7 defining G types and VP4 defining P types. At least 15 G types and 21 P types have been isolated globally with various G and P combinations (Rahman *et al.*, 2003). They also exhibit diverse and complex serotypic specificities (Hoshino and Kapikian, 2000). Avian rotaviruses are broadly distributed among birds, but only scarcely characterised at the molecular level. Interestingly, rotavirus strains or rotavirus genome segments of avian origin have been identified in calves (Brussow *et al.*, 1992), suggesting that interspecies transmission of group A rotaviruses from birds to mammals may occur in nature. There is also increasing evidence that transmission of rotaviruses from animals to humans occurs and that it significantly contributes to genetic variability of human rotaviruses (Palombo, 2002; Cook *et al.*, 2004; Müller and Johne, 2007). Adah *et al.* (2001) have reported an unexpected diversity among rotavirus strains whose VP4 segments were untypeable in Nigerian children and emphasize the need for further serological and genetic surveys on more rotavirus strains in African countries, including Nigeria. Domestic animals and humans live closely together in many African rural regions and often share the same source of water, increasing the chance of animal-human transmission and mixed rotavirus infections (Rahman *et al.*, 2003). Due to such close proximity of man and the avian species in Nigeria, it is not impossible that ARVs would reassort with the HRVs. Thus classification of avian rotaviruses is pivotal not only for investigating the genetic diversity of ARVs but also for understanding the origin of unusual strains and their zoonotic potential.

5.1.2 AIM

To characterise rotavirus detected in faeces of poultry birds in south western Nigeria.

5.1.3 OBJECTIVES

5.1.3.1 General objectives

i. To determine the groups of rotavirus present in poultry flocks.

ii. To characterise some gene segments of avian rotavirus present on some poultry farms.

5.1.3.2 Specific objectives

i. To determine the P and G types of avian rotavirus present on some poultry farms.

ii. To determine the nucleotide sequences of detected rotaviruses in order to assess their phylogenetic relationships with the known rotaviruses.

iii. To investigate the genetic diversity of avian rotavirus from south western Nigeria.

iv. To determine any relationship between avian rotaviruses from Nigeria and mammalian rotavirus.

v. To determine the presence of unusual rotavirus types in southwestern Nigeria.

5.2 MATERIALS AND METHODS

5.2.1 Location of study

Sequencing of rotavirus segments and analysis was carried out at the Institute of immunology, Laboratoire Nationale de Sante, Luxembourg.

5.2.2 cDNA preparation

- Genomed: Jet quick PCR Purification Kit
- Ethanol Molecular - Biology grade
- Eppendorf centrifuge
- 1.5 ml centrifuge tubes
- Pipettes and tips
- Heating block 70°C

5.2.3 DNA quantification

- Picogreen (molecular probes P-11496)
- Tecan Genios Fluorescence reader (ex. 480 nm; em. 520 nm)
- 96 well plates black plate (Greiner 6565076)
- Pipettes and tips

For cycle sequencing reaction, defined quantities of PCR product are required for an optimal result.

Table 12:Quantity of DNA used for sequencing

Template	Quantity
100-200 bp	1 - 3 ng
200-500 bp	3 -10 ng
500-1000 bp	5 - 20 ng
1000-2000bp	10 - 40 ng
<2000 bp	20 - 50 ng

5.2.4 Cycle sequencing PCR

Big Dye terminator v3.1 cycle sequencing kit (Applied Biosystems cat# 4337456)

- 96 well PCR plate, non flexible (Biozym cat# B70651)
- Mastercycler PCR machine (Eppendorf)
- Pipettes and tips
- 5 µM Primers
- Template PCR product
- ddH_2O

5.2.5 Post Sequencing PCR Purification

- ddH_2O
- 125 mM EDTA
  - 250 µl 500mM EDTA in 1.5 ml centrifuge tube
  - 750 µl ddH_2O
- 100 % Ethanol Molecular Biology Grade (Merck 1.08543.0250)
- 70% Ethanol;
  - 7 ml 100 % ethanol in 15 ml tube
  - 3 ml ddH_2O
- Sodium acetate 3M
  - 26.409 g NaCH3COON (Merck 1.06268.1000)
  - 100 ml ddH_2O
  - pH 5.2
  - Autoclave
  - Store at room temp for 12 months
- Centrifuge
- Pipettes and tips
- Speed-Vac

5.2.6 Sample preparation for electrophoresis

- HI-DI (highly deionised formamide) from ABI (stored at –20°C)

5.2.7 Multiple sequence and phylogenetic analyses

- BioEdit program (Hall, 1999)
- MEGA version 5.05 (Tamura *et al.*, 2011)
- Rotavirus online classification tool: RotaC (http://rotac.regatools.be/) were used for data analysis.

5.2.8 METHODS

5.2.8.1 cDNA preparation

Before running a sequencing PCR, the DNA has to be separated from primers and none incorporated nucleotides, which would otherwise interfere with the sequencing PCR reaction. This is easily performed using a DNA binding spin column. Small fragments like Primer and non-incorporated nucleotides pass through the filter while the larger PCR fragments (80 bp-20 kbp) are bound and subsequently eluted.

5.2.8.2 Procedure for cDNA preparation

Before starting, binding buffer H1 was reconstituted with isopropanol and wash buffer H2 with absolute ethanol (96–100%) according to the instructions on the label of the bottles.

5.2.8.3 Purification of PCR products by centrifugation

1. **Loading:** 4 volumes of binding buffer (H1) was added to 1 volume of a PCR sample, mixed well and placed in a JetQuick® Spin Column into a 2-ml receiver tube. Sample was pipette into the prepared column and column centrifuged at >12,000 × *g* for 1 minute.

2. **Washing:** Column was re-inserted into empty receiver tube and 500 µl wash buffer (H2) added with ethanol. Column was then centrifuged at >12,000 × *g* for 1 minute. The flow-through was discarded and column placed in the same 2-ml receiver tube. Column was again centrifuged at >12,000 × *g* for 1 minute.
3. **Elution:** The column was placed in a new 1.5-mL microcentrifuge tube. 50 µl of TRIS-EDTA (TE) buffer added to the column and centrifuged at >12,000 × *g* for 2 minutes.
4. **Storage:** The elution tube contains the purified PCR product. The purified DNA was stored at -4°C for immediate use or at -20°C for long-term storage.

5.2.8.4 Purification of PCR products by gel extraction:

PCR product was loaded on a 1% agarose gel and was then run until bands were correctly separated. Band of interest was cut with a scalpel blade on UV light. Piece of agarose was placed in 1.5-mL eppendorf tube. This was then weighed and noted on tube. Three times (3X) the weight/volume of QG buffer was added and incubated at 50°C for 10minutes. After incubation 1X volume of 100% isopropanol was then added and vortexed. The resulting fluid was loaded on the provided column and centrifuged at 13000rpm for 1minute. The flow-through was then discarded. Five hundred microlitres of QG buffer was added and centrifuged at 13000rpm for 1minute. Flow-through was again discarded and 750µl of PE buffer was added and centrifuged at 13000rpm for 1minute. Flow-through was again discarded and centrifuged at 13000rpm. Column was then placed in a labeled eppendorf tube and 30 µl of elution buffer was added. This was allowed to incubate for 1 minute and then centrifuged at 13000rpm for 1 minute.

5.2.8.5 Verification of PCR product purification

Five microlitres (5 µl) of purified PCR product mixed with 6X loading dye was loaded in 1.5% agarose gel. Gel was run in an electrophoretic tank at 120 volts for 45 minutes and then visualized on a UV illuminator. The presence of a specific band at the appropriate base pair was regarded as having a pure PCR product.

5.2.8.6 Sequencing using ABI capillary sequencer

5.2.8.7 Protocols

A. Sample preparation / PCR product purification (as described above)
B. DNA quantification
C. Cycle Sequencing PCR
D. Post PCR purification
E. Sample preparation for electrophoresis
F. Capillary electrophoresis
G. Data analysis

5.2.8.8 DNA quantification Methods

The necessary volumes for assay were calculated; 100 µl diluted test sample volume + 100 µl diluted PicoGreen. Kit components were then brought to room temperature and 20xTE

was diluted to 1x TE in autoclaved ddH_2O. 2 µg/ml stock solution of positive control dsDNA (10 µl with 490µl TE) in 96 well plate for standard was prepared.

For best accuracy, a serial pre-dilution of the standard was made in advance and stored in aliquots of 1 ml, mixed well, incubated for 5 minutes in the dark and measured in the Tecan Genios with ex. ~480 nm and em. ~520 nm filter set (Pico green protocol on the Magellan software).

5.2.8.9 Cycle sequencing PCR

The necessary template for reaction was calculated. For templates of 300 bp to 1000 bp, 10ng was used as the standard amount. The ready mix was thawed at 4°C and vortexed before use. For each sample 8uL of the following mix : 50% Big Dye, 25% ddH_2O, 25% 5X TE buffer from ABI was prepared. Columns Forward (F) and columns Reverse (R) were labelled and reaction per PCR product (Forward and a Reverse reaction in the 96 well plate) was prepared.

In each well, 4µl of reaction mix, 1µl primer, the template (max 5µl), H_2O to 10µl was dispensed in columns and closed with strip-lids. The plate was placed in centrifuge with a balance plate, spinned down for 10 sec without vacuum drying and the following program was run in the master cycler:

Table 13: Master cycler protocol for sequencing

	Temperature	**Time**
1	96°C	2 min
2	Pause-push enter	
3	96°C	1 min
4	96°C	10 sec
5	50°C	5 sec
6	60°C	4 min
7	Goto step 4	Repeat 25x
8	10°C	Hold

The samples were then stored at 4°C for post cycle reaction purification

5.2.9.0 Post Sequencing PCR Purification

The strip-lids were removed from the PCR plate. For each sample 15µl of the following mix was prepared: 1/3 125mM EDTA; 2/3 ddH2O.

Fifteen microliters was dispensed per well and mixed well. 60 µl 100% of ethanol was added to each reaction mix, and mixed well. Wells were closed with strip-lids, mixed again by inverting the plate several times. Making sure each well had a sufficient mix and incubated 15 min at room temp in the dark. This was then centrifuged for 30 min, 3000 RPM, 4°C. Immediately afterwards the strip-lids were removed and plate was inverted on tissue paper in the centrifuge plate holder, centrifuged at 900 RPM for 1 minute and 60 µl of 70% Ethanol added to each well. It was then centrifuged for 15 min, 3000 RPM, 4°C. Immediately afterwards the strip-lids were removed, plate was inverted on tissue paper in the centrifuge plate holder and centrifuge at 900 RPM for 1 minute. 15 min post centrifuge drying was carried out. Wells were then closed with strip-lids and stored at 4°C when loading on the sequencer is within hours, otherwise stored at -20°C.

5.2.9.1 Sample preparation for electrophoresis

Strip-lids were removed and 10µl HI-DI was added in each sample. One well was filled with DNA standard from ABI and wells were closed with strip-lids. Wait for lid to be above 100°C and plate loaded. This was boiled for 5 minutes at 95°C and immediately after, the plate was put on ice loaded into the sequencer.

5.2.9.2 Data analysis

5.2.9.3 Multiple sequence and phylogenetic analyses

Sequences were analysed using BioEdit program (Hall, 1999). Sequences were aligned with ClustalW multiple alignments. Phylogenetic analyses at nucleic acid and amino acid levels was conducted with the neighbour-joining method using the Kimura two-parameter model and pairwise deletion with MEGA version 5.05 (Tamura *et al.*, 2011). Bootstrap analysis of phylogenetic tree was performed with 1000 replicates and values are indicated on the tree. Previously published avian rotavirus sequences from GenBank were included in the phylogenetic analyses as reference strains.

5.3 RESULT

5.3.1 Genome sequence determination

The genome of rotavirus from this study was assembled from overlapping nucleotide sequences of PCR products amplified using primers listed in table 5. The PCR products from four segments (VP4, VP7, VP6 and NSP4) were compared with previously published sequences of rotaviruses. The rotaviruses in this study had sequence homology of 95 to 100% among themselves at the nucleotide level, whereas the sequence homology with already published rotaviruses of chicken origin ranged between 74 to 97%.

5.3.2 Phylogenetic analysis

Phylogenetic trees were constructed on the basis of the entire nucleotide sequences of the genome segments or the deduced amino acid sequences. Most of the resulting trees showed a largely similar grouping irrespective of whether nucleotide or amino acid sequences had been used for alignment.

Only partial sequences of the VP4 gene were analyzed because the full length of the segment was not detected. These were aligned to full-length (except for the VP4) avian Rotavirus sequences available in the GenBank database. Alignments were performed based on nucleotide sequences of the genome segment or the deduced amino acid residues. Generally, the phylogenetic trees established on the basis of the alignments show that for all genes the avian sequences from the same farm in this study form same branch in a cluster when compared with sequences in the GenBank database (Figures 67,69,71, and 73). The sequences encoding VP6, VP7 and NSP4 in this study cluster together with sequences derived from chicken isolates. Partial sequences of the VP4 did not cluster together with the sequences of other reference sequences from the GenBank for chicken, turkey or pigeon isolates except the Ch-06V0661, thus suggesting a reassortment event between a chicken virus and another virus of unknown source. Phylogenetic analysis of the complete nucleotide sequences of VP6, VP7 and NSP4 from different farms showed a separate clustering with high heterogenicity for different farms. This may be due to a reassortment of samples separately on different farms.

5.3.3 Sequence analysis

5.3.3.1 VP4 segment

Most of the samples were negative with the primers used to detect the VP4 gene. Only a partial sequence of the positive samples was sequenced and the partial sequence only clustered with strain Ch-06V0661 VP4 (Figure 67). The partial sequence also had 97.4% aa homology with Ch-06V0661, 69.3% with isolate Tu-03V0001E10, 72.2% with Ch-02V0002G3 and 68.7% with PO-13. The partial and complete nucleotide sequence of strains in this study and the positive control are as shown in the appendix.

```
                                            10        20        30        40        50        60        70        80        90
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|223862733[02V0002G3 complet     GYKMASLVYRQLLANSYTSELQDTIDDISAQKSQDVTINPGPFAQTGYAPVEWTHGDITTDETIEQTLDGPYTSSSIIIQPQYWILANPDTEGVI
1111924-OO-_Rotavirus249_VP4aR     -----------------------------------------------------------------------------------------------
1111925-OO-_Rotavirus256_VP4aR     ------------------.D...N......E.T.G.......................PV.....P.......A...V........V..TNQD.V
1111926-OO-_Rotavirus260_VP4aF     ---------------------------...E.T.G.......................PV.....P.......A...V........V..TNQD.V

                                           110       120       130       140       150       160       170       180       190
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|223862733[02V0002G3 complet     TDQKYARVMLLPNTADGNKQYTILGHRVTINLGNTNSNMYKFCDLVSHNGTTYTKVEELVTPHGLNAFMKDQGKLYLYNGTVPNVQQVYYTLANI
1111924-OO-_Rotavirus249_VP4aR     --X.F.C...PR..PEYDR......KQI..R....DTVN......L.ND.II...SI..I...........A....A.S.II.TIKKE.H.ID..
1111925-OO-_Rotavirus256_VP4aR     QNK.F.C...P...PESDR......KQI..R....DTVN......L.ND.II...SI..I...R.......A....A.S..I.TIKKE.H..D..
1111926-OO-_Rotavirus260_VP4aF     QNK.F.C...P...PESDR......KQI..R....DTVN......L.ND.II...SI..I...R.......A....A.S..I.TIKKE.H..D..

                                           210       220       230       240       250       260       270       280       290
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|223862733[02V0002G3 complet     NIKCNYYIVPKTQTGQLEDYIKNGLPPIQESRYIVPVNRSVINVYQSRPNEDILISKTSLWKEVRYNRDILIRFKFGNNIIKSGGLGYKWSEISF
1111924-OO-_Rotavirus249_VP4aR     ..X--------------------------------------------------------------------------------------------
1111925-OO-_Rotavirus256_VP4aR     ..T.E.....NS.----------------------------------------------------------------------------------
1111926-OO-_Rotavirus260_VP4aF     -----------------------------------------------------------------------------------------------

                                           310       320       330       340       350       360       370       380       390
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|223862733[02V0002G3 complet     EYTYERDGETVVAHTTCSVAGINNFGYNSGSLPTDLVVSTYEVLKGNSYVYIDYWDDSQAFKNMVYVRSLSAEFNAISCNGGTFNFQLPVGQWPQ
1111924-OO-_Rotavirus249_VP4aR     -----------------------------------------------------------------------------------------------
1111925-OO-_Rotavirus256_VP4aR     -----------------------------------------------------------------------------------------------
1111926-OO-_Rotavirus260_VP4aF     -----------------------------------------------------------------------------------------------

                                           410       420       430       440       450       460       470       480       490
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|223862733[02V0002G3 complet     VTLNSDAVTLSTQYTDFVSLNSLRFRFKPAIGEPSFEITRTRERRLYGLPAANPMGNXRIL*NCW*IFFNFTCSI***LSNSYSKFNYS*ARFRA
1111924-OO-_Rotavirus249_VP4aR     -----------------------------------------------------------------------------------------------
1111925-OO-_Rotavirus256_VP4aR     -----------------------------------------------------------------------------------------------
1111926-OO-_Rotavirus260_VP4aF     -----------------------------------------------------------------------------------------------

                                           510       520       530       540       550       560       570       580       590
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|223862733[02V0002G3 complet     A*RV*SVIIRNSCITIS*LSFITA*YVFNVLRN*INNRCSEIGNNLSYEEDESIDVSKVSIYNYGRIIRRSYFNFKIIINTFECISME*LNRYGN
1111924-OO-_Rotavirus249_VP4aR     -----------------------------------------------------------------------------------------------
1111925-OO-_Rotavirus256_VP4aR     -----------------------------------------------------------------------------------------------
1111926-OO-_Rotavirus260_VP4aF     -----------------------------------------------------------------------------------------------

                                           610       620       630       640       650       660       670       680       690
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|223862733[02V0002G3 complet     F**RSNTNLKNSHEIKGKRICDTDRRRIEF*RHISCCIEDENR*A*RCTTKIITYNYS*FSG*VYTIKRI*NN**RYCI*NIK*WTIFCL*SGYV
1111924-OO-_Rotavirus249_VP4aR     -----------------------------------------------------------------------------------------------
1111925-OO-_Rotavirus256_VP4aR     -----------------------------------------------------------------------------------------------
1111926-OO-_Rotavirus260_VP4aF     -----------------------------------------------------------------------------------------------

                                           710       720       730       740       750       760       770       780
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
gi|223862733[02V0002G3 complet     *CRKVR*SCYGFTSNISYNRL*NHKKSKR*FRNN*RASV*FAKI*SKSS*RIHKSE*SNYKK*NRTTHTSM*NIIENKAVYEDLT
1111924-OO-_Rotavirus249_VP4aR     -------------------------------------------------------------------------------------
1111925-OO-_Rotavirus256_VP4aR     -------------------------------------------------------------------------------------
1111926-OO-_Rotavirus260_VP4aF     -------------------------------------------------------------------------------------
```

Fig. 66: Comparison of deduced amino acid of partial sequences of VP4 protein of Avian rotavirus

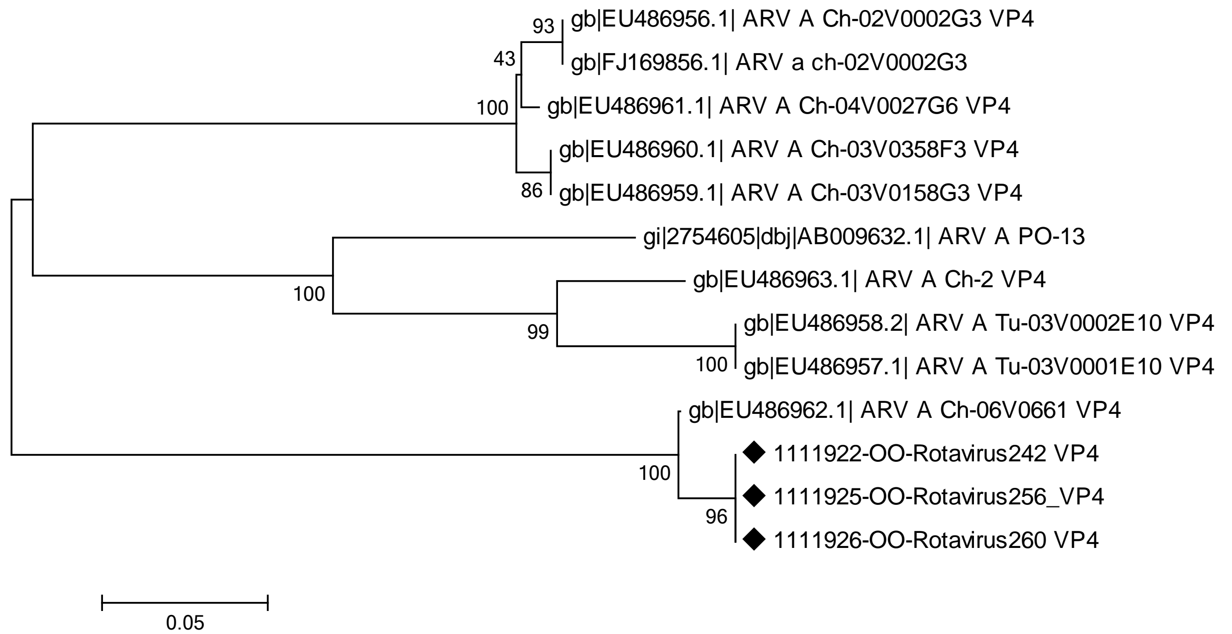


Figure 67: Phylogenetic tree of amino acid sequences of VP4 gene of Rotavirus. The marked sequences are from this study and those unmarked are GenBank sequences. Bootstrap value are shown on tree.

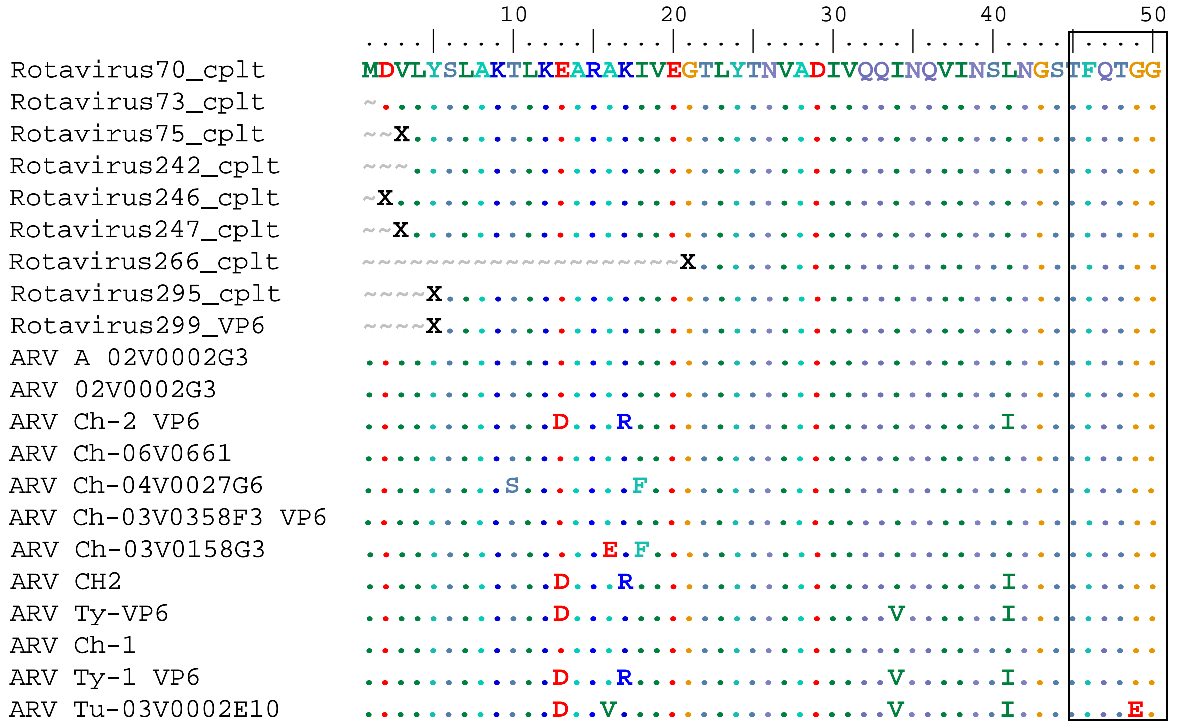
5.3.3.2 VP6 segment

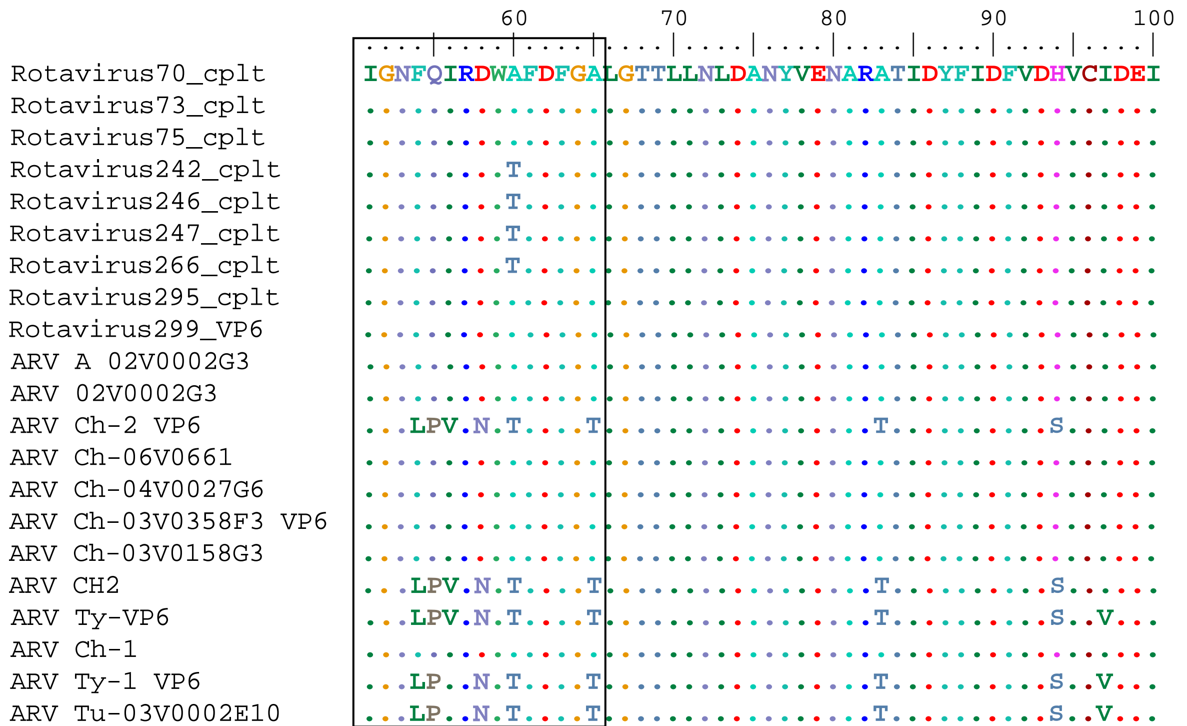
All of the isolates analyzed in this study cluster together with known avian rotavirus sequences. The VP6 nt sequence of the isolates share 74.7% to 79.3% homology with the Ch-2 strain which belong to the same genotype I4 with Tu-1E10, Tu-2E10 and PO-13 (Matthijnssens *et al.*, 2008a). Also, they have 88.1% to 95.4% homology with Ch-04V0027G6, Ch-03V0158G3, Ch-03V0358F3, and Ch-06V0661which all belong to genotype I11 (Matthijnssens *et al.*, 2008a). Isolates from the same farm had 95.5% to 100% identity. The VP6 gene sequence from the strains was 1348 nucleotides long. The ORF started at nucleotide 24 and terminated at nucleotide 1214 and codes for 397 aa as reported for most group A rotaviruses (figure 68). The histidine residue at position 153 involved in zinc binding (Erk *et al.*, 2003) is conserved in all isolates.

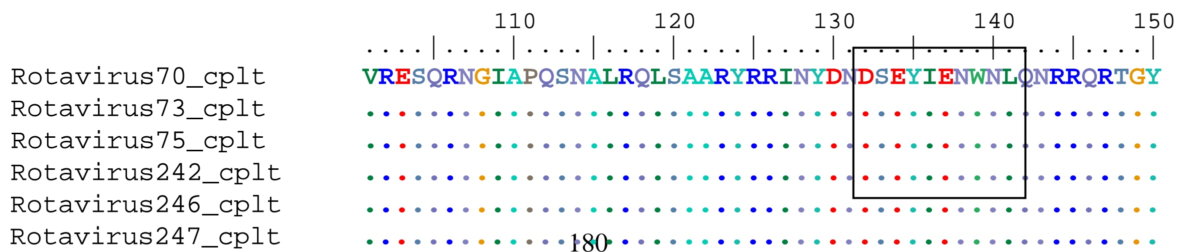
In comparison with other avian rotaviruses, one amino acid differences at positions 60 was noted between samples 242, 246, 247, 266 and the other chicken rotaviruses in the designated group A specific antigenic site I (45–65). This was changed to threonine from alanine residues as occurred in strain Ch-2 which is an I4 genotype, which makes the VP6 segment seem to be reasserting with the turkey VP6 segment. Substitution of aa is also seen in positions 218,224,242,255,262 and 264, for sample 246 with 95.5% aa identity with other samples from the same farm. In addition, two important SG specific amino acid residues Gln298 and Val310 in VP6 sequences of rotaviruses were substituted by His and Gln respectively in sample 246 which also points at a reassortment in this region.

As shown by phylogenetic tree (Figure 69) the VP6 aa sequence of this sample formed a separate cluster branch from Ch-06V0661 with which they have similar VP4 partial nt sequence.

The present results show that avian RV are divided in several types, as already shown using monoclonal antibodies against VP6 (Minamoto *et al.*, 1993). To investigate the presence of different types of avian Rotavirus it is necessary to isolate and characterise more avian rotavirus strains.


10 20 30 40 50
Rotavirus70_cplt MDVLYSLAKTLKEARAKIVEGTLYTNVADIVQQINQVINSLNGSTFQTGG
Rotavirus73_cplt
Rotavirus75_cplt
Rotavirus242_cplt
Rotavirus246_cplt
Rotavirus247_cplt
Rotavirus266_cplt
Rotavirus295_cplt
Rotavirus299_VP6
ARV A 02V0002G3
ARV 02V0002G3
ARV Ch-2 VP6
ARV Ch-06V0661
ARV Ch-04V0027G6
ARV Ch-03V0358F3 VP6
ARV Ch-03V0158G3
ARV CH2
ARV Ty-VP6
ARV Ch-1
ARV Ty-1 VP6
ARV Tu-03V0002E10


60 70 80 90 100
Rotavirus70_cplt IGNFQIRDWAFDFGALGTTLLNLDANYVENARATIDYFIDFVDHVCIDEI
Rotavirus73_cplt
Rotavirus75_cplt
Rotavirus242_cplt
Rotavirus246_cplt
Rotavirus247_cplt
Rotavirus266_cplt
Rotavirus295_cplt
Rotavirus299_VP6
ARV A 02V0002G3
ARV 02V0002G3
ARV Ch-2 VP6
ARV Ch-06V0661
ARV Ch-04V0027G6
ARV Ch-03V0358F3 VP6
ARV Ch-03V0158G3
ARV CH2
ARV Ty-VP6
ARV Ch-1
ARV Ty-1 VP6
ARV Tu-03V0002E10


110 120 130 140 150
Rotavirus70_cplt VRESQRNGIAPQSNALRQLSAARYRRINYDNDSEYIENWNLQNRRQRTGY
Rotavirus73_cplt
Rotavirus75_cplt
Rotavirus242_cplt
Rotavirus246_cplt
Rotavirus247_cplt

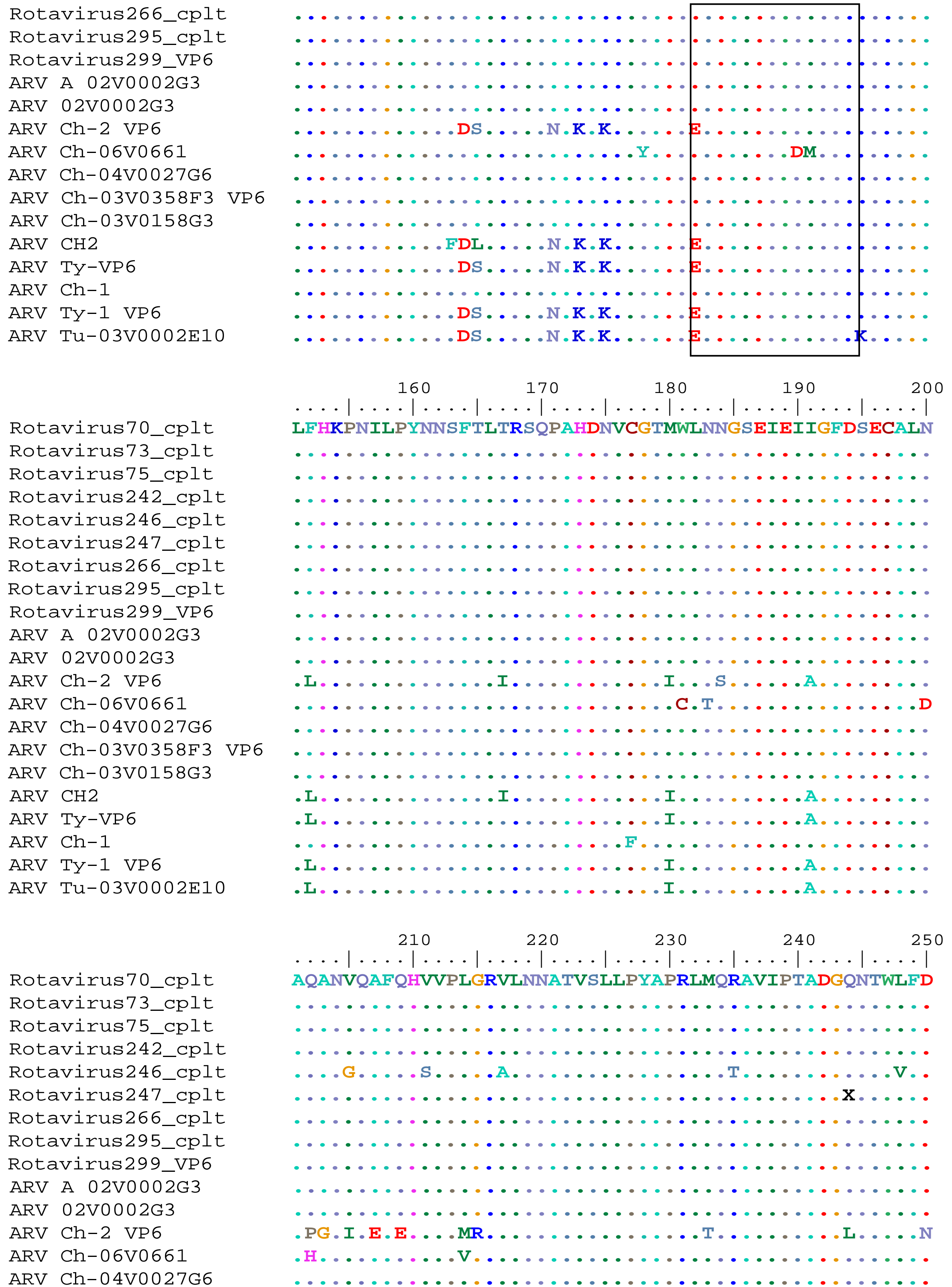


Fig. 68: Comparison of deduced amino acid sequences of VP6 protein of Avian Rotavirus. Boxes show group A specific antigenic sites I and II (aa-45-65 and aa-132-142 respectively)

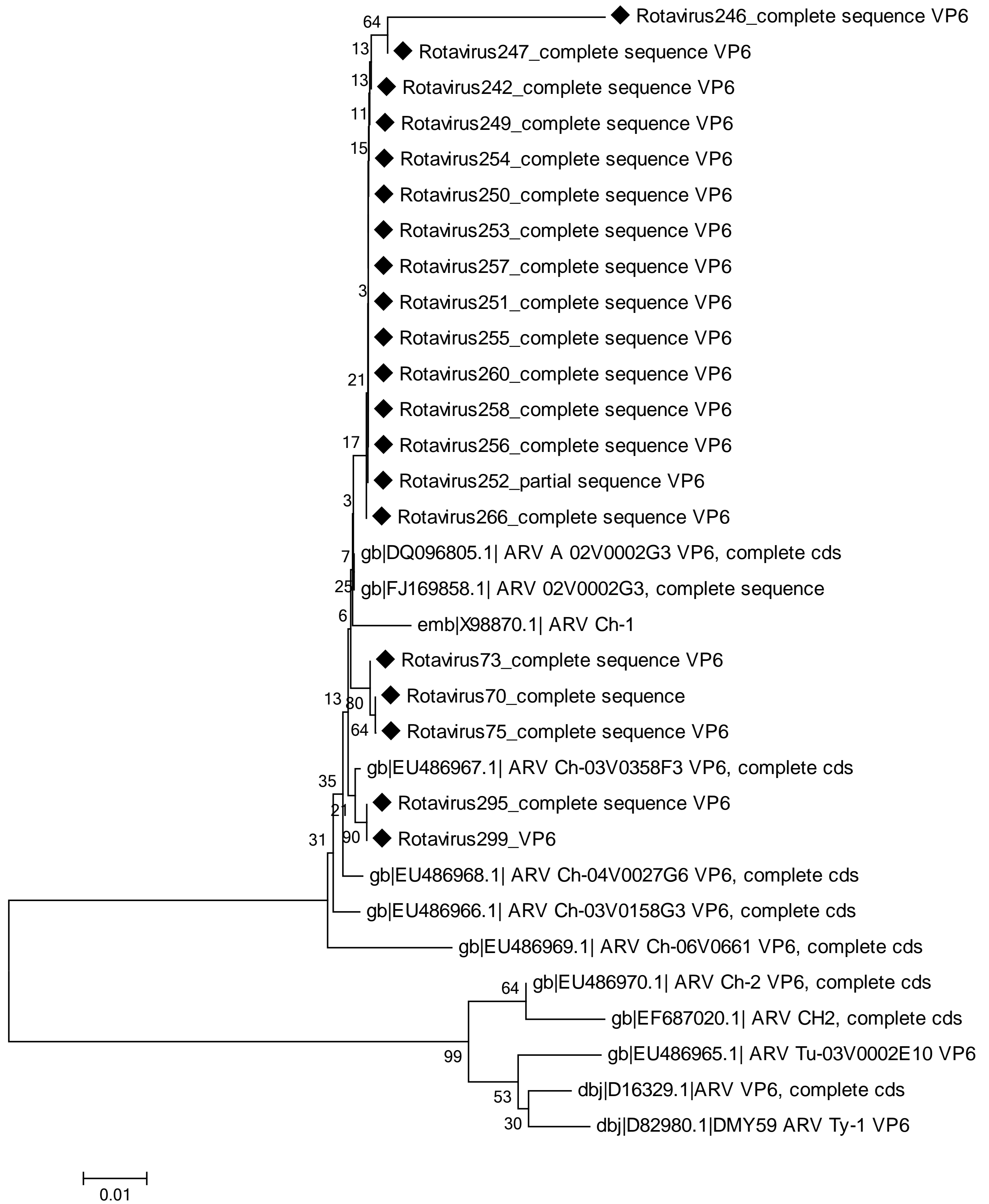


Figure 69: Phylogenetic tree of amino acid sequences of VP6 gene of Rotavirus. The marked sequences are from this study and those unmarked are GenBank sequences. Bootstrap values are shown on tree.

5.3.3.3 VP7 segment

Most of the samples cluster in separate branches from GenBank sequences (Figure 71). The sequences had 100% aa identity on the same farm. The VP7 genes show nt sequence identities between 92.6% to 96.3% and aa sequence identity of between 89.5% to 96.7% with chicken isolates Ch-04V0027G6, Ch-03V0158G3, Ch-03V0358F3, Ch-06V0661and 02V0002G3. As the samples show high nt sequence identities with chicken isolates Ch-04V0027G6, Ch-03V0158G3, Ch-03V0358F3, Ch-06V0661and 02V0002G3 which belong to genotype G19, they should be classified as G19 according to the genotyping guidelines calculated as a cut-off of 80% nt identity (Matthijnssens *et al.*, 2008b). Antigenic sites A (aa-85-95) is seen to be conserved, which means this is an authentic group A VP7 segment (Figure 70).

```
                                 10        20        30        40        50
                        ....|....|....|....|....|....|....|....|....|....|
gb|EU486976.1| ARV C    ...............................................T..
gb|EU486975.1| ARV C    ...........V.I....V..............T.............T..
gb|EU486974.1| ARV C    ...........V.I....V..............T.............T..
gb|EU486973.1| ARV T    .I.........V......S..........I...T..AN....S....TVT
gb|FJ169861.1| ARV 0    GVNVPITGSLDITVQNQTAEPIGLTSTLCLYYPAEASTEIADTEWKQPIS
gb|EU486977.1| ARV C    ...I.........I.................................T..
Rotavirus450_cplt se    ...........V.....................K.....V.......T..
Rotavirus70 cplt seq    ...........VSI....V..............K.............T..
Rotavirus73_cplt seq    ...........VSI....V..............K.............T..
Rotavirus75_ cplt se    ...........VSI....V..............K.............T..
Rotavirus242 cplt se    ...........V.I....V..............K.............TV.
Rotavirus246_ cplt s    ...........V.I....V..............K.............TV.
Rotavirus247_ cplt s    ...........V.I....V..............K.............TV.
Rotavirus249_ cplt s    ...........V.I....V..............K.............TV.
Rotavirus250 cplt se    ~~~X.......V.I....V..............K.............TV.
Rotavirus254_ cplt s    ...........V.I....V..............K.............TV.
Rotavirus255_ cplt s    ...........V.I....V..............K.............TV.
Rotavirus257_ cplt s    ...........V.I....V..............K.............TV.
Rotavirus260_ cplt s    ...........V.I....V..............K.............TV.
Rotavirus277_ cplt s    .............I....V..............K.............T..
Rotavirus298_ cplt s    ...........V.I...................K.............T..
Rotavirus299_ cplt s    ...........V.I...................K.............T..
Rotavirus300_ cplt s    ...........V.I...................K.............T..
Rotavirus362_ cplt s    ...........V.I...................K.............T..
Rotavirus432_ cplt s    ...........V.I....VG.............K.............TV.
Rotavirus441_ cplt s    ...........V.....................K.....V.......T..

                                 60        70        80        90       100
                        ....|....|....|....|....|....|....|....|....|....|
gb|EU486976.1| ARV C    ..................................................
gb|EU486975.1| ARV C    ..................................................
gb|EU486974.1| ARV C    ..................................................
gb|EU486973.1| ARV T    ..............L...T.........QL......V.V...........
gb|FJ169861.1| ARV 0    QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
gb|EU486977.1| ARV C    ......................A...........................
Rotavirus450_cplt se    ..................................................
Rotavirus70 cplt seq    ....I.............................................
Rotavirus73_cplt seq    ....I.............................................
Rotavirus75_ cplt se    ....I.............................................
Rotavirus242 cplt se    ..................................................
Rotavirus246_ cplt s    ..................................................
Rotavirus247_ cplt s    ..................................................
Rotavirus249_ cplt s    ..................................................
Rotavirus250 cplt se    ..................................................
Rotavirus254_ cplt s    ..................................................
Rotavirus255_ cplt s    ..................................................
Rotavirus257_ cplt s    ..................................................
Rotavirus260_ cplt s    ..................................................
Rotavirus277_ cplt s    ..................................................
Rotavirus298_ cplt s    ..................................................
Rotavirus299_ cplt s    ..................................................
Rotavirus300_ cplt s    ..................................................
Rotavirus362_ cplt s    ..................................................
Rotavirus432_ cplt s    ..................................................
```

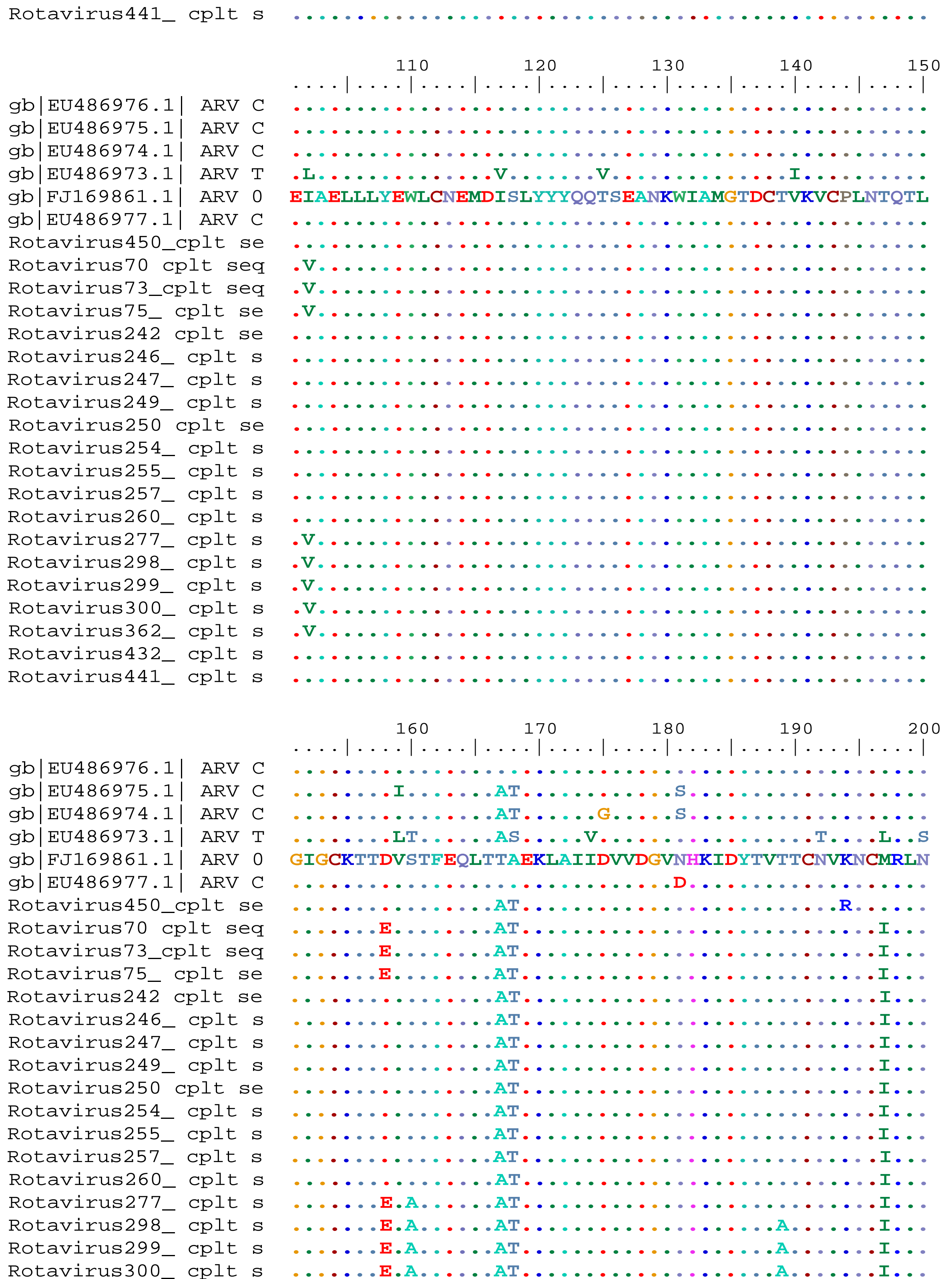


Fig. 70: Comparison of deduced amino acid sequences of VP7 protein of Avian Rotavirus. Boxes show conserved regions, antigenic sites A (aa-85-95).

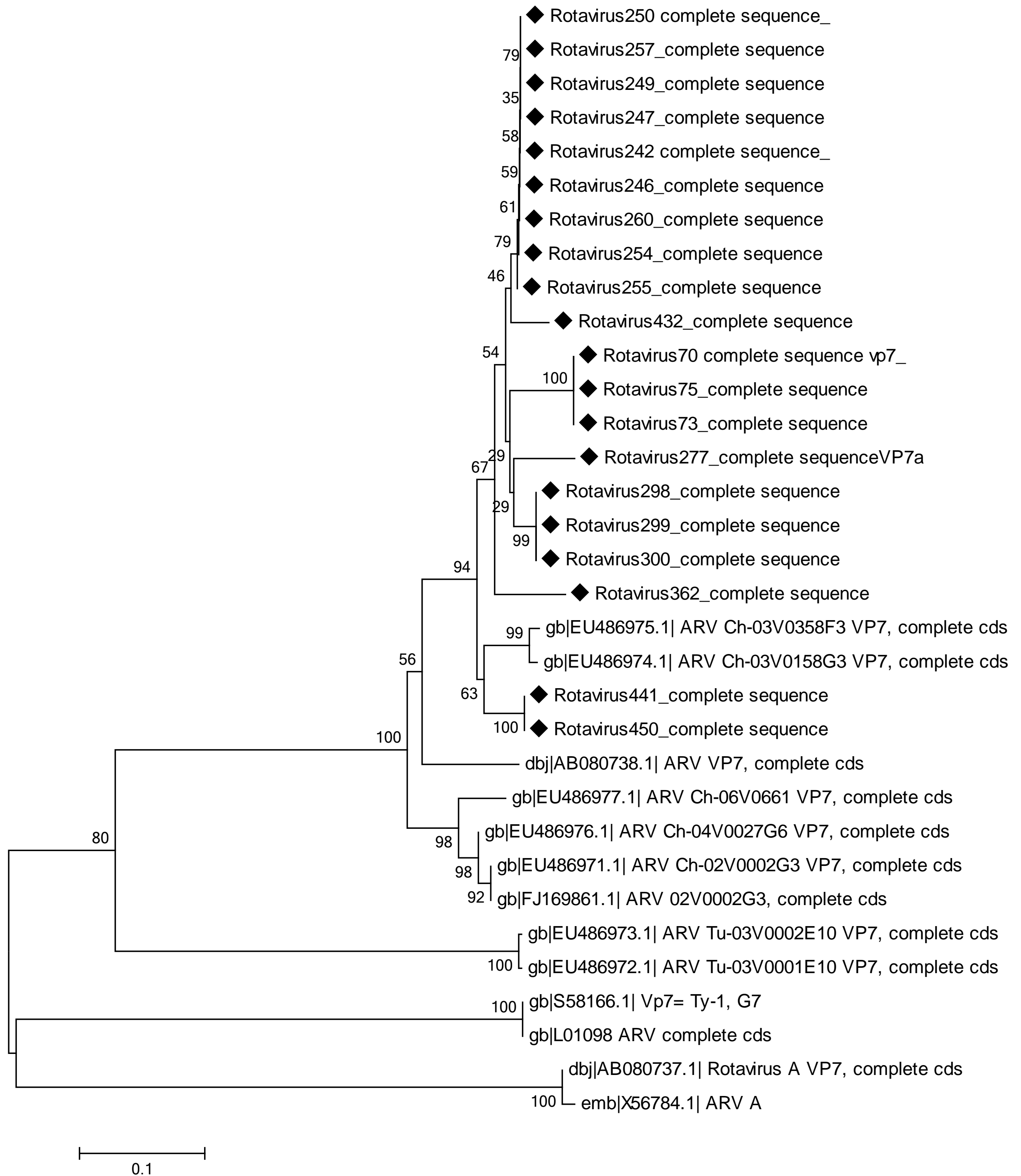


Figure 71: Phylogenetic tree of amino acid sequences of VP7 gene of Rotavirus. The marked sequences are from this study and those unmarked are GenBank sequences. Bootstrap values are shown on tree.

5.3.3.4 NSP4 segment

The rotaviruses detected assorted into four groups (Figure 73). Among all four groups there was a nt identity of between 96.1% to 97.5% and aa identity of between 91.8% to 93.3%. The sequences had 99.4% to 100% aa identity on the same farm. The ORF started at nucleotide 41 and terminated at nucleotide 547 and codes for 168 aa (figure 72). Amino acid at position 144 varied across the samples as serine, proline or threonine. The N-linked glycosylation site aa-18 and enterotoxin domain aa-109-135 are conserved in the samples of this study. Two groups clustered with Ch-1and 02V0002G3 isolates, while others formed separate branches from these. These clusters were also separated according to geographic location of the farm. Ch-1 and 02V0002G3 isolates belong to the genotype E10 (Trojnar *et al.*, 2009). Since samples from this study have an aa identity of 93.2% to 98.0% with these isolates they can be classified as genotype E10.

```
                                   10        20        30        40        50
                          ....|....|....|....|....|....|....|....|....|....|
ARV ck /Bz/NSP4           MENVSTINETLVDEVYKMTLRYFEHNVIIMKYFPFIASILTIIFTAWKMG
ARV ck /Bz/NSP4           ..................................................
ARV ck /Bz/NSP4           ..................................................
ARV ck/GA/NSP4            ....T.......E...N...G..............L..............
ARV ck/GA/NSP4            ---XTA......E...N...G..............L......T.......
ARV ck/DE/NSP4            ....T.......E...N...G..............L..............
ARV ck/AR/NSP4            ....T.......E...N...G..............L..............
ARV ck/AR/NSP4            ....T.......E...N...G..............L.........V....
ARV 02V0002G3             ....T.......E...N...G..............L..............
Rotavirus 361             -------X....E...N...G..............L..............
Rotavirus 365             -------X....E...N...G..............L..............
Rotavirus 368             -------X....E...N...G..............L..............
Rotavirus 461             -------X....E...N...G..............L..............
Rotavirus 450             -------X....E...N...G..............L......T.......
Rotavirus 246             -------X....E...N...G..............L..............
Rotavirus 249             -------X....E...N...G..............L..............
Rotavirus 254             -------X....E...N...G..............L..............
Rotavirus 257             -------X....E...N...G..............L..............
Rotavirus 73              -------X....E...N...G..............L..............
Rotavirus 75              -------X....E...N...G..............L..............
Rotavirus 247             -------X....E...N...G..............L..............
Rotavirus 253             -------X....E...N...G..............L..............
Rotavirus 258             ------------X...N...G..............L..............
Rotavirus 295             -----------------------X...........L..............
Rotavirus 256             --------------------------------------------------
rotavirus A               ------------------..G..............L..............
dbj|AB065286.1|ARV T      ...ATS....F.E...N...S..............L......A.......
gb|EF204143.1|ARV NC      ...ATS....F.E...N...S...Q..........L......V.......
gb|EF204133.1|ARV NC      ...ATS....F.E...N...S...Q..........L......V.......

                                   60        70        80        90       100
                          ....|....|....|....|....|....|....|....|....|....|
ARV ck /Bz/NSP4           RSTLKVTKTVAGSRYKVIKVVIVTIFNCVLRIFGSKAEIVPEDKMDVMAS
ARV ck /Bz/NSP4           ..................................................
ARV ck /Bz/NSP4           ..................................................
ARV ck/GA/NSP4            .............G..AV.............M..............I...
ARV ck/GA/NSP4            .............G..........................S.........
ARV ck/DE/NSP4            .............G..............................I.....
ARV ck/AR/NSP4            .............G.................M..............I...
ARV ck/AR/NSP4            .............G..A...........I..M..............I...
ARV 02V0002G3             .....M.......G.......V..V...I.......T...S.........
Rotavirus 361             .............G..........V...I..M....T...S.........
Rotavirus 365             .............G..............I..M....T...S.........
Rotavirus 368             .............G..............I..M....T...S.........
Rotavirus 461             .............G.......V..V...I..T....T...S.........
Rotavirus 450             .............G.......V......I..ML...T.V.S.........
Rotavirus 246             .............G.......V..V...I..T....T...S.........
Rotavirus 249             .............G.......V..V...I..T....T...S.........
Rotavirus 254             .............G.......V..V...I..T....T...S.........
Rotavirus 257             .............G.......V..V...I..T....T...S.........
Rotavirus 73              .............G..........V...I.KM...RT...S.........
Rotavirus 75              .............G..........V...I.KM...RT...S.........
Rotavirus 247             .............G.......V..V...I..T....T...S.........
Rotavirus 253             .............G.......V..V...I..T....T...S.........
```

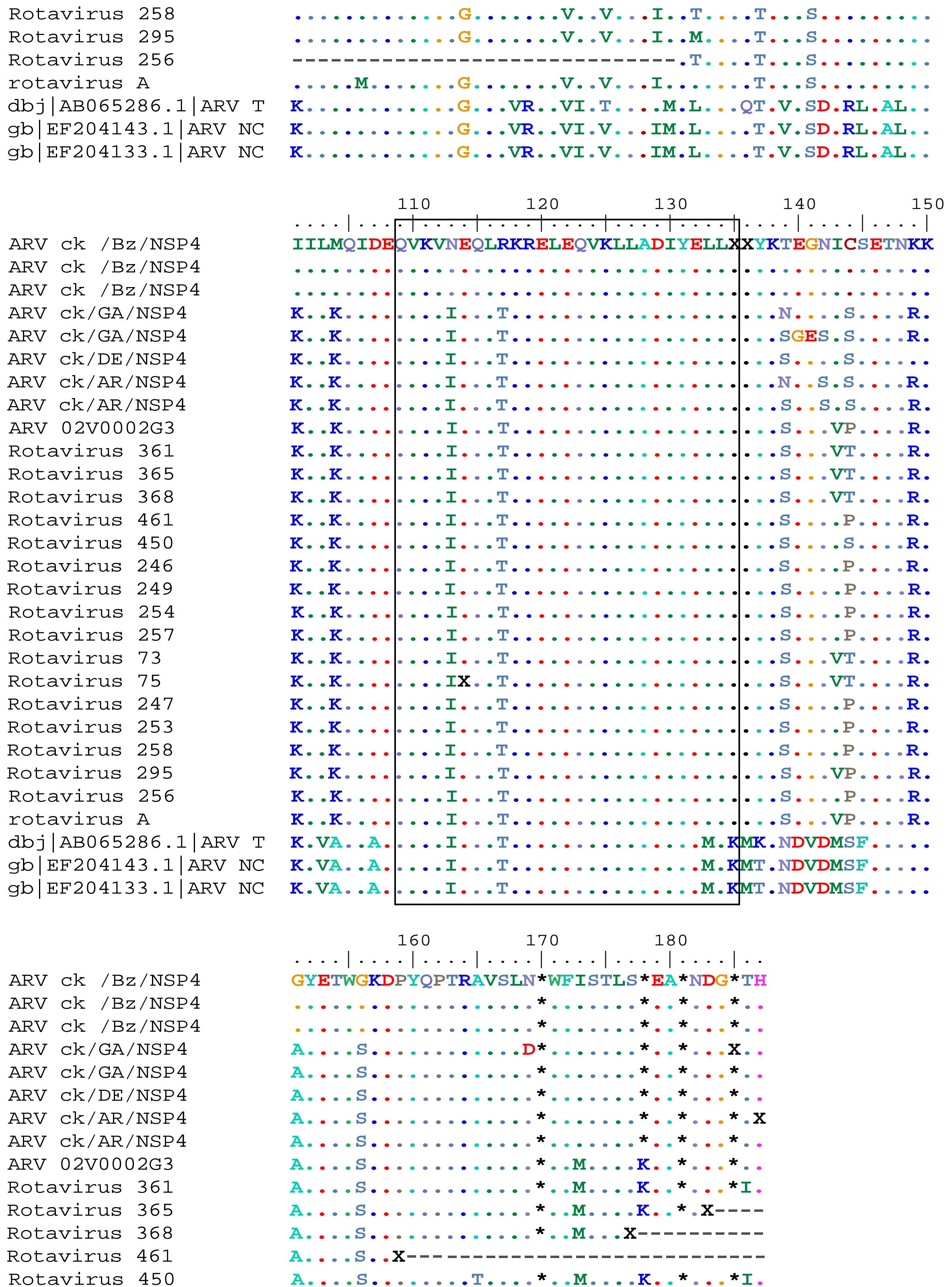


Fig. 72: Comparison of deduced amino acid sequences of NSP4 protein of Avian Rotavirus. N-linked glycosylation site aa-18 and enterotoxin domain aa-109-135 are conserved regions shown in boxes.

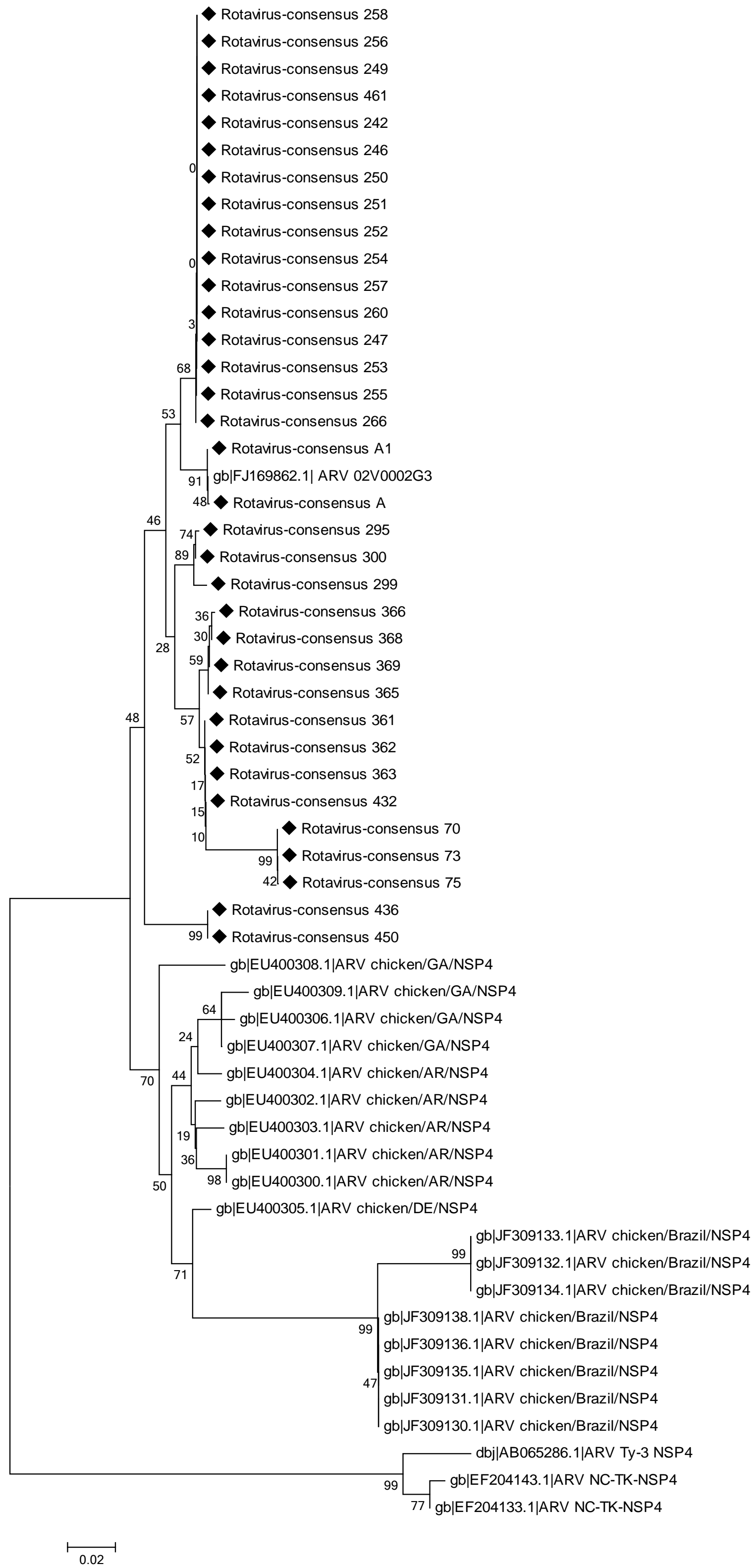


Figure 73: Phylogenetic tree of amino acid sequences of NSP4 gene of Rotavirus. The marked sequences are from this study and those unmarked are GenBank sequences. Bootstrap value are shown on tree.

5.3.4 Rotavirus online classification

Each sequence was entered into the online rotavirus classification tool, RotaC (http://rotac.regatools.be/), for further confirmation of their typing. The software confirmed them to belong to the genotypes listed above.

5.3.5 Rotavirus sequence accession numbers in GenBank

Sequences of the detected avian rotaviruses have been deposited in the GenBank with accession numbers ranging from LK932155 to LK932242. A total of eighty eight (88) sequences were deposited.

5.4 DISCUSSION

Faecal samples from chickens, crowned crane, geese and turkeys were screened for rotavirus by targeting the VP4, VP6, VP7 and NSP4 of the genome segments. These segments are critical for vaccine development because they are targets of neutralizing antibodies. Phylogenetic analysis of the different gene segments revealed separate clusters for each farm with a high degree of heterogenicity indicating a reassortment of the rotaviruses at different locations. Previously published reports also indicate assortment of rotaviruses based on different geographic locations (Pantin-Jackwood *et al.*, 2008). This may significantly contribute to genetic variability of rotaviruses by providing a reservoir of novel genotypes, especially for the antigenic determinants VP4 and VP7. In comparison with other rotavirus strains in the GenBank, strains in this study have a VP4-allele which has only low similarities to other avian rotavirus VP4 sequences. They are only closely related to the sequence of strain Ch-06V0661 (figure 67), which is stated to originate from an unknown host (Schumann *et al.*, 2009). All of the other analyzed segment sequences clearly clustered within that of chicken strains, a reassortment event is highly likely from an exchange of the VP4-encoding segment. Under experimental conditions, reassortants have been generated between pigeon strain PO-13 and turkey strain Ty3 (Mori *et al.*, 2003) as well as between turkey strain Ty-1 and simian rotaviruses (Kool *et al.*, 1992). These clusters were also separate from reference strains in the GenBank. The partial VP4 aa sequences revealed identities of 97.4% homology with Ch-06V0661, 69.3% with isolate Tu-03V0001E10, 72.2% with Ch-02V0002G3 and 68.7% with PO-13. Isolate Ch-06V0661G1 till date has an unknown host origin. The rotavirus spike protein VP4 is responsible for a number of important biological functions, such as the enhancement of infectivity by proteolytic cleavage into VP8* and VP5*, hemagglutination, restriction of growth in cell culture, virulence, initial virus attachment to cells, protease sensitivity associated to plaque formation, and host range restriction (Arias *et al.*, 1996; Bridger *et al.*, 1992; Espejo *et al.*, 1981; Greenberg *et al.*, 1983; Kalica *et al.*, 1983; Offit *et al.*, 1986; Ciarlet *et al.*, 1998, 2002). The inability to sequence the whole of this gene segment will mean that this segment is highly diverse and the functions it performs will be modified or require some other enzymes to perform its functions.

Isolation of rotavirus from field samples was attempted, but this was not successful due to the rare VP4 sequence. This is because the attachment of rotaviruses to susceptible cells is by a domain (VP8*) within the outer capsid of the unusual VP4 spike protein of the Nigerian avian rotaviruses. The inability of adapting the field samples to tissue culture is also supported by the result of the nucleotide sequence and phylogenetic analysis of the VP4 sequence, where only three field samples were partially sequenced and their clustering pattern was separate from all other rotavirus strains in the GenBank but only closely related to strain Ch-06V0661 which has an unknown host origin. To the best of our knowledge, there has been no report of isolation of rotavirus strain Ch-06V0661. In addition, virus isolation is selective for group A rotaviruses.

Rotaviruses are genetically highly diverse and can be differentiated into 7 groups according to the antigenicity and gene composition of their VP6 (Ramig *et al.*, 2005, Estes and Kapikian, 2007). The inner capsid protein VP6 of group A rotavirus possesses group and subgroup epitope specificities. Avian rotaviruses have a unique VP6 that is antigenically different from its mammalian counterpart (Buragohain *et al.*, 2008). Based on the presence or absence of SG antigens on the VP6 gene, group A rotaviruses are further classified as SGI, SGII, SGI/II and non-SGI/II. It has been proposed that primarily Ala172, Arg296–Asn299, Ala305 and Asn310 contribute to reactivity to SGI MAbs whereas Phe248, Asn305, Ala306, Gln310 and Gln315 are responsible for reactivity to SGII MAbs (Tang *et al.*, 1997; Greig *et al.*, 2006). Amino acids which contribute to SGI reactivity were conserved on our sequences.

The group A specific antigenic site I (45-65) is seen to be conserved except for the substitution of Ala60 with threonine in four samples as it occurs in the VP6 gene of turkeys. Genotyping methods targeting this site would fail due to reassortment which has occurred here and also monoclonal antibodies targeting this site would result in genotyping failures. Antigenic site II (132–142) is also conserved in these avian rotaviruses at the sequence level (figure 69). Interestingly, the antigenic site II has been specifically reported (Ito *et al.*, 1996) to be an authentic group A antigen common to both mammalian and avian rotaviruses. With the conservation of the genes in our rotavirus strains, they thus belong to group A.

The VP6 nt sequence of the isolates share 74.7% to 79.3% homology with the Ch-2 strain which belong to the same genotype I4 with Tu-1E10, Tu-2E10 and PO-13 (Matthijnssens *et al.*, 2008b. Also, they have 88.1% to 95.4% homology with Ch-04V0027G6, Ch-03V0158G3, Ch-03V0358F3, and Ch-06V0661which all belong to genotype I11 (Matthijnssens *et al.*, 2008b). Thus, they could be classified as genotype I11 as the recommended nucleotide percentage identity cut-off value is 85%.

The VP7 protein of our strains has a potential N-linked glycosylation site located at amino acid 69 (Asn), which tends to be conserved among rotavirus strains. In addition, they also have a second potential Glycosylation site at amino acid 238 (Martella *et al.*, 2003). The VP7 antigenic regions, A (aa 87–101), B (aa 143–152), C (aa 208–223), and F (aa 235–242) (Dyall-Smith *et al.*, 1986; Nishikawa *et al.*, 1989; Ciarlet *et al.*, 1998) is conserved in all the isolates, except for the substitution of Isoleucine for Valine in antigenic region A (aa 99). The VP7 genes show nt sequence identities between 92.6% to 96.3% and aa sequence identity of between 89.5% to 96.7% with chicken isolates Ch-04V0027G6, Ch-03V0158G3, Ch-03V0358F3, Ch-06V0661and 02V0002G3. As the samples show high sequence identities with chicken isolates Ch-04V0027G6, Ch-03V0158G3, Ch-03V0358F3, Ch-06V0661and 02V0002G3 which belong to genotype G19, they should be classified as G19 according to the genotyping guidelines calculated as a cut-off of 80% nt identity (Matthijnssens *et al.*, 2008b).

In analyzing the NSP4 gene, common structural features of the NSP4 protein, such as N-linked glycosylation sites at amino acids 8 and 18, and a key residue for enterotoxic activity tyrosine-131 were conserved. Tyrosine 131 NSP4 protein has been postulated to be critical for the diarrhoeagenic activity of the toxic peptide (Ball *et al.*, 1996).

Tyrosine is conserved only within genetic group I (Genotype A), whereas in genetic groups II and III histidine was more frequently found at residue 131 (Cunliffe *et al.*, 1997). The enterotoxin domain at aa 109 to 135 is also conserved in all the strains. The rotaviruses assorted into four groups (Figure 73). Among all four groups there was a nt identity of between 96.1% to 97.5% and aa identity of between 91.8% to 93.3%. The sequences had 99.4% to 100% aa identity on the same farm. Two groups clustered with Ch-1and 02V0002G3 isolates, while others formed separate branches from these. Since

samples from this study have an aa identity of 93.2% to 98.0% with these isolates they can be classified as genotype E10.

Analysis of the deduced amino acid sequences of the encoded virus proteins generally showed a close relationship to other avian group A rotavirus proteins and identified conserved functional motifs common for all group A rotaviruses; however, with the exception of VP4. Based on the genome sequence determined and analysis of sequences using the online classification tool, rotaviruses from Nigeria can be classified as group A, genotype I11, G19, E10 with an untypeable VP4 segment. However, further studies are needed to compare rotaviruses from different species in different regions of Nigeria to obtain better understanding on the types of rotaviruses circulating in avian flocks in Nigeria. This would help to relate an ancestor for some gene sequences, especially the VP4 segment which has an unknown host of origin.

The genetic variability of human rotaviruses is maintained by several mechanisms including (i) point mutations, (ii) genomic reassortment and (iii) genome rearrangements, thus leading to considerable diversity (Estes and Kapikian, 2007; Müller and Johne, 2007). Animal rotaviruses may contribute to this variability by direct transmission to humans (DeGrazia *et al.*, 2007; Matthijnssens *et al.*, 2006), or by reassortment events creating human rotaviruses which contain only parts of an animal rotavirus genome (Iturriza-Gómara *et al.*, 2002; Khamrin *et al.*, 2006; Mascarenhas *et al.*, 2007; Matthijnssens *et al.*, 2008c). One of the major drawbacks for assessment of their contribution to human rotavirus variability is the lack of sequence data for avian rotaviruses which would enable tracing of these viruses and detection of their genome fragments in human samples. Sequences generated in this study have been deposited in the GenBank and accession numbers assigned to them is as shown in the appendix.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

In summary, the present studies described the detection and molecular characterisation of the VP4, VP6, VP7, NSP4 genes of avian rotavirus strains. They also provided evidence for the circulation of group A rotavirus with a rare and untypeable P type in Nigerian birds. The findings also highlight some variations in these rotavirus strains. In view of this, vigilance on the intragenotypic diversity in VP4 genes would provide a key to understanding the evolution and genetic/antigenic differences in the rotavirus genotypes. Characterisation of additional strains may reveal the distribution of this VP4 type and help to clarify its species of origin. This study also identified so far that all the Nigerian ARV strains detected are group A, SG I and belong to genotype I11, G19 and E10 with an untypeable P type.

A better understanding of the rotavirus epidemiology will contribute to the optimization of current prevention programs of rotavirus diarrhoea in humans and animals. Furthermore, precise evaluation of antigenic or molecular diversity among domestic animal herd populations is of critical importance for the development of an effective vaccine because animal rotaviruses may be involved in interspecies transmission or reassortment in humans. Likewise, the inability of vaccines produced against human rotavirus in developing countries to confer protection against this disease may be due to reassortment of human rotavirus and animal rotaviruses of rare origin as seen in this study. The availability of the genome sequences in this study would also promote the development of sensitive diagnostic tests for the detection of rotaviruses in poultry as well as for tracing genetic material of avian rotaviruses in samples derived from mammalian animals and humans.

Taking into account the widespread use of chickens for food production and the high incidence of rotavirus infections in this animal species, a relatively high exposure of humans with avian rotaviruses would be expected. Considering the animal species and humans together, rotavirus diarrhoea has to be considered as the proximal cause of

enteritis and diarrhoea in infants and young ones of domestic animals. Several case studies have indicated infection of humans by animal rotaviruses. Comparison of genetic sequences of human and animal rotaviruses often reveals close identity. Surveillance of circulating rotaviruses in the human population has revealed the presence of several uncommon genotypes. Many of these have been found in domestic animals, and it is possible that they arose in the human population through zoonotic transmission. There may be some measure of environmental contamination through livestock excrement. In many developing countries, there is close contact between humans and domestic livestock. In areas prone to flooding, or with a monsoon climate, this can increase the chances of contact with animal faeces. This exposure may not result in high levels of infection, but some infection could occur. There may be a continual input of rotavirus strains or sequences into the human population from the animal population albeit at a very low level.

Rotaviruses have extreme genetic diversity and are resistant to many common disinfection methods, thus adding to the difficulty of implementing suitable preventive measures against the infection. However, to prevent the losses associated with rotavirus infections, multivariate approaches including good management and strict biosecurity have to be followed.

6.2 AREAS OF FURTHER RESEARCH

Exciting scientific information should continue to emerge if the challenges of sequencing and isolation of rotaviruses are overcome. As such it is important that further studies be focused on a unique methodology for isolation of all strains of rotaviruses and concurrent sequencing of the VP4 segment. This will probably give new insights into virus–host–environment interactions and viral evolution, because the VP4 is responsible for the enhancement of infectivity, virulence, initial virus attachment to cells, and host range restriction. Further, it is important to analyze humans, animals and other breed of bird's rotavirus genome, by serotype specific-RT-PCR or gene sequencing, for assessing the interspecies transmission of these viruses between other mammalian and avian species.

6.3 RECOMMENDATIONS

As the rotaviruses are known to exhibit extreme genetic diversity and resist disinfection procedures, eradication of the pathogen is often difficult. Hence, for prevention, good management practices have to be carried out on farm premises. In cases of disease, diarrhoea may contribute to poor litter conditions that may be controlled by increasing the ventilation rate and temperature, and adding fresh litter. New reassortants continue to emerge by acquisition of genes from rotaviruses of different animals and man. The evolved virus becomes introduced to the environment through international trades and become transmitted through an array of food vehicles mostly of animal origin. It is thus important to carry out continuous surveillance in order to prevent outbreak of diarrhoea in man and animals and aside diarrhoea, a drop in production through decreased weight gain and egg loss in poultry production.

The following specific actions are recommended:

- Establishment of standard reference laboratory equipped with state of the art technologies and equipment for exhaustive studies on rotavirus of man and animal.
- To establish the relationships between rotavirus strains infecting humans and animals in Nigeria, a national periodic surveillance for rotavirus in clinical and non-clinical samples from man, animals and birds should be performed for concomitantly circulating strains. This would help to obtain better understanding on the types of rotaviruses circulating in avian flocks in Nigeria. One of such

survey is carried out by African Rotavirus Surveillance Network (AFR RSN) in some other countries of Africa.

- The evidence for cross-species infection raises interesting questions about the genetic and cellular basis of species specificity, and this should be established. This would provide an element of prediction of replication in heterologous species.
- Avian rotaviruses could potentially be transmitted by foods directly or indirectly, as such, efforts to reduce contaminations during poultry processing or packaging should be encouraged.
- Studies on compounds that can protect gut integrity should be carried out in order to reduce diarrhoea.
- Studies on co-infection of rotavirus with other organisms should be carried out in order to determine the severity of the disease with these co-infecting agents.
- There is also a need to design specific primers to detect other rotavirus of poultry.
- Proper disposal of poultry waste should be encouraged.
- Thorough cleaning and disinfection of facilities between flocks, in order to reduce environmental contamination and degree of exposure of young poultry should be ensured.

REFERENCES

Aboudy, Y., Shif, I., Zilberstein, I. and Gotlieb-Stematsky, T. (1988). Use of polyclonal and monoclonal antibodies and analysis of viral RNA in the detection of unusual group A human rotaviruses. J. Med. Virol. 25:351-359.

Adah, M.I., Nagashima, S., Wakuda, M. and Taniguchi, K. (2003). Close relationship between G8-serotype bovine and human rotaviruses isolated in Nigeria. Journal of Clinical Microbiology, 41, 3945–3950

Adah, M.I, Olaleye, R. A. and Werchau, H. (1997). Nigerian rotavirus serotypoe G8 could not be typed by PCR due to nucleotide mutation at the 3' end of the primer site. Arch Virol :142:1881–7.

Adah, M. I., Wade, A. and Taniguchi, K. (2001). Molecular epidemiology of rotaviruses in Nigeria: detection of unusual strains with G2P[6] and G8P[1] specificities. J. Clin. Microbiol. 39:3969-3975.

Adams, W. R. and Kraft, L. M. (1963). Epizootic diarrhoea of infant mice: identification of the etiologic agent. Science 141:359-60.

Adene, D.F. (2004). Poultry health and production. Principles and Practices. Stirling-Horden publishers (Nig.) Ltd.

Adene, D.F. and Oguntade, A.E. (2006). Overview of poultry production in Nigeria. In: The structure and importance of the commercial and village based poultry industry in Nigeria. FAO study. Pp 4-27.

Afrikanova, I., Miozzo, M. C., Giambiagi, S. and Burrone, O. (1996). Phosphorylation generates different forms of rotavirus NSP5. J Gen Virol 77 (Pt 9):2059-65.

Alfieri, A. F., Resende, M., Resende, J. S. and Alfieri, A. A. (1989). Atypical rotavirus infections among broiler chickens in Brazil. Arq Bras Med Vet Zoot 41:81—82.

Aminu, M., Esona, M.D., Geyer, A., Steele, A.D. (2008). Epidemiology of rotavirus and astrovirus infections in children in northwestern Nigeria. Annals of African Medicine Vol. 7, No.4: 168 – 174

Andral, B. and Toquin, D. (1984). Observations au microscope electronique a partir de prelevements de dindes presentant des troubles pathologiques. Avian Pathol 13:389—417.

Arias, C. F., Romero, P, Alvarez, V. and Lopez, S. (1996). Trypsin activation pathway of rotavirus infectivity. J Virol 70:5832-9.

Arista, S., Giovannelli, L., Pistoia, D., Cascio, A., Parea, M. and Gerna, G. (1990). Electropherotypes, subgroups and serotypes of human rotavirus strains causing gastroenteritis in infants and young children in Palermo, Italy, from 1985 to 1989. Res Virol 141, 435–448.

Arnold, M., Patton, J.T. and McDonald, M. (2009). Culturing, storage and quantification of Rotaviruses. Current protocols in microbiology 15C.3.1-15C.3.24

Au, K. S., Chan, W. K., Burns, J. W. and Estes, M. K. (1989). Receptor activity of rotavirus nonstructural glycoprotein NS28. J Virol 63:4553-62.

Au, K. S., Mattion, N. M. and Estes, M. K. (1993). A subviral particle binding domain on the rotavirus nonstructural glycoprotein NS28. Virology 194:665-73.

Bakulin, V., Aliev, A. S., Dzhavadov, E. D., Tul'skaya, I. I. , Vashukova, S. S., Gorbachev, E. N. and Verbor, S. N. (1991). Morphology of avian rotavirus and the lesions it produces in chicks. Veterinariya (Moskva) 1:36—37.

Ball, J. M., Tian, P., Zeng, C. Q., Morris, A. P. and Estes, M. K. (1996). Age-dependent diarrhoea induced by a rotaviral nonstructural glycoprotein. Science 272:101-4.

Banatvala, J.E.; Totterdell, B.; Chrystie , I.L.; Woode, G.N. (1975). *In-vitro* detecting of human rotavirus. Lancet, p.821.

Banyai, K., Gentsch, J.R., Griffin, D.D., Holmes, J.L., Glass, R.I., Szucs, G. (2003) Genetic variability among serotype G6 human rotaviruses: identification of a novel lineage isolated in Hungary. J Med Virol: 71:124–34.

Barnes, H. J. (1997). In: Diseases of Poultry. 10th ed. Iowa State University Press, Ames, IA. Pages 685–686

Barnes, H. J., and Guy, J. S. (2003). Poult enteritis-mortality syndrome. In: Diseases of poultry, 11th ed. Y. M. Saif, ed. Iowa State University Press, Ames, IA. pp. 1171–1180.

Barnes, H. J., Guy, J. S. and Vaillancourt, J. P. (2000) Poult enteritis complex. Rev. Sci. Tech. 19:565–588.

Baxendale, W. and Mebatsion, T. (2004). The isolation and characterisation of astroviruses from chickens. Avian Pathol. 33:364–370.

Bellinzoni, R., Mattion, N., Vallejos, L., La Torre, J. L. and Scodeller, E.A. (1987). Atypical rotavirus in chickens in Argentina. Res Vet Sci 43:130—131.

Bergeland, M. E., McAdaragh, J. P. and Stotz, I. (1977). Rotaviral enteritis in turkey poults. Pages 129 – 130. Proceedings of the 26th Western Poultry Diseases Conference.

Bishop, R. F., Davidson, G. P., Holmes, I. H. and Ruck, B. J. (1973). Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. Lancet 2:1281-3.

Bishop, R. F., Davidson, G. P., Holmes, I. H. and Ruck, B. J. (1974). Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis. Lancet 1:149-51.

Boom, R., Sol, C.J.A., Salismans, M.M.M., Jansen, C.L., Werthiem-van Dillen, P.M.E., van den Noordaa, J. (1990). Rapid and simple method for the purification of nucleic acids. J Clin Microbiol, 28:495-503.

Breslauer, K.J., Ronald, F., Blocker, H. and Marky, L.A. (1986). Predicting DNA duplex stability from the base sequence. Proc. Natl. Acad. Sci. 83: 3746-3750.

Bridger, J., Burke, B., Beards, G.M. and Desselberger, U. (1992). The pathogenicity of two porcine rotaviruses differing in their *in-vitro* growth characteristics and genes 4. J. Gen. Virol. 73, 3011–3015.

Bridger, J. C., Dhaliwal, W., Adamson, M. J. and Howard, C. R. (1998). Determinants of rotavirus host range restriction--a heterologous bovine NSP1 gene does not affect replication kinetics in the pig. Virology 245:47-52.

Bridger, J.C., Woode, G.N., Jones, J.M., Flewett, T.H., Bryden, A.S. and Davies, H. (1975). Transmission of human rotaviruses to gnotobiotic piglets. Journal of Medical Microbiology, 8, 565–569

Broome, R. L., Vo, P. T., Ward, R. L., Clark, H. F. and Greenberg, H. B. (1993). Murine rotavirus genes encoding outer capsid proteins VP4 and VP7 are not major determinants of host range restriction and virulence. J Virol 67:2448-55.

Brussow, H., Nakagomi, O., Gerna, G. and Eichhorn, W. (1992). Isolation of an avianlike group A rotavirus from a calf with diarrhoea. Journal of Clinical Microbiology, 30, 67–73

Burke, B. and Desselberger, U. (1996). Rotavirus pathogenicity. Virology 218:299-305.

Buragohain, M., Cherian, S.S., Prabhakar, G. and Chitambar, S.D. (2008). VP6 capsid protein of chicken rotavirus strain CH2: sequence, phylogeny and in silico antigenic analyses. Virus Res. 137, 173–178.

Castro, A. E., Moore, J., Hammami, S., Manalac, R. B. and Chin, R. P. (1992). Direct isolation of rotaviruses from turkeys in embryonating chicken eggs. Vet Rec 130:379—380.

Castrucci, G., Frigeri, F., Ferrari, M., Cilli, V., Aldrovandi, V., Caleffi, F. and Gatti, R. (1984). Comparative study of rotavirus strains of bovine and rabbit origin. Comparative Immunology and Microbiology, 7, 171–178

Castrucci, G., Frigeri, F., Ferrari, M., Cilli, V., Aldronvandi, V., Gatti, R. and Rampichini, L. (1985). A comparison of rotavirus strains of bovine, simian and porcine origin. European Journal of Epidemiology, 1, 274–28

Cattoli, G., Toffan, A., De Battisti, C., Salviato, A., Terregino, C. and Capua, I. (2005). Astroviruses found in the intestinal contents of guinea fowl chicken astrovirus, avian nephritis virus, and avian rotavirus. Avian Dis. 51, 681–684.

Chang, K. O., Kim, Y. J. and Saif, L. J. (1999). Comparisons of nucleotide and deduced amino acid sequences of NSP4 genes of virulent and attenuated pairs of group A and C rotaviruses. Virus Genes 18:229-33.

Chemello, M. E., Aristimuno, O. C., Michelangeli, F. and Ruiz, M. C. (2002). Requirement for vacuolar H+ -ATPase activity and Ca2+ gradient during entry of rotavirus into MA104 cells. J Virol 76:13083-7.

Chen, D., Luongo, C. L., Nibert, M. L. and Patton, J. T. (1999). Rotavirus open cores catalyze 5'-capping and methylation of exogenous RNA: evidence that VP3 is a methyltransferase. Virology 265:120-30.

Chizhikov, V. and Patton, J.T. (2000). A four nucleotide translation enhancer in the 3’-terminal consensus sequence of the nonpolyadenylated mRNAs of Rotavirus. *RNA* 2000, 6:814-825.

Chou, Q., Russell, M., Birch, D.E., Raymond, J. and Bloch, W. (1992). Prevention of pre-PCR mis-priming and primer dimerization improves low-copy number amplifications. Nucleic Acids Res. 20: 1717-1723.

Ciarlet, M., Conner, M. E., Finegold, M. J. and Estes, M. K. (2002). Group A Rotavirus Infection and Age-Dependent Diarrhoeal Disease in Rats: a New Animal Model To Study the Pathophysiology of RotavirusInfection. J Virol 76:41-57.

Ciarlet, M. and Estes, M. K. (1999). Human and most animal rotavirus strains do not require the presence of sialic acid on the cell surface for efficient infectivity. J Gen Virol 80:943-8.

Ciarlet, M. and Estes, M. (2002) Rotaviruses: basic biology, epidemiology and methodologies. In: Britton G (ed) Encyclopedia of environmental microbiology. Wiley, New York, pp 2573–2773

Ciarlet, M., Estes, M.K., Barone, C., Ramig, R.F. and Conner, M.E. (1998). Analysis of host range restriction determinants in the rabbit model: comparison of homologous and heterologous rotavirus infections. J. Virol. 72, 2341–2351.

Ciarlet, M., Ia, P., Conner, M.E. and Liprandi, F. (2001). Antigenic and molecular analyses reveal that the equine rotavirus strain H-1 is closely related to porcine, but not equine, rotaviruses: interspecies transmission from pigs to horses? Virus Genes, 22, 5–20

Ciarlet M., Liprandi F., Conner M.E., Estes M.K. (2000). Species specificity and interspecies relatedness of NSP4 genetic groups by comparative NSP4 sequence analyses of animal rotaviruses. Arch Virol 145:371–383

Cohen, J. (1977). Ribonucleic acid polymerase activity associated with purified calf rotavirus. J. Gen. Virol. 36:395-402.

Collins, J., Starkey, W. G., Wallis, T. S., Clarke, G. J., Worton, K. J., Spencer, A. J., Haddon, S. J., Osborne, M. P., Candy, D. C. and Stephen, J. (1988). Intestinal enzyme profiles in normal and rotavirus-infected mice. J Pediatr Gastroenterol Nutr 7:264-72.

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

Coulson, B.S. (1992). Typing of human rotavirus VP4 by an enzyme immunoassay using monoclonal antibodies. J Clin Microbiol 31:1-8.

Coulson, B.S., Gentsch, J.R., Das, B.K., Bhan, M.K., Glass, R.I. (1999). Comparison of enzyme immunoassay and reverse transcriptase PCR for identification of serotype G9 rotaviruses. J Clin Microbiol 37:3187-93.

Coulson, B.S. and Kirkwood, C. (1991). Relation of VP7 amino acid sequence to monoclonal antibody neutralization of rotavirus and rotavirus monotype. J Virol 65:5968-74.

Coulson B., Unicomb, L.E., Putson, G.A. and Bishop, R.F. (1987) Simple and specific enzyme immunoassay using monoclonal antibodies for serotyping human retroviruses. J Clin Microbiol 25:509-15.

Crawford, S. E., Labbe, M., Cohen, J., Burroughs, M. H., Zhou, Y. J. and Estes, M. K. (1994).

Characterisation of virus-like particles produced by the expression of rotavirus capsid proteins in insect cells. J Virol 68:5945-52.

Crawford, S. E., Mukherjee, S. K., Estes, M. K., Lawton, J. A., Shaw, A. L., Ramig, R. F. and Prasad, B. V. (2001). Trypsin cleavage stabilizes the rotavirus VP4 spike. J Virol 75:6052-61.

Cunliffe, N.A., Gondwe, J. S., Broadhead R. L., Molyneux M.E., Woods, P.A., Bresee, J. S. (1999). Rotavirus G and P types in children with acute diarrhoea in Blantyre, Malawi, from 1997 to 1998: predominance of novel P[6]G8 strains. J Med Virol; 57:308–12.

Cunliffe, N. A., Gondwe, J. S., Graham, S.M., Thindwa, B. D., Dove, W., Broadhead, R.L., Molyneux, M. E. and Hart C.A. (2001). Rotavirus strain diversity in Blantyre, Malawi, from 1997 to 1999. J Clin Microbiol; 39:836-43.

Cunliffe, N.A.,Woods, P.A., Leite, J.P.G., Das, B.K., Ramachandran, M., Bhan, M.K., Hart, C.A., Glass, R.I. and Gentsch, J.R. (1997). Sequence analysis of NSP4 gene of human rotavirus allows classification into two main genetic groups. J. Med. Virol. 53, 41–50.

Das, M, Dunn, S.J, Woode, G.N, Greenberg, H.B. and Rao, C.D. (1993). Both surface proteins (VP4 and VP7) of an asymptomatic neonatal rotavirus strain (I321) have high levels of sequence identity with the homologous proteins of a serotype 10 bovine rotavirus. Virology 194:374–9.

Das, B.K, Gentsch, J.R., Cicirello, H.G., Woods, P.A., Gupta, A., Ramachandran, M., Kumar, R., Bhan, M.K. and Glass, R.I. (1994). Characterisation of rotavirus strains from newborns in New Delhi, India. J Clin Microbiol 32:1820-2.

Day, J.M., Spackman, E., Pantin-Jackwood, M. (2007). A multiplex RT-PCR test for the differential identification of turkey astrovirus type 1, turkey astrovirus type 2, demonstration of distinct serotypes and RNA electropherotypes. Avian Pathol. 9, 363–375.

De Grazia, S., Martella, V., Giammanco, G.M., Gomara, M.I., Ramirez, S., Cascio, A., Colomba, C. and Arista, S. (2007). Canine-origin G3P[3] rotavirus strain in child with acute gastroenteritis. Emerging Infectious Diseases, 13, 1091–1093.

Delorme, C., Brussow, H., Sidoti, J., Roche, N., Karlsson, K. A., Neeser, J. R. and Teneberg, S. (2001). Glycosphingolipid binding specificities of rotavirus: identification of a sialic acid-binding epitope. J Virol 75:2276-87.

Despins, J. L., Axtell, R. C., Rives, D. V., Guy, J. S. and Ficken, M. D. (1994). Transmission of enteric pathogens of turkeys by darkling beetle larva (Alphitobius diaperinus). J Appl Poult Res 3:61—65.

Desselberger, U., Iturriza-Gomara, M. and Gray, J.J. (2001a). Rotavirus epidemiology and surveillance. In: Chadwick D., Goode J.A., (eds), Gastroenteritis viruses, Wiley, New York, USA, 82–100.

Desselberger, U., Iturriza-Gomara, M. and Gray, J.J. (2001b). Rotavirus epidemiology and surveillance. Novartis Foundation Symposium 238:125-47.

Desselberger, U., Wolleswinkel-van den Bosch, J., Mrukowicz, J., Rodrigo, C., Giaquinto, C. and Vesikari, T. (2006). Rotavirus types in Europe and their significance for vaccination. Pediatr Infect Dis J 25:S30–41.

Devitt, C. M. and Reynolds, D. L. (1993). Characterisation of a group D rotavirus. Avian Dis 37:749—755.

Dieffenbach, C.W., Lowe, T. M. and Dveksler, G. S. (1993). General concepts for PCR primer design. Genome Res. 3: S30-S37

Dormitzer, P. R. and Greenberg, H. B. (1992). Calcium chelation induces a conformational change in recombinant herpes simplex virus-1-expressed rotavirus VP7. Virology 189:828-32.

Duan, Z., Li, D., Zhang, Q., Liu, N., Huang, C., Jiang, X., Jiang, B., Glass, R., Steele, D., Tang, J., Wang, Z. and Fang, Z. (2007). Novel human rotavirus of genotype G5P[6] identified in a stool specimen from a Chinese girl with diarrhoea. Journal of Clinical Microbiology, 45, 1614–1617

Dveksler, G.S., Dieffenbach, C.W., Cardellichio, C.B., McCuaig, K., Pensiero, M.N., Jiang, G.-S., Beauchemin, N. and Holmes, K.V. (1993). Several members of the mouse carcinoembryonic antigen-related glycoprotein family are functional receptors for the coronavirus mouse hepatitis virus-A59. J. Virol. 67: 1-8.

Dyall-Smith, M. L., Azad, A. A. and Holmes, I. H. (1986). Gene mapping of rotavirus double-stranded RNA segments by Northern blot hybridization: application to segments 7, 8, and 9. J. Virol. 46:317-320.

Els, H. J. and Josling, D. (1998). Viruses and virus-like particles identified in ostrich gut contents. J S African Vet Assoc 69:74—80.

Elschner, M., Prudlo, J., Hotzel, H., Otto, P. and Sachse, K. (2002). Nested reverse transcriptase-polymerase chain reaction for the detection of group A rotaviruses. J. Vet. Med. B 49, 77–81.

Erk, I., Huet, J.C., Duarte, M., Duquerroy, S., Rey, F., Cohen, J. and Lepault, J. (2003). A zinc ion controls assembly and stability of the major capsid protein of rotavirus. J. Virol. 77, 3595–3601.

Esona, M.D, Armah, G.E, Geyer, A. and Steele, A.D. (2004). Detection of an unusual human rotavirus strain with G5P[8] specificity in a Cameroonian child with diarrhoea. J Clin Microbiol 42:441–4.

Esparza, J. and Gil, F. (1978). A study on the ultrastructure of human rotavirus. Virology 91:141-150.

Espejo, R. T., Lopez, S. and Arias, C. (1981). Structural polypeptides of simian rotavirus SA11 and the effect of trypsin. J Virol 37:156-60.

Estes, M. K. (2001). Rotaviruses and their replication, p. 1747-1785. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology. Lippencott-Raven Publishers, Philadelphia.

Estes, M. K. and Cohen, J. (1989). Rotavirus structure and function. Microbiological reviews 53:410-449.

Estes, M. K., Crawford, S. E., Penaranda, M. E., Petrie, B. L., Burns, J. W., Chan, W. K., Ericson, B., Smith, G. E. and Summers, M. D. (1987). Synthesis and immunogenicity of the rotavirus major capsid antigen using a baculovirus expression system. J Virol 61:1488-94.

Estes, M.K. and Desselberger U. (2012). Rotaviruses: cause of vaccine-preventable disease yet many fundamental questions remain to be explored. Current Opinion in Virology 2012, 2:369–372

Estes M. K., Graham, D. Y. and Dimitrov, D. H. (1984). The molecular epidemiology of rotavirus gastroenteritis. Progress in Medical Virology, 29: 1 – 22.

Estes, M. K., Graham, D. Y. and Mason, B. B. (1981). Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. J Virol 39:879-88.

Estes, M.K. and Kapikian A.Z. (2007) Rotaviruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds) Fields virology, 5th edn. Lippincott Williams and Wilkins, Philadelphia, pp 1917–1974.

Fabbretti, E., Afrikanova, I., Vascotto, F. and Burrone, O. R. (1999). Two non-structural rotavirus proteins, NSP2 and NSP5, form viroplasm-like structures *in-vivo*. J Gen Virol 80 (Pt 2):333-9.

Ferrari, M., Gualandi, G.L. and Gelmetti, D. (1986). Isolation of cytopathic strains of rotavirus from pigs. Microbiological, v.9, p.287-295.

Fiore, L., Greenberg, H. B. and Mackow, E. R. (1991). The VP8 fragment of VP4 is the rhesus rotavirus hemagglutinin. Virology 181:553-63.

Fischer, T.K., Eugen-Olsen, J., Pedersen, A.G., Molbak, K., Bottiger, B. and Rostgaard K. (2005). Characterisation of rotavirus strains in a Danish population: high frequency of mixed infections and diversity within the VP4 gene of P[8] strains. J Clin Microbiol 43:1099–104.

Fitzgerald, S.D. (2008). Adenovirus infections. In: Saif, Y.M., Fadley, A.M., Glission, J.R., Mc Dougald, L.R., Nolan, L.K., Swayne, D.E. (Eds.), Diseases of Poultry. 12th ed. Blackwell Publishing Professional, Ames, IA, USA, pp. 251–252.

Flewett, T. H., Bryden, A. S. and Davies, H. (1973). Letter: Virus particles in gastroenteritis. Lancet 2:1497.

Flewett, T. H., Bryden, A. S., Davies, H., Woode, G. N., Bridger, J. C. and Derrick, J. M. (1974). Relation between viruses from acute gastroenteritis of children and newborn calves. Lancet 2:61-3.

Foni, E., Gelmetti, D., Nigrelli, A. D., Gatti, R. and Carra, V. (1989). Transmissible enteritis syndrome in pheasants for restocking: Experimental reproduction of the disease. Isolation of rotavirus. Sel Vet 30:879—888.

Fraga, M., Frias, M. T., Moreno, A. and L'Haridon, R. (1985). Diagnosis of rotavirus in broilers. Revista de Salud Animal 1:13—18.

Freier, S.M., Kierzek, R., Jaeger, J.A., Sugimoto, N., Caruthers, M.H., Neilson, T. and Turner, D.H. (1986). Improved free-energy parameters for predictions of RNA duplex stability. Proc. Natl. Acad. Sci. 83: 9373-9377.

Gentsch J.R, Laird A.R, Bielfelt B, Griffin D.D, Ba´nyai K, Ramachandran M, Jain V, Cunliffe N.A, Nakagomi O, Kirkwood C.D, Fischer T.K, Parashar U.D, Bresee J.S, Jiang B. and Glass R.I. (2005) Serotype diversity and reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs. J Infect Dis 192(Suppl 1):S146–159

Gentsch, J.R., Glass, R.I., Woods, P., Gouvea, V., Gorziglia, M., Flores, J., Das, B.K, Bhan, M.K. (1992). Identification of group A rotavirus gene 4 types by polymerase chain reaction. J Clin Microbiol: 30:1365-73.

Gentsch, J.R., Woods, P.A., Ramachandran, M., Das, B.K., Leite, J.P., Alfieri, A., Kumar, R., Bhan, M.K. and Glass, R.I. (1996). Review of G and P typing results from a global collection of strains: implications for vaccine development. J Infect Dis 174(Suppl 1):S30-6.

Golantsova, N. E., Gorbunova, E. E. and Mackow, E. R. (2004). Discrete domains within the rotavirus VP5* direct peripheral membrane association and membrane permeability. J Virol 78:2037-44.

Gonzalez, R. A., Torres-Vega, M. A., Lopez, S. and Arias, C. F. (1998). *In-vivo* interactions among rotavirus nonstructural proteins. Arch Virol 143:981-96.

Gorziglia, M., Larralde, G., Kapikian, A.Z., Chanock, R.M. (1990). Antigenic relationships among human rotaviruses as determined by outer capsid protein VP4. Proc. Natl. Acad. Sci. U.S.A. 87, 7155–7159.

Gough, R. E., Collins, M. S., Alexander, D. J. and Cox, W. J. (1990). Viruses and virus-like particles detected in samples from diseased game birds in Great Britain during 1988. Avian Pathol 19:331—342.

Gough, R. E., Collins, M. S., Wood, G. W. and Lister, S. A. (1988). Isolation of a chicken embryo-lethal rotavirus from a lovebird (Agapornis species). Vet Rec 122:363—364.

Gough, R. E., Cox, W. J. and Davis, C. (1999). Studies with an inactivated pheasant rotavirus vaccine. Vet Rec 144:423—424.

Gough, R. E., Cox, W. J. and Devoy, J. (1992). Isolation and identification of rotavirus from racing pigeons. Vet Rec 130:273.

Gough, R.E., Wood, G.W., Collins, M.S., Spackman, D., Kemp, J. and Gibson, L.A (1985) Rotavirus infection in pheasant poults. Vet Rec 116:295

Gough, R. E., Wood, G. W. and Spackman, D. (1986). Studies with an atypical avian rotavirus from pheasants. Vet Rec 118:611—612.

Gouvea, V., Allen, J.R., Glass, R.I., Fang, Z.Y., Bremont, M., Cohen, J., McCrae, M.A., Saif, L.J., Sinarachatanant, P. and Caul, E.O. (1991). Detection of group B and C rotaviruses by polymerase chain reaction. J Clin Microbiol 29:519-23.

Gouvea, V., de Castro, L., Timenetsky, M.D.C., Greenberg, H. and Santos, N. (1994). Rotavirus serotype G5 associated with diarrhoea in Brazilian children [published erratum appears in J Clin Microbiol 32(7):1834.

Gouvea, V., Glass, R.I., Woods, P., Taniguichi, K., Clark, H.F., Forrester, B., Fang, Z.Y. (1990) Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J Clin Microbiol 28:276-82.

Graham, D.Y., Estes, M.K. (1985) Proposed working serologic classification system for rotaviruses. Annales de l'Institut Pasteur de Virologie 136:5–12

Graham, K. L., Halasz, P., Tan, Y., Hewish, M. J., Takada, Y., Mackow, E. R., Robinson, M. K. and Coulson, B. S. (2003). Integrin-using rotaviruses bind alpha2beta1 integrin alpha2 I domain via VP4 DGE sequence and recognize alphaXbeta2 and alphaVbeta3 by using VP7 during cell entry. J Virol 77:9969-78.

Green, K.Y., Sears, J.F, Taniguchi, K., Midthun, K., Hoshino, Y., Gorziglia, M., Nishikawa, K., Urasawa, S., Kapikian, A.Z., Chanock, R.M. and Flores, J. (1988)

Prediction of human rotavirus serotype by nucleotide sequence analysis of the VP7 protein gene. J Virol 62:1819-23.

Greenberg, H.B., Flores, J., Kalika, A.R., Wyatt, R.G., Jones, R. (1983). Gene coding assignments for growth restriction neutralization and subgroup specificities of the Wa and DS-1 strains of human rotavirus. J. Gen. Virol. 64, 313–320.

Greig, S.L., Berriman, J.A., O'Brien, J.A., Taylor, J.A., Bellamy, A.R., Yeager, M.J., Mitra, A.K. (2006). Structural determinants of rotavirus subgroup specificity mapped by cryo-electron microscopy. J. Mol. Biol. 356, 209–221.

Guerrero, C. A., Bouyssounade, D., Zarate, S., Isa, P., Lopez, T., Espinosa, R., Romero, P., Mendez, E., Lopez, S. and Arias, C. F. (2002). Heat shock cognate protein 70 is involved in rotavirus cell entry. J Virol 76:4096-102.

Gunasena, S., Nakagomi, O., Isegawa, Y., Kaga, E., Nakagomi, T., Steele, A.D., Flores, J., Ueda, S. (1993). Relative frequency of VP4 gene alleles among human rotaviruses recovered over a 10 year period (1982-1991) from Japanese children with diarrhoea. J Clin Microbiol 31:2195-7.

Gunson, R. N., Miller, J., Leonard, A. and Carman, W. F. (2003). Importance of PCR in the diagnosis and understanding of rotavirus illness in the community. Commun. Dis. Public Health 6:63–65.

Halaihel, N., Lievin, V., Alvarado, F. and Vasseur, M. (2000a). Rotavirus infection impairs intestinal brushborder membrane Na(+)-solute cotransport activities in young rabbits. Am J Physiol Gastrointest Liver Physiol 279:G587-96.

Halaihel, N., Lievin, V., Ball, J. M., Estes, M. K., Alvarado, F. and Vasseur, M. (2000b). Direct inhibitory effect of rotavirus NSP4(114-135) peptide on the Na(+)-D-glucose symporter of rabbit intestinal brush border membrane. J Virol 74:9464-70.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41:95–98.

Hamilton, J. R. and Gall, D. G. (1982). Pathophysiological and clinical features of viral enteritis. In D. A. J. Tyrrell and A. Z. Kapikian (eds.). Virus infections of the gastrointestinal tract. Marcel Dekker: New York, 227—238.

Hayhow, C. S. and Saif, Y. M. (1993). Experimental infection of specific-pathogen-free turkey poults with single and combined enterovirus and group A rotavirus. Avian Dis 37:546—557.

Haynes, J. S., Reynolds, D. L., Fagerland, J. A. and Fix, A. S. (1994). Morphogenesis of enteric lesions induced by group D rotavirus in ringneck pheasant chicks (*Phasianus colchicus).* Vet Pathol 31:74—81.

Heinrich, H.W., Schirrmeier, H., Lange, E. and Granzow, H. (1983). Cross infection with rotavirus of different species. Archives Experimental Veterinamedicine, 37, 55–60

Herring, A.J., Inglis, N.F., Ojeh, C.K., Snodgrass, D.R., Menzies, J.D. (1982). Rapid diagnosis of rotavirus infection by direct detection of viral nucleic acid in silver-stained polyacrylamide gels. J Clin Microbiol 16:473-7.

Hines, I.I., Styer, E. L., Baldwin, C. A. and Cole, Jr., J. R. (1995). Combined adenovirus and rotavirus enteritis with Escherichia coli septicemia in an emu chick (Dromaius novaehollandiae). Avian Dis 39:646—651.

Horrox, N. E. (1980). Some observations and comments on rotaviruses in turkey poults. Proc 29th West Poult Dis Conf, 162—164.

Hoshino, Y. and Kapikian, A.Z. (2000) Rotavirus serotypes: classification and importance in epidemiology, immunity, and vaccine development. J Health Pop Nutr 18:5-14.

Hoshino, Y., Saif, L. J., Kang, S. Y., Sereno, M. M., Chen, W. K. and Kapikian, A. Z. (1995). Identification of group A rotavirus genes associated with virulence of a porcine rotavirus and host range restriction of a human rotavirus in the gnotobiotic piglet model. Virology 209:274-80.

http://en.m.wikipedia.org/wiki/primer_(molecular_biology) (2014)

http://rotac.regatools.be/. (2012). RotaC automated genotyping tool for Group A rotaviruses

Imai, M; Akatani, K; Ikegami, N; Furuichi, Y. (1983). Capped and conserved terminal structures in human rotavirus genome doublestranded RNA segments. Journal of Virology 47:125-136.

Ito, H., Sugiyama, M., Masubuchi, K., Mori, Y., and Minamoto, N. (2001) Complete nucleotide sequence of a group A avian rotavirus genome and a comparison with its counterparts of mammalian rotaviruses. Virus Res 75:123–138

Ito, H., Minamoto, N., Goto, H., Luo, T.R., Sugiyama, M. and Kinjo, T. (1996). Mapping of antigenic sites on the major inner capsid protein of avian rotavirus using an *Escherichia coli* expression system. Arch. Virol. 141, 2129–2138.

Iturriza-G´omara, M., Desselberger, U. and Gray, J. (2003). Molecular epidemiology of rotaviruses: genetic mechanisms associated with diversity. In: Desselberger U, Gray J, editors. Viral gastroenteritis,vol. 9. Amsterdam: Elsevier p. 317–44.

Iturriza-Gomara, M., Green, J., Brown, D.W.G., Desselberger, U., Gray, J.J. (1999). Comparison of specific and random priming in the reverse transcription polymerase chain reaction for genotyping group A rotaviruses. J Virol Meth 78:93-103.

Iturriza-G´omara, M., Green, J., Brown, D.W., Desselberger, U., Gray, J.J. (2000c). Diversity within the VP4 gene of rotavirus P[8] strains: implications for reverse transcription-PCR genotyping. J Clin Microbiol: 38:898– 901.

Iturriza-G´omara, M., Green, J., Brown, D., Ramsay, M., Desselberger, U. and Gray, J. (2000a) Molecular epidemiology of human group A rotavirus infections in the UK between 1995 and 1998. J Clin Microbiol 38:4094–401.

Iturriza-Gomara, M., Green, J. and Gray J. (2000b). Methods of rotavirus detection, sero- and genotyping, sequencing, and phylogenetic analysis. In: Gray J, Desselberger U, eds. Rotaviruses: methods and protocols. Human Press, Totowa, New Jersey p. 189-216.

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

Iturriza-G´omara, M., Kang, G. and Gray, J.J. (2004a) Rotavirus genotyping: keeping up with an evolving population of human rotaviruses. J Med Virol 31:259–65.

Iturriza-G´omara, M., Kang, G., Mammen, A., Jana, A.K., Abraham, M. and Desselberger, U. (2004b). Characterisation of G10P[11] rotaviruses causing acute gastroenteritis in neonates and infants in Vellore, India. J Clin Microbiol 42:2541–7.

Iturriza Go´mara, M., Wong, C., Blome, S., Desselberger, U. and Gray, J. (2002). Molecular characterisation of VP6 genes of human rotavirus isolates: correlation of

genogroups with subgroups and evidence of independent segregation. J Virol 76:6596–6601

Johne, R., Otto, P., Roth, B., Lo¨ hren, U., Belnap, D., Reetz, J., Trojnar, E. (2011). Sequence analysis of the VP6-encoding genome segment of avian group F and G rotaviruses. Virology 412, 384–391.

Jones, C. (2008). Reovirus infections. In: Saif, Y.M., Fadley, A.M., Glission, J.R., Mc Dougald, L.R., Nolan, L.K., Swayne, D.E. (Eds.), Diseases of Poultry. 12th ed. Blackwell Publishing Professional, Ames, IA, USA, pp. 309–328.

Jones, R. C., Hughes, C. S. and Henry, R. R. (1979). Rotavirus infection in commercial laying hens. Vet Rec 104:22.

Kabcenell, A. K. and Atkinson, P. H. (1985). Processing of the rough endoplasmic reticulum membrane glycoproteins of rotavirus SA11. J Cell Biol 101:1270-80.

Kalendar, R., Lee D., Schulman, A.H. (2009). FastPCR Software for PCR Primer and Probe Design and Repeat Search. Genes, Genomes and Genomics, 3(1): 1-14.

Kalica, A. R., Flores, J. and Greenberg, H. B. (1983). Identification of the rotaviral gene that codes for hemagglutination and protease-enhanced plaque formation. Virology 125:194-205.

Kaljot, K. T., Shaw, R. D., Rubin, D. H. and Greenberg, H. B. (1988). Infectious rotavirus enters cells by direct cell membrane penetration, not by endocytosis. J Virol 62:1136-44.

Kang, S. Y., Nagaraja, K. V. and Newman, J. A. (1988). Electrophoretic analysis of rotavirus isolated from turkeys. Avian Dis. 30, 794–801.

Kang, G., Iturriza-Gomara, M., Wheeler, J.G., Crystal, P., Monica, B., Ramani, S., Primrose, B., Moses, P.D., Gallimore, C.I., Brown, D.W. and Gray, J. (2004) Quantitation of group A rotavirus by real-time reverse-transcription-polymerase chain reaction: correlation with clinical severity in children in South India. J Med Virol 73:118-22.

Kapikian, A. Z. (1993). Viral gastroenteritis. Vet Res Commun (2009) 33:1–23

Kapikian, A. Z. and Chanock, R. M. (1990). Rotaviruses, p. 1353-1404. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, and B. Roizman (ed.), Virology, 2nd ed. Raven press, NewYork.

Kapikian, A., Hoshino, Y. and Chanock, R. (2001). Rotaviruses. In: Knipe DM, Howley PM, eds. Fields Virology. Vol. 2. 4th ed. Philadelphia: Lippincott-Raven Publishers, p.1787-1834.

Kapahnke, R., Rappold, W., Desselberger, U. and Riesner, D. (1986). The stiffness of dsRNA: hydrodynamic studies on fluorescence- labelled RNA segments of bovine rotavirus. Nucleic Acids Res. 14:3215-3228.

Katyal, R., Rana, S. V., Vaiphei, K., Ohja, S., Singh, K. and Singh, V. (1999). Effect of rotavirus infection on small gut pathophysiology in a mouse model. J Gastroenterol Hepatol 14:779-84.

Khamrin, P., Maneekarn, N., Peerakome, S., Yagyu, F., Okitsu, S. and Ushijima, H. (2006). Molecular characterisation of a rare G3P[3] human rotavirus reassortant strain reveals evidence for multiple human animal interspecies transmissions. Journal of Medical Virology, 78, 986–994

Kirkwood, C., Bogdanovic-Sakran, N., Palombo, E., Masendycz, P., Bugg, H., Barnes, G. and Bishop, R. (2003). Genetic and antigenic characterisation of rotavirus serotype G9 strains isolated in Australia between 1997 and 2001. J Clin Microbiol 41:3649-54.

Kool, D.A., Matsui, S.M., Greenberg, H.B. and Holmes, I.H. (1992). Isolation and characterisation of a novel reassortant between avian Ty-1 and simian RRV rotaviruses. J. Virol.66, 6836–6839.

Koshimura, Y., Nakagomi, T. and Nakagomi, O. (2000). The relative frequencies of G serotypes of rotaviruses recovered from hospitalized children with diarrhoea: a 10-year survey (1987-1996) in Japan with a review of globally collected data. Microbiol Immunol 44:499-510.

Lee, C. N., Wang, Y. L., Kao, C. L., Zao, C. L., Lee, C. Y. and Chen, H. N. (2000). NSP4 gene analysis of rotaviruses recovered from infected children with and without diarrhoea. J Clin Microbiol 38, 4471–4477.

Legrottaglie, R., Rizzi, V. and Agrimi, P. (1997). Isolation and identification of avian rotavirus from pheasant chicks with signs of clinical enteritis. Comp Immunol Microbiol Infect Dis 20:205–210

Liang, P. and Pardee, A. (1992). Differential display of eukaryotic mRNAs by PCR. Science 257: 967-971.

Lin, S. L. and Tian, P. (2003). Detailed computational analysis of a comprehensive set of group A rotavirus NSP4 proteins. Virus Genes 26, 271–282.

Lin, P.K.T. and Brown, D.M. (1992). Synthesis of oligodeoxyribonucleotides containing degenerate bases and their use as primers in the polymerase chain reaction. *Nucleic Acids Res.* 19: 5149-5152.

Liu, M., Mattion, N. M. and Estes, M. K. (1992). Rotavirus VP3 expressed in insect cells possesses guanyltransferase activity. Virology 188:77-84.

Lopez, S. and C.F. Arias (2006). Early steps in rotavirus cell entry. Current Topics in Microbiology and Immunology 309:39-66.

Lopez, S.; Arias, C. F; Bell, J. R; Strauss, J. H. and Espejo, R. T. (1985). Primary structure of the cleavage site associated with trypsin enhancement of rotavirus SAl infectivity. Virology 144:11-19.

Ludert, J.E., Feng, N., Yu, J.H., Broome, R.L., Hoshino, Y., Greenberg, H.B., 1996. Genetic mapping indicates that VP4 is the rotavirus cell attachment protein in vitro and in vivo. J. Virol. 70, 487–493.

Lundgren, O., Peregrin, A. T., Persson, K., Kordasti, S., Uhnoo, I. and Svensson, L. (2000). Role of the enteric nervous system in the fluid and electrolyte secretion of rotavirus diarrhoea. Science 287:491-5.

Maass, D. R. and Atkinson, P. H. (1990). Rotavirus proteins VP7, NS28, and VP4 form oligomeric structures. J Virol 64:2632-41.

Mack, D.H. and Sninsky, J.J. (1988). A sensitive method for the identification of uncharacterized viruses related to known virus groups: Hepadnavirus model system. Proc. Natl. Acad. Sci. 85: 6977-6981.

Mackay, I.M., Arden, K.E. and Nitsche, A. (2002). Real-time PCR in virology. Nucleic Acids Res. 30, 1292–1305.

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

Martella, V., Ciarlet, M., Camarda, A., Pratelli, A., Tempesta, M., Greco, G., Cavalli, A., Elia, G., Decaro, N., Terio, V., Bozzo, G., Camero, M. and Buonavoglia, C. (2003). Molecular characterisation of the VP4, VP6, VP7, and NSP4 genes of lapine rotaviruses identified in Italy: emergence of a novel VP4 genotype. Virology 314:358–370.

Martella, V., Terio, V., Arista, S., Elia, G., Corrente, M. and Madio, A. (2004). Nucleotide variation in the VP7 gene affects PCR genotyping of G9 rotaviruses identified in Italy. J Med Virol 72:143–8.

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

Masendycz, P.J., Palombo, E.A., Gorrell, R.J. and Bishop, R.F. (1997). Comparison of enzyme immunoassay, PCR, and type-specific cDNA probe techniques for identification of group A rotavirus gene 4 types. J Clin Microbiol 35:3104-8.

Matsui, S.M., Mackow, E.R., Matsuno, S., Paul, P.S. and Greenberg, H.B. (1990). Sequence analysis of gene 11 equivalents from “short” and “super short” strains of rotavirus. J Virol 64:120-4.

Matthijnssens, J., Ciarlet, M., Heiman, E., Arijs, I., Delbeke, T., McDonald, S.M., Palombo, A.E., Iturriza-Go´mara, M., Maes, P., Patton, J.T., Rahman, M. and Van Ranst, M. (2008a). Full genome-based classification of rotaviruses reveals common origin between human Wa-like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. J Virol 82:3204–3219

Matthijnssens, J., Ciarlet, M., Rahman, M., Attoui, H., Ba´nyai, K., Estes, M.K., Gentsch, J.R., Iturriza-Go´mara, M., Kirkwood, C.D., Martella, V., Mertens, P.P., Nakagomi, O., Patton, J.T., Ruggeri, F.M., Saif, L.J., Santos, N., Steyer, A., Taniguchi, K., Desselberger, U. and Van Ranst, M. (2008b). Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. Arch Virol 153:1621–1629

Matthijnssens, J., Rahman, M. and Van Ranst, M. (2008c). Two out of 11 genes of an unusual human G6P[6] rotavirus isolate are of bovine origin. J. Gen. Virol. 89, 2630–2635.

Matthijnssens, J., Rahman, M., Martella, V., Xuelei, Y., De Vos, S., De Leener, K., Ciarlet, M., Buonavoglia, C. and Van Ranst, M. (2006). Full genomic analysis of human rotavirus strain B4106 and lapine rotavirus strain 30/96 provides evidence for interspecies transmission. J Virol 80:3801–3810.

Mattion, N. M., Cohen, J., Aponte, C. and Estes, M. K. (1992). Characterisation of an oligomerization domain and RNA-binding properties on rotavirus nonstructural protein NS34. Virology 190:68-83.

Mattion, N.M., Cohen, J. and Estes, M.K. (1994). The Rotavirus proteins. In: Kapikian AZ., ed: Viral infections of the gastrointestinal tract, 2nd ed. New York: Marcel Dekker; 1994: 169-249.

McNulty, M.S. (2003). Rotavirus infections. In: Saif, Y.M. (Ed.), Diseases of Poultry. Iowa State University press, Ames, IA, pp. 308–320.

McNulty, M. S., Allan, G. M. and McCracken, R. M. (1983). Experimental infection of chickens with rotaviruses: Clinical and virological findings. Avian Pathol 12:45—54.

McNulty, M. S., Allan, G. M. and Stuart, J. C. (1978). Rotavirus infection in avian species. Vet Rec 103:319—320.

McNulty, M. S., Allan, G. M.., Todd, D. and McFerran, J. B. (1979a). Isolation and cell culture propagation of rotaviruses from turkeys and chickens. Arch Virol 61:13—21.

McNulty, M. S., Allan, G. M.., Todd, D. and McFerran, J. B. and McCracken, R. M. (1981). Isolation from chickens of a rotavirus lacking the rotavirus group antigen. J Gen Virol 55:405—413.

McNulty, M.S., Curran, W.L., Todd, D., McFerran, J.B. (1979). Detection of viruses in avian faeces by direct electron microscopy. Avian Pathol. 8, 239–247.

McNulty, M.S., Allan, G.M., Todd, D., McFerran, J.B., McKillop, E.R., Collins, D.S. and McCracken, R.M. (1980). Isolation of rotaviruses from turkeys and chickens:

demonstration of distinct serotypes and RNA electropherotypes. Avian Pathol. 9, 363–375.

McNulty, M.S., Todd, D., Allan, G.M., Mc Ferran, J.B. and Greene, J.A. (1984a). Epidemiology of rotavirus infection in broiler chickens: recognition of four serogroups. Arch. Virol. 81, 113–121.

McNulty, M.S., Allan, G.M. and Mc Ferran, J.B. (1984b). Prevalence of antibody to conventional and atypical rotaviruses in chickens. Vet. Rec. 114, 219.

Mebus, C.A., Underdahl, N.R., Rhodes, M.B. and Twiehaus, M.J. (1969).Calf diarrhoea (scours) reproduced with a virus from a field outbreak. Univ. Nebraska Agric. Exp. Stn. Res. Bul., v.223, p.1-16.

Mebus, C. A., Kono, M., Underdahl, N. R. and Twiehaus, M. J. (1971). Cell culture propagation of neonatal calf diarrhoea (scours) virus. Can Vet J 12:69-72.

Mebus, C.A., Wyatt, R.G. and Kapikian, A.Z. (1977). Intestinal lesions induced in gnotobiotic calves by the virus of human infantile gastroenteritis. Veterinary Pathology, 14, 273–282

Mertens, P. P. C., Arella, M., Attoui, H., Belloncik, S., Bergoin, M., Boccardo, G., Booth, T. F., Chiu, W., Diprose, J. M., Duncan, R., Estes, M. K., Gorziglia, M., Gouet, P., Gould, A. R., Grimes, J. M., Hewat, E., Holmes, I. H., Hoshino, Y., Hill, C., Joklik, W. K., Knowles, N., Ferber, M. L., Malby, R., Marzachi, C., McCrae, M. A., Milne, R. G., Nibert, M., Nunn, M., Omura, T., Prasad, B. V. V., Pritchard, I., Roy, P., Samal, S. K., Schoehn, G., Shikata, E., Stoltz, D. B., Stuart, D. I., Suzuki, N., Upadhyaya, N., Uyeda, I., Waterhouse, P., Williams, C. F., Winton, J. R. and Zhou, H. Z. (2000). Reoviridae. In M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeogh, C. R. Pringle, and R. B. Wickner (eds.). Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press: San Diego, 395—480.

Meulemans, G., Charlier, G. and Halen, P. (1985a). Detection de rotavirus aviaire et adaptation a la culture cellulaire. Ann Med Vet 127:43—48.

Meulemans, G., Peeters, J. E. and Halen, P. (1985b). Experimental infection of broiler chickens with rotavirus. Br Vet J 141:69—73.

Minamoto, N., Oki, K., Tomita, M., Kinjo, T. and Suzuki, Y. (1988a). Isolation and characterisation of rotavirus from feral pigeon in mammalian cell cultures. Epidemiol Infect 100:481—492.

Minamoto, N., Sugimoto, Y. M., Kinjo T. and Suzuki, Y. (1988). Isolation and characterisation of rotavirus from feral pigeon in mammalian cell cultures. Epidermiol. Infect 100, 481-492.

Minamoto, N., Sugimoto, Y. M., Tomita,, H., Goto, M., Sugiyama, and Kinjo, T. (1993). Antigenic analysis of avian rotavirus VP6 using monoclonal antibodies. ArchVirol 131:293-305.

Mirazimi, A., Nilsson, M. and Svensson, L. (1998). The molecular chaperone calnexin interacts with the NSP4 enterotoxin of rotavirus *in-vivo* and *in-vitro*. J Virol 72:8705-9.

Mori, Y., Borgan, M. A., Ito, N., Sugiyama, M. and Minamoto, N. (2002). Diarrhoea-inducing activity of avian rotavirus NSP4 glycoproteins, which differ greatly from mammalian rotavirus NSP4 glycoproteins in deduced amino acid sequence in suckling mice. J Virol 76:5829-34.

Mori, Y., Borgan, M.A., Takayama, M., Ito, N., Sugiyama, M., Minamoto, N. (2003). Roles of outer capsid proteins as determinants of pathogenicity and host range of avian rotaviruses in a suckling mouse model. Virology 316, 126–134.

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

Mota-Hernandez, F., Calva, J. J., Gutierrez-Camacho, C., Villa-Contreras, S., Arias, C. F., Padilla- Noriega, L., Guiscafre-Gallardo, H., de Lourdes Guerrero, M., Lopez, S., Munoz, O., Contreras, J. F., Cedillo, R., Herrera, I. and Puerto, F. I. (2003). Rotavirus diarrhoea severity is related to the VP4 type in Mexican children. J Clin Microbiol 41:3158-62.

Muller, H. and Johne, R. (2007). Rotaviruses: diversity and zoonotic potential-a brief review. Berlin Munch Tierarztl Wochenschr, 120, 108–112

Myers, T.J. and Schat, K.A. (1990). Natural killer cell activity of chicken intraepithelial leukocytes against rotavirus-infected target cells. Veterinary Immunology and Immunopathology, 26, 157–170

Nakagomi, O. and Nakagomi, T. (1991). Genetic diversity and similarity among mammalian rotaviruses in relation to interspecies transmission of rotavirus. Archives of Virology, 120, 43–55

Nakagomi, O. and Nakagomi, T. (2002). Genomic relationships among rotaviruses recovered from various animal species as revealed by RNA-RNA hybridization assays. Res Veterin Sci 73:207-214.

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

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

Ngoveni H.G., Seheri, L.M. and Mphahlele, M.J. (2012). Genetic evidence of avian rotaviruses in limpopo province of South Africa: an unusual detection of group a rotaviruses in chickens. In: Proceedings of the 11th International Symposium on Double-Stranded RNA Viruses, San Juan, Puerto-rico.

Nibert, M. L., Furlong, D. B. and Fields, B. N. (1991). Mechanisms of viral pathogenesis. Distinct forms of reoviruses and their roles during replication in cells and host. J Clin Invest 88:727-34.

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

Niture G.S., Karpe, A.G., Prasad, M., Bhonsle, A.V. and Patil, S.V. (2010). Detection of Group D Avian Rotaviruses among Layer Poultry from Western India International Journal of Poultry Science 9 (1): 72-76.

Offit, P.A., Blavat, G., Greenberg, H.B. and Clark, H.F. (1986). Molecular basis of rotavirus virulence: role of gene segment 4. J. Virol. 57, 46–49.

Ojeh, C.K., Atti, D.J., Omotade, O.O. (1995). Comparison of the genome dsRNA of human rotavirus strains in parts of Ibadan, Nigeria. Afr J Med Med Sci. 24:359-63.

Okada, J., Kobayashi, N., Taniguchi, K. and Urasawa, S. (1999). Analysis on reassortment of rotavirus NSP1 genes lacking coding region for cysteine-rich zinc finger motif. Arch Virol 144:345-53.

Olomola, A.S. (2007). "Strategies for managing the opportunities and challenges of the current Agricultural Commodity Booms in SSA" In seminar papers on managing Commodity booms in Sub-sahara Africa: A publication of the AERC senior policy seminar IX. African Economic Research Consortium (AERC), Nairobi, Kenya.

O'Neill, H. J., McCaughey, C., Coyle, P. V., Wyatt, D. E. and Mitchell, F. (2002). Clinical utility of nested multiplex RT-PCR for group F adenovirus, rotavirus and Norwalk-like viruses in acute viral gastroenteritis in children and adults. J. Clin. Virol. 25:335–343.

Osborne, M. P., Haddon, S. J., Spencer, A. J., Collins, J., Starkey, W. G., Wallis, T. S., Clarke, G. J., Worton, K. J., Candy, D. C. and Stephen, J. (1988). An electron microscopic investigation of time-related changes in the intestine of neonatal mice infected with murine rotavirus. J Pediatr Gastroenterol Nutr 7:236-48.

Otto, P., Liebler-Tenorio, E.M., Elschner, M., Reetz, J., Löhren, U. and Diller, R. (2006). Detection of rotaviruses and intestinal lesions in broiler chicks from flocks with runting and stunting syndrome (RSS). Avian Dis. 50, 411–418.

Otto, P., Liebler-Tenorio, E.M., Löhren, U., Bachmeier, J. (2007). Occurrence of rotaviruses in broiler flocks from Northern and Southern Germany. Referatesammlung 72. Fachgespräch, Fachgruppe "Geflügelkrankheiten", Deutsche Veterinärmedizinische Gesellschaft e.V.

Ou, C.Y., Kwok, S., Mitchell, S.W., Mack, D.H., Sninsky, J.J., Krebs, J.W., Feorino, P., War field, D. and Schochetman, G. (1988). DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science 280: 295-297.

Padilla-Noriega, L., Werner-Eckert, R., Mackow, E.R., Gorziglia, M., Larralde, G., Taniguchi, K., Greenberg, H.B. (1993). Serologic analysis of human rotavirus serotypes P1A and P2 by using monoclonal antibodies. J Clin Microbiol 31:622-8.

Padilla-Noriega, L., Mendez-Toss, M., Menchaca, G., Contrearas, J.F., Romero-Guido, P., Puerto, F.I., Guiscafre, H., Mota. F., Herrera, I., Cedillo, R., Munoz, O., Calva, J., Guerrero, M.L., Coulson, B.S., Greenberg, H.B., Lopez, S., Arias, C.F. (1998). Antigenic and genomic diversity of human rotavirus VP4 in two consecutive epidemic seasons in Mexico. J Clin Microbiol 36:1688-92. Manual of Rotavirus detection and characterisation methods

Palombo, E.A. (2002). Genetic analysis of Group A rotaviruses: evidence for interspecies transmission of rotavirus genes. Virus Genes, 24, 11–20

Palombo, E.A. (2003). Genetic reassortment and interspecies transmission of rotaviruses. In: Kobayashi N, ed. Research signpost 37, p661. Kerala

Pang, X.L., Lee, B., Boroumand, N., Leblanc, B., Preiksaitis, J.K., Yu, I.C.C. (2004). Increased detection of rotavirus using a real time reverse transcriptionpolymerase chain reaction (RT-PCR) assay in stool specimens fromchildren with diarrhoea. J. Med. Virol. 72, 496–501.

Parra, G.I., Bok, K., Martinez, M., Gomez, J.A. (2004) Evidence of rotavirus intragenic recombination between two sublineages of the same genotype. J Gen Virol 85:1713–1716

Parra, G.I., Vidales, G., Gomez, J.A., Fernandez, F.M., Parreno, V. and Bok, K. (2008). Phylogenetic analysis of porcine rotavirus in Argentina: Increasing diversity of G4 strains and evidence of interspecies transmission. Veterinary Microbiology, 126, 243–250

Pascucci, S. and Lavazza, A. (1994). A survey of enteric viruses in commercial avian species: Experimental studies of transmissible enteritis of guinea fowl. In M. S. McNulty and J. B. McFerran (eds.). New and Evolving Virus Diseases of Poultry. Commission of the European Communities: Brussels, Belgium, 225—241.

Pantin-Jackwood, M.J., Day, J.M., Jackwood, M.W., Spackman, E. (2008). Enteric viruses detected by molecular methods in commercial chicken and turkey flocks in the United States between 2005 and 2006. Avian Diseases 52, 235–244.

Pantin-Jackwood, M. J., Spackman, E., Michael D. J. and Rives, D. (2007). Periodic monitoring of commercial turkeys for enteric viruses indicates continuous presence of Astrovirus and Rotavirus on the farms. Avian Disease, 51: 674 – 680.

Patton, J. T. (1996). Rotavirus VP1 alone specifically binds to the 3' end of viral mRNA, but the interaction is not sufficient to initiate minus-strand synthesis. J Virol 70:7940-7.

Patton, J. T., Jones, M. T., Kalbach, A. N., He, Y. W. and Xiaobo, J. (1997). Rotavirus RNA polymerase requires the core shell protein to synthesize the double-stranded RNA genome. J Virol 71:9618-26.

Petrie, B. L., Estes, M. K. and Graham, D. Y. (1983). Effects of tunicamycin on rotavirus morphogenesis and infectivity. J Virol 46:270-4.

Phan, T.G., Okitsu, S., Maneekarn, N., Ushijima, H. (2007a). Genetic heterogeneity, evolution and recombination in emerging G9 rotaviruses. Infect Genet Evol 7:656–663

Phan, T.G., Okitsu, S., Maneekarn, N., Ushijima, H. (2007b). Evidence of intragenic recombination in G1 rotavirus VP7 genes. J Virol 81:10188–10194

Pizarro, J. M., Pizarro, J. L., Fernandez, J., Sandino, A. M. and Spencer, E. (1991). Effect of nucleotide analogues on rotavirus transcription and replication. Virology 184:768-72.

Poncet, D., Laurent, S. and Cohen, J. (1994). Four nucleotides are the minimal requirement for RNA recognition by rotavirus non-structural protein NSP3. Embo J 13:4165-73.

Poncet, D., Lindenbaum, P., L'Haridon, R. and Cohen, J. (1997). *In-vivo* and *in-vitro* phosphorylation of rotavirus NSP5 correlates with its localization in viroplasms. J Virol 71:34-41.

Poruchynsky, M. S., and Atkinson, P. H. (1991). Rotavirus protein rearrangements in purified membraneenveloped intermediate particles. J Virol 65:4720-7.

Poruchynsky, M. S., Tyndall, C., Both, G. W., Sato, F., Bellamy, A. R. and Atkinson, P. H. (1985). Deletions into an NH2-terminal hydrophobic domain result in secretion of rotavirus VP7, a resident endoplasmic reticulum membrane glycoprotein. J Cell Biol 101:2199-209.

Prasad, B. V., J. W. Burns, E. Marietta, M. K. Estes, and W. Chiu. (1990). Localization of VP4 neutralization sites in rotavirus by three-dimensional cryo-electron microscopy. Nature 343:476-9.

Prasad, B. V., Rothnagel, R., Zeng, C. Q., Jakana, J., Lawton, J. A., Chiu, W. and Estes, M. K. (1996). Visualization of ordered genomic RNA and localization of transcriptional complexes in rotavirus. Nature 382:471-3.

Prasad, B. V., Wang, G. J., Clerx, J. P. M. and Chiu, W. (1988). Three-dimensional structure of rotavirus. J. Mol. Biol. 199:269-275.

Rahman, M., De Leener, K., Goegebuer, T., Wollants, E., Van der Donck, I., Van Hoovels, L. and Van Ranst, M. (2003). Genetic Characterisation of a Novel, Naturally Occurring Recombinant Human G6P[6] Rotavirus. Journal of Clinical Microbiology, p. 2088–2095

Rahman, M., Matthijnssens, J., Nahar, S., Podder, G., Sack, D.A, Azim, T., Van Ranst, M. (2005). Characterisation of a novel P[25], G11 human group a rotavirus. J Clin Microbiol 43:3208–3212

Rahman, M., Matthijnssens, J., Yang, X., Delbeke, T., Arijs, I., Taniguchi, K., Iturriza-Gomara, M., Iftekharuddin, N., Azim, T., Van Ranst, M. (2007). Evolution history and global spread of the emerging G12 human rotaviruses. J. Virol. 81, 2382–2390.

Ramani, S. and Kang, P. (2007). Burden of disease and molecular epidemiology of group A rotavirus infections in India. Indian Journal of Medical Research, 125, 619–632

Ramig, R.F., Ciarlet, M., Mertens, P.P.C., Dermody, T.S. (2005). Rotavirus. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) Virus taxonomy: eight report of the International Committee on Taxonomy of Viruses. Elsevier, Amsterdam, pp 484–496

Reynolds, D. L., Theil, K. W. and Saif, Y. M. (1987a). Demonstration of rotavirus and rotavirus-like virus in the intestinal contents of diarrheic pheasant chicks. Avian Dis 31:376—379.

Reynolds, D.L., Saif, Y.M., Theil, K.W. (1987b). A survey of enteric viruses of turkey poults. Avian Dis. 31, 89–98. Reynolds, D.L., Saif, Y.M., Theil, K.W. Enteric viral infections of turkey poults: incidence of infection. Avian Dis. 31, 272–276.

Reynolds, D.L., Theil, K.W., and Saif, Y.M. (1987c). Demonstration of rotavirus and rotavirus-like virus from the intestinal contents of diarrheic pheasant chicks. Avian Dis. 31, 376–379.

Reynolds, D.L. and Schultz-Cherry, S.L. (2008). Astrovirus infections. In: Saif, Y.M., Fadley, A.M., Glission, J.R., Mc Dougald, L.R., Nolan, L.K., Swayne, D.E. (Eds.), Diseases of Poultry. 12th ed. Blackwell Publishing Professional, Ames, IA, USA, pp. 351–355.

Richards, A. F., Lopman, B., Gunn, A., Curry, A., Ellis, D., Cotterill, H., Ratcliffe, S., Jenkins, M. Appleton, H., Gallimore, C. I., Gray, J. J. and Brown, D. W. (2003). Evaluation of a commercial ELISA for detecting Norwalk-like virus antigen in faeces. J. Clin. Virol. 26:109–115.

Richardson, S., Grimwood, K., Gorrell, R., Palombo, E., Barnes, G. and Bishop, R. (1998) Extended excretion of rotavirus after severe diarrhoea in young children. Lancet 351:1844-8.

Rixon, F., Taylor, P. and Desselberger, U. (1984). Rotavirus RNA segments sized by electron microscopy. J. Gen. Virol. 65:233-239.

Rohwedder, A., Schutz, K.I., Minamoto, N. and Brussow, H. (1995). Sequence analysis of pigeon, turkey, and chicken rotavirus VP8* identifies rotavirus 993/83, isolated from calf faeces, as a pigeon rotavirus. Virology, 210, 231–235

Rollo, E. E., Kumar, K. P., Reich, N. C., Cohen, J., Angel, J., Greenberg, H. B., Sheth, R., Anderson, J., Oh, B., Hempson, S. J., Mackow, E. R. and Shaw, R. D. (1999). The epithelial cell response to rotavirus infection. J Immunol 163:4442-52.

Roseto, A., Esgaig, J., Delain, E., Cohen, J. and Scherrer, R. (1979). Structure of rotaviruses as studied by the freeze-dryingtechnique. Virology 98:471-475.

Ruggeri, F. M. and Greenberg, H. B. (1991). Antibodies to the trypsin cleavage peptide VP8 neutralize rotavirus by inhibiting binding of virions to target cells in culture. J Virol 65:2211-9.

Ruiz-Palacios, G.M., Pe´ rez-Schael, I., Vela´ zquez, F.R., Abate, H., Breuer, T., Clemens, S.C., Cheuvart, B., Espinoza, F., Gillard, P.and Innis, B.L. (2006). Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. N Engl J Med 354:11-22.

Saif, Y.M. (2008). Viral enteric infections. Introduction. In: Saif, Y.M., Fadley, A.M., Glission, J.R., Mc Dougald, L.R., Nolan, L.K., Swayne, D.E. (Eds.), Diseases of Poultry. 12th ed. Blackwell Publishing Professional, Ames, IA, USA, p. 329.

Saif, L. J., Saif, Y. M. and Theil, K. W. (1985). Enteric viruses in diarrrhoeic turkey poults. Avian Diseases, 29: 798 – 811.

Salim, A. F., Phillips, A. D. and Farthing, M. J. (1990). Pathogenesis of gut virus infection. Baillieres Clin Gastroenterol 4:593-607.

Sanchez-San Martin, C., Lopez, T., Arias, C. F. and Lopez, S. (2004). Characterisation of rotavirus cell entry. J Virol 78:2310-8.

Sandino, A. M., Jashes, M., Faundez, G. and Spencer, E. (1986). Role of the inner protein capsid on *in-vitro* human rotavirus transcription. J Virol 60:797-802.

Santos, N. and Hoshino, Y. (2005). Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine. Rev Med Virol 15:29-56.

Santos, N., Lima, R.C., Pereira, C.F. and Gouvea V. (1998). Detection of rotavirus types G8 and G10 among Brazilian children with diarrhoea. J Clin Microbiol; 36:2727–9.

Santos, N., Volotao, E.M., Soares, C.C., Albuquerque, M.C., da Silva, F.M. and Chizhikov, V. (2003). VP7 gene polymorphism of serotype G9 rotavirus strains and its impact on G genotype determination by PCR. Virus Res 93:127–38.

Sato, K., Inaka, Y., Shinozaki, T. and Fujii, R. (1981a). Matumoto. M. Isolation of human rotavirus in cell cultures. Arch. Virol., v.69, p.155-160.

Sato, K., Inaka, Y., Shinozaki, T. and Matumoto, M. (1981b). Neutralizing antibody to bovine rotavirus in various animal species. Veterinary Microbiology, 6, 259–261

Schumann, T., Hotzel, H., Otto, P. and Johne, R. (2009). Evidence of interspecies transmission and reassortment among avian group A rotaviruses. Virology 386, 334–343.

Schwers, A., Hoyois, P., Chappuis, G., Dagenais, L. and Pastoret, P.P. (1982). Propagation of bovine rotavirus by cats and dogs. Annals Research in Veterinary Science, 13, 303–308

Seheri, L. M, Peenze, I., Nemarude, L., Netshifhefhe, L., Nyaga, M., Ngobeni, H., Maake L L, Kiulia, N., Nyangao, J., Esona, M. D., Page, N. A., Mphahlele, M. J., Mwenda, J. M., Steele, A.D. (2012). The circulating rotavirus strains in africa from

2009 to 2011. In: Proceedings of the 11th International Symposium on Double-Stranded RNA Viruses, San Juan, Puerto-rico.

Shawky, S. A., Saif, Y. M. and Swayne, D. E. (1993). Role of circulating maternal anti-rotavirus IgG in protection of intestinal mucosal surface in turkey poults. Avian Dis 37:1041—1050.

Shawky, S. A., Saif, Y. M. and McCormick, J. (1994). Transfer of maternal anti-rotavirus IgG to the mucosal surfaces and bile of turkey poults. Avian Dis 38:409—417.

Shepherd, R. W., Butler, D. G., Cutz, E., Gall, D. G. and Hamilton, J. R. (1979). The mucosal lesion in viral enteritis. Extent and dynamics of the epithelial response to virus invasion in transmissible gastroenteritis of piglets. Gastroenterology 76:770-7.

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

Steele, A.D; Geyer, A. and Gerdes, G. (2004). Rotavirus infections. In: Coetzer JT, ed. Infectious diseases of livestock, 2nd ed. Cape Town: Oxford University Press p.1256-64.

Sudhir, B. and Gagandeep, K. (2012). Rotavirus vaccination in developing countries. Current Opinion in Virology 2012, 2:443–448

Suggs, S.V., Hirose, T., Myake, E.H., Kawashima, M.J., Johnson, K.I. and Wallace, R.B. (1981). ICN-UCLA symposium for developmental biology using purified gene. (ed. D.D. Brown), Vol. 23, pp. 683-693. Academic Press, New York.

Sugiyama, M., Goto, K., Uemukai, H., Mori, Y., Ito, N., Minamoto, N. (2004). Attachment and infection to MA104 cells of avian rotaviruses require the presence of sialic acid on the cell surface. Journal of Veterinary Medical Science, 66, 461–463.

Svensson, L. (2000). Diagnosis of foodborne viral infection in patients. International Journal of Food Microbiology, 59, 117–126

Takase, K., Nonaka, F., Sakaguchi, M. and Yamada, S. (1986). Cytopathic avian rotavirus isolated from duck faeces in chicken kidney cell cultures. Avian Pathol 15:719—730.

Takase, K., Uchimura, T., Katsuki, N. and Yamamoto, M. (1990). A survey of chicken sera for antibody to atypical avian rotavirus of duck origin, in Japan. Jap J Vet Sci 52:1319—1321.

Tamehiro, C.Y., Alfieri, A.F., Medici, K.C. and Alfieri, A.A. (2003). Segmented double-stranded genomic RNA viruses in faecal samples from broiler chicken. Braz. J. Microbiol., 34: 349-53.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 28(10):2731–2739.

Tang, B., Gilbert, J. M., Matsui, S. M. and Greenberg, H. B. (1997). Comparison of the rotavirus gene 6 from different species by sequence analysis and localization of subgroup-specific epitopes using site-directed mutagenesis. Virology 237, 89–96.

Taniguchi, K., Urasawa, T., Morita, Y., Greenberg, H.B. and Urasawa, S. (1987). Direct serotyping of human rotavirus in stools using serotype 1-, 2-, 3-, and 4-specific monoclonal antibodies to VP7. J Infect Dis 155:1159-66.

Taraporewala, Z., Chen, D. and Patton, J. T. (1999). Multimers formed by the rotavirus nonstructural protein NSP2 bind to RNA and have nucleoside triphosphatase activity. J Virol 73:9934-43.

Taylor, J. A. and Bellamy, A. R. (2003). Interaction of the rotavirus nonstructural glycoprotein NSP4 with the viral and cellular components. In Viral Gastroenteritis, pp. 225–235. Edited by U. Desselberger & J. Gray. Amsterdam: Elsevier Science.

Taylor, J. A., O'Brien, J. A. and Yeager, M. (1996). The cytoplasmic tail of NSP4, the endoplasmic reticulum-localized non-structural glycoprotein of rotavirus, contains distinct virus binding and coiled coil domains. Embo J 15:4469-76.

Teodoroff, T.A., Tsunemitsu, H., Okamoto, K., Katsuda, K., Kohmoto, M., Kawashima, K., Nakagomi, T. and Nakagomi, O. (2005). Predominance of porcine rotavirus G9

in Japanese piglets with diarrhoea: close relationship of their VP7 genes with those of recent human G9 strains. Journal of Clinical Microbiology, 43, 1377–1378

Theil, K. W. (1987). A modified genome electropherotyping procedure for detecting turkey rotaviruses in small volumes of intestinal contents. Avian Dis 31:899—903.

Theil, K.W., Reynolds, D.L. and Saif, Y.M. (1986). Comparison of immune electron microscopy and genome electropherotyping techniques for detection of turkey rotaviruses and rotaviruslike viruses in intestinal contents. J. Clin. Microbiol. 23, 695–699.

Theil, K. W. and Saif, Y. M. (1987). Age-related infections with rotavirus, rotavirus-like virus, and atypical rotavirus in turkey flocks. J Clin Microbiol 25:333—337.

Tian, P., Ball, J. M., Zeng, C. Q. and Estes, M. K. (1996). The rotavirus nonstructural glycoprotein NSP4 possesses membrane destabilization activity. J Virol 70:6973-81.

Todd, D. and McNulty, M.S. (1986). Electrophoretic variation of avian rotavirus RNA in polyacrylamid gels. Avian Pathol. 15, 149–159.

Trojnar, E., Otto, P. and Johne, R. (2009). The first complete genome sequence of a chicken group A rotavirus indicates independent evolution of mammalian and avian strains. Virology 386:325–333.

Trojnar, E., Otto, P., Roth, B., Reetz, J. and Johne, R. (2010). The genome segments of a group D rotavirus possess group A-like conserved termini but encode group-specific proteins. J. Virol. 84, 10254–10265.

Torres-Vega, M. A., Gonzalez, R. A., Duarte, M., Poncet, D., Lopez, S. and Arias, C. F. (2000). The Cterminal domain of rotavirus NSP5 is essential for its multimerization, hyperphosphorylation and interaction with NSP6. J Gen Virol 81:821-30.

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

Varley, J., Jones, R. C., Bradbury, J. M. and Jordan, F. T. W. (1993). A survey of the viral flora of two commercial Pekin duck flocks. Avian Pathol 22:703—714.

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

Vende, P., Piron, M., Castagne, N. and Poncet, D. (2000). Efficient translation of rotavirus mRNA requires simultaneous interaction of NSP3 with the eukaryotic translation initiation factor eIF4G and the mRNA 3' end. J Virol 74:7064-71.

Vesikari, T., Matson, D.O., Dennehy, P., Van Damme, P., Santosham, M., Rodriguez, Z., Dallas, M.J., Heyse, J.F., Goveia, M.G. and Black, S.B. (2006). Safety and efficacy of a pentavalent humanbovine (WC3) reassortant rotavirus vaccine. N Engl J Med 354:23-33.

Villarreal, L.Y.B., Uliana, G., Valenzuela, C., Chacon, J.L.V., Saidenberg, A.B.S., Sanches, A.A., Brandao, P. E., Jerez, J.A. and Ferreira, A.J.P. (2006). Rotavirus detection and isolation from chickens with or without symptoms. Brazilian Journal of Poultry Science 3, 187–191.

Vindevogel, H., Dagenais, L., Lansival, B. and Pastoret, P. P. (1981). Incidence of rotavirus, adenovirus and herpesvirus in pigeons. Vet Rec 109:285—286.

Wang, Z. D., Lu, S. W., Huang, J. S., Zhang, F. P. and Liu, M. J. (1995). Investigation and identification of atypical rotavirus from animals and humans. Chinese J Virol 11:336—341.

Ward, R.L., Nakagomi, D.R., Knowlton, D.R., McNeal, M.M., Nakagomi, T., Clemens, J.D., Sack, D.A. and Schiff, G.M. (1990). Evidence for natural reassortments of human rotaviruses belonging to different genogroups. J. Virol. 64, 3219–3225.

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

Wilde, J., Yolken, R., Willoughby, R. and Eiden, J. (1991). Improved detection of rotavirus shedding by polymerase chain reaction. Lancet 337:323-6.

Williams, J.G.K. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18" 6531-6535.

Woode, G.N. and Crouch, C.F. (1978). Naturally occurring and experimentally induced rotavirus infection of domestic and laboratory animals. Journal of American Veterinary Medical Association, 173, 522–526.

www.chickenvet.co.uk 2010-2013 Health and common diseases

www.niaid.nih.gov/topics/rotavirus

Yason, C. V. and Schat, K. A. (1985). Isolation and characterisation of avian rotaviruses. Avian Dis 29:499—508.

Yason, C. V. and Schat, K. A. (1986a). Experimental infection of specific-pathogen-free chickens with avian rotaviruses. Avian Dis 30:551—556.

Yason, C. V. and Schat, K. A. (1986b). Pathogenesis of rotavirus infection in turkey poults. Avian Pathol 15:421—435.

Yason, C. V. and Schat, K. A. (1987). Pathogenesis of rotavirus infection of chickens and turkeys: Clinical signs and virology. Am J Vet Res 48:977—983.

Yason, C. V., Summers, B. A. and Schat, K. A. (1987). Pathogenesis of rotavirus infection in various age groups of chickens and turkeys: Pathology. Am J Vet Res 48:927—938.

Yeager, M., Berriman, J. A., Baker, T. S. and Bellamy, A. R. (1994). Three-dimensional structure of the rotavirus haemagglutinin VP4 by cryo-electron microscopy and difference map analysis. Embo J 13:1011-8.

Yegani, M. and Korve, D.R. (2008). Factors affecting intestinal health in poultry. Poultry Science 87, 2052–2063.

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

Yolken, R. and Wilde, J. (1994). Assays for detecting human rotavirus. In: Kapikian, A.Z., ed. Viral Infections of the Gastrointestinal Tract, 2nd ed. New York: Marcel Dekker; 1994: 251-278.

Zarate, S., Romero, P., Espinosa, R., Arias, C. F. and Lopez, S. (2004). VP7 Mediates the Interaction of Rotaviruses with Integrin {alpha}v{beta}3 through a Novel Integrin-Binding Site. J Virol 78:10839-10847.

Zeng, C. Q., Estes, M. K., Charpilienne, A. and Cohen, J. (1998). The N terminus of rotavirus VP2 is necessary for encapsidation of VP1 and VP3. J Virol 72:201-8.

Zhang, M., Zeng, C. Q., Dong, Y., Ball, J. M., Saif, L. J., Morris, A. P. and Estes, M. K. (1998). Mutations in rotavirus nonstructural glycoprotein NSP4 are associated with altered virus virulence. J Virol 72:3666-72.

APPENDIX I

Hank's balanced salt solution

Penicillin

Streptomycin

Fungizone

QIAamp Viral RNA minikit

Buffer AVL (Lysis buffer)

Ethanol (96-100%)

Buffer AW1 and AW2 (washing buffer)

Buffer AE (Elution buffer)

Spin columns

Medium 199

Penicillin……………….. 2000U/ml

Streptomycin…………… 200mg/ml

polymyxin B …………….2000U/ml

Gentamycin………………250mg/ml

Ofloxacin…………………60mg/ml

Sulfamethoxazole………...200mg/ml

Amphotericin B…………..2.5mg/ml

Laboratory reagents for extraction

Lysis buffer:

Guanidine m-isothiocyanate…………………… 120g

0.1M Tris-HCL…………………………………100ml

Triton X-100……………………..……………….2.6g

0.2M EDTA……………………………………..22ml

Proteinase K digestion buffer:

0.2M Tris-HCL (pH 8)…………………………20ml of 1M

25mM EDTA (pH 8)………………………….5ml of 0.5M

0.3M NaCl…………………………………….10ml of 3M

2% SDS……………………………………….10ml of 10% SDS

Water………………………………………….55ml

Tris-HCL Buffer

<u>20X Tris-HCL (0.5M Tris Base, pH7.6):</u>

Trizma Base…………………………..122g

Distilled water………………………..1000ml

Adjust to pH 7.6 using concentrated HCL

<u>10X Tris-HCL Tween 20 (0.5M Tris Base, 0.5% Tween 20, pH 7.6)</u>

Trizma Base…………………………..61g

Distilled water………………………..1000ml

Adjust to pH 7.6 using concentrated HCL and then 5ml of Tween 20

EDTA 0.5 M pH 8.0

- EDTA 93g
- ddH2O 500 ml
- pH 8.0
- Autoclave
- Store at room temp for 12 months

TBE 5x

• Trisma base	108 g
• Boric acid	55 g
• EDTA	7.73 or 20 ml 0.5M EDTA
• ddH2O	2 liter

- pH8.2
- Autoclave
- Store at room temp for 6 months

APPENDIX II

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                       10        20        30        40        50
                  ....|....|....|....|....|....|....|....|....|....|
Rotavirus 361     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 362     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 363     ---------XLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 365     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 366     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 368     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 369     --------ETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 461     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 432     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 436     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTITFTAWKMG
Rotavirus 450     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTITFTAWKMG
Rotavirus 242     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 246     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 249     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 250     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 251     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 252     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 254     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 257     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 260     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 70      -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 73      -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 75      -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus A1      -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 247     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 253     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 255     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 258     ------------XEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 266     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 295     -----------------------XHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 299     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 300     -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 256     --------------------------------------------------
rotavirus A       -----------------MTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG

                       60        70        80        90       100
                  ....|....|....|....|....|....|....|....|....|....|
Rotavirus 361     RSTLKVTKTVAGSGYKVIKVVIVTVFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 362     RSTLKVTKTVAGSGYKVIKVVIVTVFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 363     RSTLKVTKTVAGSGYKVIKVVIVTVFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 365     RSTLKVTKTVAGSGYKVIKVVIVTIFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 366     RSTLKVTKTVAGSGYKVIKVVIVTIFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 368     RSTLKVTKTVAGSGYKVIKVVIVTIFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 369     RSTLKVTKTVAGSGYKVIKVVIVTIFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 461     RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 432     RSTLKVTKTVAGSGYKVIKVVIVTVFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 436     RSTLKVTKTVAGSGYKVIKVVVVTIFNCILRMLGSKTEVVSEDKMDVMAS
Rotavirus 450     RSTLKVTKTVAGSGYKVIKVVVVTIFNCILRMLGSKTEVVSEDKMDVMAS
Rotavirus 242     RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 246     RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 249     RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 250     RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 251     RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 252     RSTLKVTKTVAGSGYKVI VVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
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                    10        20        30        40        50
           ....|....|....|....|....|....|....|....|....|....|
Rotavirus 361  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 362  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 363  ---------XLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 365  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 366  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 368  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 369  --------ETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 461  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 432  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 436  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTITFTAWKMG
Rotavirus 450  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTITFTAWKMG
Rotavirus 242  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 246  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 249  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 250  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 251  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 252  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 254  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 257  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 260  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 70   -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 73   -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 75   -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus A1   -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 247  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 253  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 255  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 258  ------------XEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 266  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 295  -----------------------XHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 299  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 300  -------XETLVEEVYNMTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG
Rotavirus 256  --------------------------------------------------
rotavirus A    -----------------MTLGYFEHNVIIMKYFPFLASILTIIFTAWKMG

                    60        70        80        90       100
           ....|....|....|....|....|....|....|....|....|....|
Rotavirus 361  RSTLKVTKTVAGSGYKVIKVVIVTVFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 362  RSTLKVTKTVAGSGYKVIKVVIVTVFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 363  RSTLKVTKTVAGSGYKVIKVVIVTVFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 365  RSTLKVTKTVAGSGYKVIKVVIVTIFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 366  RSTLKVTKTVAGSGYKVIKVVIVTIFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 368  RSTLKVTKTVAGSGYKVIKVVIVTIFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 369  RSTLKVTKTVAGSGYKVIKVVIVTIFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 461  RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 432  RSTLKVTKTVAGSGYKVIKVVIVTVFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 436  RSTLKVTKTVAGSGYKVIKVVVVTIFNCILRMLGSKTEVVSEDKMDVMAS
Rotavirus 450  RSTLKVTKTVAGSGYKVIKVVVVTIFNCILRMLGSKTEVVSEDKMDVMAS
Rotavirus 242  RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 246  RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 249  RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 250  RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 251  RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 252  RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
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Rotavirus 254   RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 257   RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 260   RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 70    RSTLKVTKTVAGSGYKVIKVVIVTVFNCILKMFGSRTEIVSEDKMDVMAS
Rotavirus 73    RSTLKVTKTVAGSGYKVIKVVIVTVFNCILKMFGSRTEIVSEDKMDVMAS
Rotavirus 75    RSTLKVTKTVAGSGYKVIKVVIVTVFNCILKMFGSRTEIVSEDKMDVMAS
Rotavirus A1    RSTLKMTKTVAGSGYKVIKVVVVTVFNCILRIFGSKTEIVSEDKMDVMAS
Rotavirus 247   RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 253   RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 255   RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 258   RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 266   RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRTFGSKTEIVSEDKMDVMAS
Rotavirus 295   RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 299   RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 300   RSTLKVTKTVAGSGYKVIKVVVVTVFNCILRMFGSKTEIVSEDKMDVMAS
Rotavirus 256   ------------------------------RTFGSKTEIVSEDKMDVMAS
rotavirus A     RSTLKMTKTVAGSGYKVIKVVVVTVFNCILRIFGSKTEIVSEDKMDVMAS

                       110       120       130       140       150
                ....|....|....|....|....|....|....|....|....|....|
Rotavirus 361   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVTSETNRK
Rotavirus 362   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVTSETNRK
Rotavirus 363   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVTSETNRK
Rotavirus 365   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVTSETNRK
Rotavirus 366   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVTSETNRK
Rotavirus 368   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVTSETNRK
Rotavirus 369   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVTSETNRK
Rotavirus 461   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 432   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVTSETNRK
Rotavirus 436   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNISSETNRK
Rotavirus 450   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNISSETNRK
Rotavirus 242   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 246   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 249   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 250   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 251   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 252   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 254   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 257   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 260   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 70    KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVTSETNRK
Rotavirus 73    KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVTSETNRK
Rotavirus 75    KILKQIDEQVKVIXQLTKRELEQVKLLADIYELLXXYKSEGNVTSETNRK
Rotavirus A1    KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVPSETNRK
Rotavirus 247   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 253   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 255   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 258   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 266   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
Rotavirus 295   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVPSETNRK
Rotavirus 299   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVPSETNRK
Rotavirus 300   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVPSETNRK
Rotavirus 256   KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNIPSETNRK
rotavirus A     KILKQIDEQVKVIEQLTKRELEQVKLLADIYELLXXYKSEGNVPSETNRK

                       160       170       180
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                 ....|....|....|....|....|....|....|..
Rotavirus 361    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*IH
Rotavirus 362    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*IH
Rotavirus 363    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*IH
Rotavirus 365    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NX----
Rotavirus 366    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*IH
Rotavirus 368    AYETWSKDPYQPTRAVSLN*WFMSTLX----------
Rotavirus 369    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*X-----
Rotavirus 461    AYETWSKDX----------------------------
Rotavirus 432    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*IH
Rotavirus 436    AYETWSKDPYQPTRTVSLN*WFMSTLSKEA*NDG*IH
Rotavirus 450    AYETWSKDPYQPTRTVSLN*WFMSTLSKEA*NDG*IH
Rotavirus 242    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 246    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 249    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*T-
Rotavirus 250    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 251    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 252    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 254    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 257    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 260    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 70     AYEAWSKDPYQPTRTVPLN*WFMSTLSKEA*NDG*IH
Rotavirus 73     AYEAWSKDPYQPTRTVPLN*WFMSTLSKEA*NDG*IH
Rotavirus 75     AYEAWSKDPYQPTRTVPLN*WFMSTLSKEA*NDG*IH
Rotavirus A1     AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 247    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 253    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 255    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 258    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 266    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
Rotavirus 295    AYETWNKDPYQPTRAVSLN*WFMSTLSKEA*NDG*IH
Rotavirus 299    AYETWNKDPYQPARAVSLN*WFMSTLSKEA*NDG*IH
Rotavirus 300    AYETWNKDPYQPTRAVSLN*WFMSTLSKEA*NDG*IH
Rotavirus 256    AYETWSKDPYQPTRAVSLN*WFMSTLSKEA*NDG*TH
rotavirus A      AYETWSKDPYQPTRAVSLN*WFMSTLSKEAYNDG*TH
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Figure 74: Deduced amino acid sequences of NSP4 protein of Avian Rotavirus.

```
                           10        20        30        40        50
                   ....|....|....|....|....|....|....|....|....|....|
Rotavirus242 VP4   SAEKTQGVTINPGPFAQTGYAPVEWTHGDIPVDETIEPTLDGPYTASSIV
Rotavirus256_VP4   SAEKTQGVTINPGPFAQTGYAPVEWTHGDIPVDETIEPTLDGPYTASSIV
Rotavirus260 VP4   SAEKTQGVTINPGPFAQTGYAPVEWTHGDIPVDETIEPTLDGPYTASSIV

                           60        70        80        90       100
                   ....|....|....|....|....|....|....|....|....|....|
Rotavirus242 VP4   IQPQYWILVNPTNQDVVIEADAQNKKFACVMLPPNTPESDRQYTILGKQI
Rotavirus256_VP4   IQPQYWILVNPTNQDVVIEADAQNKKFACVMLPPNTPESDRQYTILGKQI
Rotavirus260 VP4   IQPQYWILVNPTNQDVVIEADAQNKKFACVMLPPNTPESDRQYTILGKQI

                          110       120       130       140       150
                   ....|....|....|....|....|....|....|....|....|....|
Rotavirus242 VP4   TIRLGNTDTVNYKFCDLLSNDGIIYTKSIELITPHRLNAFMKDAGKLYAY
Rotavirus256_VP4   TIRLGNTDTVNYKFCDLLSNDGIIYTKSIELITPHRLNAFMKDAGKLYAY
Rotavirus260 VP4   TIRLGNTDTVNYKFCDLLSNDGIIYTKSIELITPHRLNAFMKDAGKLYAY

                          160       170
                   ....|....|....|....|
Rotavirus242 VP4   SGTIPTIKKEYHTLDNIENV
Rotavirus256_VP4   SGTIPTIKKEYHTLDNIENV
Rotavirus260 VP4   SGTIPTIKKEYHTLDNIENV
```

Figure 75: Deduced amino acid sequences of VP4 protein of Avian Rotavirus.

```
                          10        20        30        40        50
                  ....|....|....|....|....|....|....|....|....|....|
Rotavirus70_cplt  MDVLYSLAKTLKEARAKIVEGTLYTNVADIVQQINQVINSLNGSTFQTGG
Rotavirus73_cplt  ~DVLYSLAKTLKEARAKIVEGTLYTNVADIVQQINQVINSLNGSTFQTGG
Rotavirus75_cplt  ~~XLYSLAKTLKEARAKIVEGTLYTNVADIVQQINQVINSLNGSTFQTGG
Rotavirus242_cplt ~~~LYSLAKTLKEARAKIVEGTLYTNVADIVQQINQVINSLNGSTFQTGG
Rotavirus246_cplt ~XVLYSLAKTLKEARAKIVEGTLYTNVADIVQQINQVINSLNGSTFQTGG
Rotavirus247_cplt ~~XLYSLAKTLKEARAKIVEGTLYTNVADIVQQINQVINSLNGSTFQTGG
Rotavirus266_cplt ~~~~~~~~~~~~~~~~~~~~XTLYTNVADIVQQINQVINSLNGSTFQTGG
Rotavirus295_cplt ~~~~XSLAKTLKEARAKIVEGTLYTNVADIVQQINQVINSLNGSTFQTGG
Rotavirus299_VP6  ~~~~XSLAKTLKEARAKIVEGTLYTNVADIVQQINQVINSLNGSTFQTGG

                          60        70        80        90       100
                  ....|....|....|....|....|....|....|....|....|....|
Rotavirus70_cplt  IGNFQIRDWAFDFGALGTTLLNLDANYVENARATIDYFIDFVDHVCIDEI
Rotavirus73_cplt  IGNFQIRDWAFDFGALGTTLLNLDANYVENARATIDYFIDFVDHVCIDEI
Rotavirus75_cplt  IGNFQIRDWAFDFGALGTTLLNLDANYVENARATIDYFIDFVDHVCIDEI
Rotavirus242_cplt IGNFQIRDWTFDFGALGTTLLNLDANYVENARATIDYFIDFVDHVCIDEI
Rotavirus246_cplt IGNFQIRDWTFDFGALGTTLLNLDANYVENARATIDYFIDFVDHVCIDEI
Rotavirus247_cplt IGNFQIRDWTFDFGALGTTLLNLDANYVENARATIDYFIDFVDHVCIDEI
Rotavirus266_cplt IGNFQIRDWTFDFGALGTTLLNLDANYVENARATIDYFIDFVDHVCIDEI
Rotavirus295_cplt IGNFQIRDWAFDFGALGTTLLNLDANYVENARATIDYFIDFVDHVCIDEI
Rotavirus299_VP6  IGNFQIRDWAFDFGALGTTLLNLDANYVENARATIDYFIDFVDHVCIDEI

                         110       120       130       140       150
                  ....|....|....|....|....|....|....|....|....|....|
Rotavirus70_cplt  VRESQRNGIAPQSNALRQLSAARYRRINYDNDSEYIENWNLQNRRQRTGY
Rotavirus73_cplt  VRESQRNGIAPQSNALRQLSAARYRRINYDNDSEYIENWNLQNRRQRTGY
Rotavirus75_cplt  VRESQRNGIAPQSNALRQLSAARYRRINYDNDSEYIENWNLQNRRQRTGY
Rotavirus242_cplt VRESQRNGIAPQSNALRQLSAARYRRINYDNDSEYIENWNLQNRRQRTGY
Rotavirus246_cplt VRESQRNGIAPQSNALRQLSAARYRRINYDNDSEYIENWNLQNRRQRTGY
Rotavirus247_cplt VRESQRNGIAPQSNALRQLSAARYRRINYDNDSEYIENWNLQNRRQRTGY
Rotavirus266_cplt VRESQRNGIAPQSNALRQLSAARYRRINYDNDSEYIENWNLQNRRQRTGY
Rotavirus295_cplt VRESQRNGIAPQSNALRQLSAARYRRINYDNDSEYIENWNLQNRRQRTGY
Rotavirus299_VP6  VRESQRNGIAPQSNALRQLSAARYRRINYDNDSEYIENWNLQNRRQRTGY

                         160       170       180       190       200
                  ....|....|....|....|....|....|....|....|....|....|
Rotavirus70_cplt  LFHKPNILPYNNSFTLTRSQPAHDNVCGTMWLNNGSEIEIIGFDSECALN
Rotavirus73_cplt  LFHKPNILPYNNSFTLTRSQPAHDNVCGTMWLNNGSEIEIIGFDSECALN
Rotavirus75_cplt  LFHKPNILPYNNSFTLTRSQPAHDNVCGTMWLNNGSEIEIIGFDSECALN
Rotavirus242_cplt LFHKPNILPYNNSFTLTRSQPAHDNVCGTMWLNNGSEIEIIGFDSECALN
Rotavirus246_cplt LFHKPNILPYNNSFTLTRSQPAHDNVCGTMWLNNGSEIEIIGFDSECALN
Rotavirus247_cplt LFHKPNILPYNNSFTLTRSQPAHDNVCGTMWLNNGSEIEIIGFDSECALN
Rotavirus266_cplt LFHKPNILPYNNSFTLTRSQPAHDNVCGTMWLNNGSEIEIIGFDSECALN
Rotavirus295_cplt LFHKPNILPYNNSFTLTRSQPAHDNVCGTMWLNNGSEIEIIGFDSECALN
Rotavirus299_VP6  LFHKPNILPYNNSFTLTRSQPAHDNVCGTMWLNNGSEIEIIGFDSECALN

                         210       220       230       240       250
                  ....|....|....|....|....|....|....|....|....|....|
Rotavirus70_cplt  AQANVQAFQHVVPLGRVLNNATVSLLPYAPRLMQRAVIPTADGQNTWLFD
Rotavirus73_cplt  AQANVQAFQHVVPLGRVLNNATVSLLPYAPRLMQRAVIPTADGQNTWLFD
Rotavirus75_cplt  AQANVQAFQHVVPLGRVLNNATVSLLPYAPRLMQRAVIPTADGQNTWLFD
Rotavirus242_cplt AQANVQAFQHVVPLGRVLNNATVSLLPYAPRLMQRAVIPTADGQNTWLFD
Rotavirus246_cplt AQANGQAFQHSVPLGRALNNATVSLLPYAPRLMQTAVIPTADGQNTWVFD
Rotavirus247_cplt AQANVQAFQHVVPLGRVLNNATVSLLPYAPRLMQRAVIPTADGXNTWLFD
```

Figure 76: Deduced amino acid sequences of VP6 protein of Avian Rotavirus.

```
                              10        20        30        40        50
                     ....|....|....|....|....|....|....|....|....|....|
Rotavirus450_cplt se GVNVPITGSLDVTVQNQTAEPIGLTSTLCLYYPKEASTEVADTEWKQTIS
Rotavirus70 cplt seq GVNVPITGSLDVSIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTIS
Rotavirus73_cplt seq GVNVPITGSLDVSIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTIS
Rotavirus75_ cplt se GVNVPITGSLDVSIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTIS
Rotavirus242 cplt se GVNVPITGSLDVTIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTVS
Rotavirus246_ cplt s GVNVPITGSLDVTIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTVS
Rotavirus247_ cplt s GVNVPITGSLDVTIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTVS
Rotavirus249_ cplt s GVNVPITGSLDVTIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTVS
Rotavirus250 cplt se ~~~XPITGSLDVTIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTVS
Rotavirus254_ cplt s GVNVPITGSLDVTIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTVS
Rotavirus255_ cplt s GVNVPITGSLDVTIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTVS
Rotavirus257_ cplt s GVNVPITGSLDVTIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTVS
Rotavirus260_ cplt s GVNVPITGSLDVTIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTVS
Rotavirus277_ cplt s GVNVPITGSLDITIQNQTVEPIGLTSTLCLYYPKEASTEIADTEWKQTIS
Rotavirus298_ cplt s GVNVPITGSLDVTIQNQTAEPIGLTSTLCLYYPKEASTEIADTEWKQTIS
Rotavirus299_ cplt s GVNVPITGSLDVTIQNQTAEPIGLTSTLCLYYPKEASTEIADTEWKQTIS
Rotavirus300_ cplt s GVNVPITGSLDVTIQNQTAEPIGLTSTLCLYYPKEASTEIADTEWKQTIS
Rotavirus362_ cplt s GVNVPITGSLDVTIQNQTAEPIGLTSTLCLYYPKEASTEIADTEWKQTIS
Rotavirus432_ cplt s GVNVPITGSLDVTIQNQTVGPIGLTSTLCLYYPKEASTEIADTEWKQTVS
Rotavirus441_ cplt s GVNVPITGSLDVTVQNQTAEPIGLTSTLCLYYPKEASTEVADTEWKQTIS

                              60        70        80        90       100
                     ....|....|....|....|....|....|....|....|....|....|
Rotavirus450_cplt se QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus70 cplt seq QLFLIKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus73_cplt seq QLFLIKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus75_ cplt se QLFLIKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus242 cplt se QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus246_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus247_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus249_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus250 cplt se QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus254_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus255_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus257_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus260_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus277_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus298_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus299_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus300_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus362_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus432_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS
Rotavirus441_ cplt s QLFLTKGWPTTSIYFNEYQDLQTFSNNPSINCDYNIILIKYDGNQGLDIS

                             110       120       130       140       150
                     ....|....|....|....|....|....|....|....|....|....|
Rotavirus450_cplt se EIAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus70 cplt seq EVAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus73_cplt seq EVAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus75_ cplt se EVAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus242 cplt se EIAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus246_ cplt s EIAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus247_ cplt s EIAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus249_ cplt s EIAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
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Rotavirus250 cplt se   EIAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus254_ cplt s   EIAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus255_ cplt s   EIAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus257_ cplt s   EIAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus260_ cplt s   EIAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus277_ cplt s   EVAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus298_ cplt s   EVAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus299_ cplt s   EVAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus300_ cplt s   EVAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus362_ cplt s   EVAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus432_ cplt s   EIAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL
Rotavirus441_ cplt s   EIAELLLYEWLCNEMDISLYYYQQTSEANKWIAMGTDCTVKVCPLNTQTL

                                      160       170       180       190       200
                       ....|....|....|....|....|....|....|....|....|....|
Rotavirus450_cplt se   GIGCKTTDVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVRNCMRLN
Rotavirus70 cplt seq   GIGCKTTEVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus73_cplt seq   GIGCKTTEVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus75_ cplt se   GIGCKTTEVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus242 cplt se   GIGCKTTDVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus246_ cplt s   GIGCKTTDVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus247_ cplt s   GIGCKTTDVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus249_ cplt s   GIGCKTTDVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus250 cplt se   GIGCKTTDVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus254_ cplt s   GIGCKTTDVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus255_ cplt s   GIGCKTTDVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus257_ cplt s   GIGCKTTDVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus260_ cplt s   GIGCKTTDVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus277_ cplt s   GIGCKTTEVATFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus298_ cplt s   GIGCKTTEVATFEQLTATEKLAIIDVVDGVNHKIDYTVATCNVKNCIRLN
Rotavirus299_ cplt s   GIGCKTTEVATFEQLTATEKLAIIDVVDGVNHKIDYTVATCNVKNCIRLN
Rotavirus300_ cplt s   GIGCKTTEVATFEQLTATEKLAIIDVVDGVNHKIDYTVATCNVKNCIRLN
Rotavirus362_ cplt s   GIGCKTTDVSTFEQLTAAEKLAIIDVVDGVNHKIDYTVTTCNVKNCMRLN
Rotavirus432_ cplt s   GIGCKTTDVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVKNCIRLN
Rotavirus441_ cplt s   GIGCKTTDVSTFEQLTATEKLAIIDVVDGVNHKIDYTVTTCNVRNCMRLN

                                      210       220       230       240       250
                       ....|....|....|....|....|....|....|....|....|....|
Rotavirus450_cplt se   QRENVAIIQVGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQVFYTIVDYI
Rotavirus70 cplt seq   QRENVAIIQVGGPEIIDVSEDPMVVPKMQRVTRINWX~~~~~~~~~~~~~
Rotavirus73_cplt seq   QRENVAIIQVGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQVFYTIVDYI
Rotavirus75_ cplt se   QRENVAIIQVGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQVFYTIVDYI
Rotavirus242 cplt se   QRENVAIIQIGGPEIIDVSEDPMVVPKMQRVTRINWKRX~~~~~~~~~~~
Rotavirus246_ cplt s   QRENVAIIQIGGPEIIDVSEDPMVVPKMQRVTRINWKRX~~~~~~~~~~~
Rotavirus247_ cplt s   QRENVAIIQIGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQVFYTIVDYI
Rotavirus249_ cplt s   QRENVAIIQIGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQVFYTIVDYI
Rotavirus250 cplt se   QRENVAIIQIGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQVFYTIVDYI
Rotavirus254_ cplt s   QRENVAIIQIGGPEIIDVSEDPMVVPKMQRVTRINWKRWWX~~~~~~~~~
Rotavirus255_ cplt s   QRENVAIIQIGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQX~~~~~~~~
Rotavirus257_ cplt s   QRENVAIIQIGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQVFYTIVDYI
Rotavirus260_ cplt s   QRENVAIIQIGGPEIIDVSEDPMVVPKMQRVTRINWKRWX~~~~~~~~~~
Rotavirus277_ cplt s   QRENVAIIQVGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQX~~~~~~~~
Rotavirus298_ cplt s   QRENVAIIQVGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQVFYTIVDYI
Rotavirus299_ cplt s   QRENVAIIQVGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQVFYTIVDYI
Rotavirus300_ cplt s   QRENVAIIQVGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQVFYTIVDYI
Rotavirus362_ cplt s   QRENVAIIQVGGPEIIDVSEDPMVVPKMQRVTRINWKRWWQVFYTIVDYI
```

Figure 77: Deduced amino acid sequences of VP7 protein of Avian Rotavirus.

APPENDIX III

NUCLEOTIDE SEQUENCE OF SOME DETECTED AVIAN ROTAVIRUS AND POSITIVE CONTROL

>1111924-OO-Rotavirus--_Rotavirus249_VP4aR

AGAAATTTGCTTGTGTTATGTTACCACGTAATACTCCTGAATATGATAGACAGTATACGATATT
AGGGAAACAAATAACGATTAGATTAGGGAATACTGATACAGTGAATTATAAATTCTGTGACTT
ATTGAGTAATGATGGAATAATTTACACAAAATCAATAGAGTTAATTACTCCGCATGGTTTAAAT
GCATTCATGAAAGATGCTGGTAAATTATATGCATATAGTGGTATTATACCAACAATAAAAAAA
GAATACCATACGATAGATAATATAGAAAATGTGCAACCCAATATAAC-----

>1111925-OO-Rotavirus--_Rotavirus256_VP4aR

TCAGATTTGCAGGATAATATAGATGATATTTCTGCTGAAAAAACTCAAGGAGTAACGATTAATC
CGGGACCGTTCGCTCAAACAGGTTATGCGCCAGTGGAATGGACTCATGGAGACATACCAGTTG
ATGAAACAATTGAACCAACACTTGATGGACCATATACAGCTTCATCAATTGTAATTCAACCACA
ATATTGGATATTAGTGAATCCAACAAATCAGGATGTCGTGATTGAAGCCGATGCACAGAATAA
GAAATTTGCTTGTGTTATGTTACCACCTAATACTCCTGAATCTGATAGACAGTATACGATATTA
GGGAAACAAATAACGATTAGATTAGGGAATACTGATACAGTGAATTATAAATTCTGTGACTTA
TTGAGTAATGATGGAATAATTTACACAAAATCAATAGAGTTAATTACTCCGCATCGTTTAAATG
CATTCATGAAAGATGCTGGTAAATTATATGCATATAGTGGTACTATACCAACAATAAAAAAAG
AATACCATACGCTAGATAATATAGAAAATGTGCAAACCAATATAACATGTGAGTATTATATAG
TGCCAAATAGTCAA--------------------------------------

>1111926-OO-Rotavirus--_Rotavirus260_VP4aF

ATTTCTGCTGAAAAAACTCAAGGAGTAACGATTAATCCGGGACCGTTCGCTCAAACAGGTTAT
GCGCCAGTGGAATGGACTCATGGAGACATACCAGTTGATGAAACAATTGAACCAACACTTGAT
GGACCATATACAGCTTCATCAATTGTAATTCAACCACAATATTGGATATTAGTGAATCCAACAA
ATCAGGATGTCGTGATTGAAGCCGATGCACAGAATAAGAAATTTGCTTGTGTTATGTTACCACC
TAATACTCCTGAATCTGATAGACAGTATACGATATTAGGGAAACAAATAACGATTAGATTAGG
GAATACTGATACAGTGAATTATAAATTCTGTGACTTATTGAGTAATGATGGAATAATTTACACA
AAATCAATAGAGTTAATTACTCCGCATCGTTTAAATGCATTCATGAAAGATGCTGGTAAATTAT
ATGCATATAGTGGTACTATACCAACAATAAAAAAAGAATACCATACGCTAGATAATATAGAAA
ATGTGCAAAC-----------------------

>gi|186702943|gb|EU486956.1| Rotavirus A isolate Ch-02V0002G3 VP4 gene, complete cds-----
ATGGCTTCTCTCGTATATAGACAGCTTTTAGCTAATTCTTATACTTCTGAATTACAAGATACAAT
TGATGATATTTCTGCCCAGAAATCACAGGACGTAACAATCAATCCAGGACCATTTGCTCAAACA
GGTTATGCGCCAGTAGAATGGACGCATGGCGATATAACAACTGATGAAACTATAGAACAAACA
CTCGATGGACCTTATACGTCTTCATCAATAATAATACAACCACAGTATTGGATATTAGCAAATC
CTGATACTGAAGGAGTGATAGTCGAAGCGGACGCCACAGACCAAAAATATGCTCGTGTGATGT
TACTACCAAATACGGCAGATGGTAATAAACAATATACAATACTAGGTCACCGAGTGACAATTA
ATCTAGGGAATACGAATTCAAACATGTATAAATTTTGTGACTTGGTTAGTCACAACGGAACGAC
ATATACTAAGGTTGAAGAGTTGGTTACACCACACGGATTAAATGCATTTATGAAAGACCAAGG
TAAATTATATTTATATAATGGAACAGTGCCAAATGTACAACAAGTATATTATACACTTGCTAAT
ATAGAAAATGTTCAAACTAACATCAAATGTAATTATTATATCGTACCTAAAACTCAAACGGGA
CAACTAGAAGATTATATTAAGAATGGTCTACCTCCAATTCAAGAGTCCAGATATATAGTTCCAG
TGAATAGATCTGTCATAAATGTATATCAATCCAGACCAAATGAAGATATATTAATCTCTAAGAC
GTCATTGTGGAAGGAAGTACGATATAATAGAGATATTCTTATTAGATTCAAATTCGGTAATAAT
ATAATAAAATCAGGTGGATTAGGTTATAAATGGTCTGAAATTTCTTTTAAGCCAATGAACTATG
AGTACACGTATGAACGTGATGGTGAAACAGTTGTAGCACATACTACGTGTTCAGTTGCTGGTAT
AAATAATTTTGGCTATAATTCTGGTTCTTTGCCAACAGATTTAGTAGTTTCAACATATGAAGTAT
TGAAAGGTAATTCATATGTATACATCGATTACTGGGATGATTCACAAGCATTTAAAAATATGGT
GTATGTGAGATCATTAAGTGCGGAATTTAATGCCATTAGTTGTAATGGAGGCACGTTTAATTTC
CAGTTACCAGTTGGTCAATGGCCACAAATGCGTGGCGGAAATGTAACATTAAACTCCGACGCT
GTAACATTATCAACACAATATACAGATTTTGTATCACTTAATTCACTACGATTCAGGTTTAAGC
CAGCTATTGGTGAGCCATCATTCGAAATAACACGGACGCGTGAACGAAGACTATATGGCCTAC
CAGCTGCAAATCCAATGGGTAATCAAGAATACTATGAAACTGCTGGTAGATTTTCTTTAATTTC
ACTTGTTCCATCTAATGATGACTATCAAACTCCTATTCAAAATTCAACTACAGTTAGGCAAGAT
TTAGAGCAACAAATAACAAATTTGCGTGAAGAGTTTAATCAGTTATCATCAGAAATAGCTGTAT
CACAATTAGTTGACTTAGCTTTATTACCGCTTGATATGTTTTCAATGTTCTCAGGAATTAAATCA
ACAATAGATGCAGTGAAATCGGTAACAACCTCAGTTATGAAGAAGATGAAAGCATCGACGTTA
GCAAAGTCAGTATCTACAATTACGGAAGAATTATCAGACGCAGCTACTTCAATTTCAAGATCAT
CATCAATACGTTCGAATGCATCAGTATGGAATGACTTAATAGATACGGCAACACAAACTTCAT
ATGCTTCTAGTGACGTAGCAACACAAACCTCAAAAATAGCCACGAAATTAAGGGTAAAAGAAT
TTGCGACACAGACAGACGGAGGATTGAATTTTAACGACATATCAGCTGCTGTATTGAAGACGA
AAATCGATAAGCTTGACGCTGTACAACCAAAATTATTACCTACAATTATAGCTGATTCAGTGGA
TAAGTTTATACCATCAAGAGAATATAGAATAATTAATAAAGATATTGCATATGAAATATCAAA
TAGTGGACGATATTTTGCTTATAAAGTGGATACGTTAGAAGAAGTGGTATTTGATGTAGAAAA
GTTCGCTAATCTTGTTACGGATTCACCAGTAATATCAGCTATAATAGACTTTAGAACCATAAAA
AATCTAAACGATAATTTCGGAATAACTGGAGAGCAAGCGTATAATAATCTAAGATCTGATCCA
AGAGTTCTTAAAGAATTCATAAATCAGAATAATCCAATTATAAAAAATAGAATAGAACAACTC
ATACTTCAATGTAGAATATAA------------------------------

>Rotavirus70_complete sequence

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CGT

>Rotavirus246_complete sequence VP6

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CGT

>Rotavirus254_complete sequence VP6

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>Rotavirus300_complete sequence VP6

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>gi|69957891|gb|DQ096805.1| Avian rotavirus A isolate 02V0002G3 VP6 gene, complete cds

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CCGT

>gi|223862743|gb|FJ169861.1| Rotavirus 02V0002G3 complete
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>Rotavirus 70 complete sequence vp7
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>Rotavirus250 complete sequence
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>Rotavirus277_complete sequenceVP7a
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>Rotavirus441_complete sequence
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>Rotavirus-NSP4-consensus 258
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>Rotavirus-NSP4-consensus 461
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>Rotavirus-NSP4-consensus 254
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APPENDIX IV

ACCESSION NUMBERS OF AVIAN ROTAVIRUS ES FROM SOUTHWESTERN NIGERIA IN GenBank

1. LK932155 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/070/2011
2. LK932156 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/073/2011
3. LK932157 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/075/2011
4. LK932158 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/242/2011
5. LK932159 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/246/2011
6. LK932160 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/247/2011
7. LK932161 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/249/2011
8. LK932162 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/250/2011
9. LK932163 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/251/2011
10. LK932164 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/252/2011
11. LK932165 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/253/2011
12. LK932166 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/254/2011
13. LK932167 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/255/2011
14. LK932168 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/257/2011
15. LK932169 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/260/2011

16. LK932170 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/299/2011

17. LK932171 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/361/2011

18. LK932172 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/362/2011

19. LK932173 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/363/2011

20. LK932174 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/365/2011

21. LK932175 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/366/2011

22. LK932176 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/369/2011

23. LK932177 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/432/2011

24. LK932178 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/436/2011

25. LK932179 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/450/2011

26. LK932180 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/461/2011

27. LK932181 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/258/2011

28. LK932182 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/266/2011

29. LK932183 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/295/2011

30. LK932184 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/300/2011

31. LK932185 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/256/2011

32. LK932186 NSP4 gene for nonstructural protein 4, strain RVA/chicken/Nigeria/NIE11/368/2011

33. LK932187 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/070/2011

34. LK932188 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/073/2011

35. LK932189 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/075/2011

36. LK932190 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/242/2011

37. LK932191 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/249/2011

38. LK932192 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11-250/2011

39. LK932193 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11-251/2011

40. LK932194 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11-252/2011

41. LK932195 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11-253/2011

42. LK932196 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11-254/2011

43. LK932197 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11-255/2011

44. LK932198 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/256/2011

45. LK932199 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/266/2011

46. LK932200 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/257/2011

47. LK932201 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/258/2011

48. LK932202 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/260/2011

49. LK932203 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/295/2011

50. LK932204 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/299/2011

51. LK932205 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/300/2011

52. LK932206 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/432/2011

53. LK932207 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/441/2011

54. LK932208 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/450/2011

55. LK932209 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/246/2011

56. LK932210 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/247/2011

57. LK932211 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/277/2011

58. LK932212 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/298/2011

59. LK932213 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/361/2011

60. LK932214 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/362/2011

61. LK932215 VP6 gene for inner capsid VP6 protein, strain RVA/chicken/Nigeria/NIE11/366/2011

62. LK932216 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/070/2011

63. LK932217 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/073/2011

64. LK932218 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/075/2011

65. LK932219 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/242/2011

66. LK932220 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/247/2011

67. LK932221 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/249/2011

68. LK932222 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/251/2011

69. LK932223 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/252/2011

70. LK932224 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/253/2011

71. LK932225 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/254/2011

72. LK932226 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/255/2011

73. LK932227 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/256/2011

74. LK932228 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/257/2011

75. LK932229 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/258/2011

76. LK932230 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/260/2011

77. LK932231 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/266/2011

78. LK932232 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/298/2011

79. LK932233 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/299/2011

80. LK932234 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/300/2011

81. LK932235 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/366/2011

82. LK932236 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/432/2011

83. LK932237 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/441/2011

84. LK932238 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/246/2011

85. LK932239 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/250/2011

86. LK932240 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/277/2011

87. LK932241 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/295/2011

88. LK932242 VP7 gene for outer capsid glycoprotein VP7, strain RVA/chicken/Nigeria/NIE11/450/2011