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MOLECULAR DETECTION, GENETIC CHARACTERIZATION AND ZOONOTIC POTENTIAL OF PORCINE ROTAVIRUSES

Joshua O Amimo, *University of Nairobi*

MOLECULAR DETECTION, GENETIC CHARACTERIZATION AND ZOONOTIC POTENTIAL OF PORCINE ROTAVIRUSES

A THESIS SUBMITTED TO THE UNIVERSITY OF NAIROBI IN FULFILMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN ANIMAL GENETICS AND BREEDING

JOSHUA OLUOCH AMIMO (BVM, MSc)

DEPARTMENT OF ANIMAL PRODUCTION
FACULTY OF VETERINARY MEDICINE
UNIVERSITY OF NAIROBI

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DECLARATION

This thesis is my own original work and to my knowledge it has not been presented for award of degree or diploma in any university.

Signed: J. Amimo Date 24/09/2014

Joshua Oluoch Amimo, *BVM, MSc*
(Reg. No. J80/95095/2014)

This thesis has been submitted for examination with our approval as University supervisors

Signed: Date 30/09/2014

Dr. Joseph O. Junga (PhD)
Depart. of Animal Production, University of Nairobi

Signed: Date 08.10.2014

Prof. William O. Ogara (PhD)
Depart. of Public Health Pharmacology & Toxicology, University of Nairobi

Signed: Date October 06, 2014

Dr. Appolinaire Djikeng (PhD)
Biosciences of east and central Africa- International Livestock Research Institute Hub

Signed: Linda J. Saif Date 1st October 2014

Prof. Linda J. Saif (PhD)
Food Animal Health Research Program, College of Vet. Medicine, The Ohio State University

ABSTRACT

Virus induced enteritis is one of the serious problems accounting for high mortality rates in neonatal animals and human throughout the world. The absence of appropriate surveillance programs and laboratory facilities have resulted in a scarcity of accessible data on virus associated diarrheas (especially rotavirus) in pigs in the East African region. The aim of this study was to provide detailed molecular epidemiological information on the prevalence and genetic diversity of rotaviruses (RVs) and also the distribution of genotypes of group A RVs (RVA) circulating in high intensive commercial pig production systems in the USA and smallholder pig production systems (1-25 pigs per farm) in Kenya and Uganda. Fecal samples obtained from the state of Ohio, US**A** (n=380), western Kenya (n=239) and eastern Uganda (n=207) from nursing and weaned piglets were screened for the presence of RVs (USA and East Africa) and other enteric viruses (kobuviruses and astroviruses in East African pig fecal samples).

Of the 380 samples obtained from 5 swine herds in Ohio, USA, 371 samples were examined for the presence of RVA of which 9.4% (35/371) were positive. A total of 23 positive samples were analyzed for RVA G and P genotypes. The dominant G-P combination was G9P[13] found in 60.9% of sequenced strains. The other combinations were G9P[7] (8.7%), G4P[13] (8.7%), G11P[13] (4.3%) and G11P[7] (4.3%). Sequence analysis of partial VP7 genes of selected strains revealed that the G4 strains were closely related to one another (95%) and to a less extent to human (82-84%) and porcine (84-86%) G4 strains. The G11 strains detected shared identical VP7 gene sequence (100%) and were closely related to human (85-86%) and other porcine (83%) G11 strains. The G9 strains identified were closely related to one another, human and other porcine strains (96-97%, 89-91% and 89-91% nucleotide identities, respectively). The VP4 gene analysis revealed that P[7] strains were closely related to each other and to P[7] strains isolated from porcine, bovine and panda (91-99%, 92-99% and 92-99%, respectively). The P[13] strains showed a higher diversity among themselves and with other porcine P[13] strains ranging from 83% to 99% and 82-97%, respectively.

A total of 380 USA samples were also tested for group C rotavirus (RVC) and 19.5% were positive. Of the 128 samples collected in 2012, 23.5% from nursing piglets and 8.5% from weaned piglets were RVC positive, with a higher RVC frequency in diarrheic (28.4%) than in

non-diarrheic (6.6%) piglets. Two strains (RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6Px) from two different farms were characterized genetically to gain information on virus diversity based on full length sequences of the inner capsid VP6, enterotoxin NSP4 and the outer capsid VP7 and VP4 genes. The results of this study indicate high genetic heterogeneity in RVA and RVC genes and the concurrent co-circulation of different RV genogroups and genotypes at the same time in the USA. The RVA genotyping results suggest the possibility of genetic reassortment between different RVA genotypes within these US farms. These findings are useful for the development of more accurate diagnostic tools and to provide information for vaccine development.

Of the 446 samples obtained from East Africa, 31.6% (141/446) were positive for RVs, of which 73.8% (104/141) were positive for RVA alone, and 17% (24/141) were positive for RVC alone and 9.2% (13/141) were of RVA/RVC mixed infection. More nursing piglets (78.7%) shed RVA than weaned (32.9%) and grower (5.8%) pigs. RVA incidence was higher in pigs that were either housed_free-range (77.8%) or tethered_free-range (29.0%) than those that were free-range or housed or housed-tethered pigs. The farms with larger herd size (>10 pigs) had higher RVA prevalence (56.5%) than farms with smaller herd size (24.1-29.7%). This study revealed that age, management system and pig density significantly ($p<0.01$) influenced the incidence of RVA infections, with housed_free-range management system and larger herd size showing higher risks for RVA infection. Partial (811-1604nt region) sequence of the VP4 gene of selected positive samples revealed that different genotypes (P[6], P[8] and P[13]) are circulating in the study area with P[8] being predominant. The P[6] strain shared nucleotide (nt) and amino acid (aa) sequence identity of 84.4-91.3% and 95.1-96.9%, respectively, with known porcine and human P[6] strains. The P[8] strains shared high nt and aa sequence identity with known human P[8] strains ranging from 95.6-100% and 92-100%, respectively. The P[13] strains shared nt and aa sequence identity of 83.6-91.7% and 89.3-96.4%, respectively, with known porcine P[13] strains. The study in Kenya and Uganda demonstrates that infection with RV is frequent in East African piggeries. The P[6] and P[8] RVA genotypes detected were genetically closely related to human strains suggesting the possibility of interspecies transmission and zoonotic potential.

Additionally, of the 446 samples from East Africa, 251 samples were screened for the presence of other enteric viruses (caliciviruses and kobuviruses), of which 12.7% were positive for caliciviruses and 13.1% positive for kobuviruses. Genetic analysis of the *kobuvirus* RdRp partial

sequence revealed that they are more diverse, sharing nucleotide identity ranging from 89.7-99.1% among them. This study also detected porcine astroviruses in few samples belonging to type 2 and type 3 *Mamastroviruses*.

To my knowledge this study reports the first detection and molecular analysis of group A RVs, kobuviruses and astroviruses in the Kenyan and Ugandan swine population. The presence of these gastroenteritis-producing viruses in clinically healthy pigs represents a source of infection of pigs, and possibly to humans, and hence further studies are required to determine their role in gastrointestinal infections of pigs in this region and to determine their genetic diversity in-order to develop accurate diagnostic tools and implement appropriate control strategies to improve pig health. Improved pig health will lead to improved production and ultimately improved livelihood.

DEDICATION

This thesis work is dedicated to my beloved wife, Noel Malesi, who has been a constant source of support and encouragement during the challenges of doctorate program and life. I am truly thankful for having you in my life. I also dedicate this work to my son (J.C Nyandat) and daughter (J.N Achieng') for being there for me throughout the entire process. Both of you have been my best cheerleaders.

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Finally, I dedicate this work to all my friends (especially Nyasena Welfare members) and relatives (especially Eng. David Orenya Nyandat) for their support and whose good examples have taught me to work hard for the things that I aspire to achieve.

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LIST OF PUBLICATIONS RELATED TO THE THESIS

1. **Amimo, J.O**, A.N Vlasova and L.J Saif (2013) Detection and genetic diversity of porcine group A rotaviruses in historic (2004) and recent (2011/12) swine fecal samples in Ohio, USA: Predominance of G9P[13] genotype in nursing piglets. Journal of Clinical Microbiology, 51(4):1142-51: http://www.ncbi.nlm.nih.gov/pubmed/23363823
2. **Amimo, J.O**, A.N Vlasova and L.J Saif (2013) Prevalence and genetic heterogeneity of porcine group C rotaviruses in nursing and weaned piglets in Ohio, USA and identification of a potential new VP4 genotype: Veterinary Microbiology, 164 (1-2): 27-38 http://www.ncbi.nlm.nih.gov/pubmed/23428382
3. **Joshua O. Amimo**, Edward Okoth, Joseph O. Junga, William O. Ogara, Moses N. Njahira, Qiuhong Wang, Anastasia N. Vlasova, Linda J. Saif, Appolinaire Djikeng (2014) Molecular detection and genetic characterization of kobuviruses and astroviruses in asymptomatic local pigs in East Africa. Archives of Virology (2014) 159:1313–1319. http://www.ncbi.nlm.nih.gov/pubmed/24327095
4. **Amimo, J.O**, E. Okoth, J.O Junga, W.O Ogara, M.N Njahira, A.N Vlasova, L.J Saif, A. Djikeng (2014) Detection and genetic characterization of porcine group A rotaviruses in asymptomatic pigs in smallholder farms in East Africa: Predominance of P[8] genotype resembling human strains (***Submitted to Veterinary Microbiology journal***)

LIST OF PRESENTATIONS (ORAL AND POSTERS) RELATED TO THE THESIS

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Poster presentations

1. **J O Amimo**, A N Vlasova and L J Saif. 2012. Prevalence and genetic diversity of group A and non-group A porcine rotaviruses in selected US farms. American Society of Virology (**ASV**) Annual Meeting, July 21-25, Madison, WI, USA
2. **J.O. Amimo**, E. Okoth, J.O. Junga, W.O. Ogara, M.N. Njahira, Q Wang, A.N. Vlasova, L.J. Saif, A. Djikeng (2013) Prevalence and genetic diversity of rotaviruses in swine raised under small-scale production system in East Africa. 2nd International Congress on Pathogens at the Human-Animal Interface (**ICOPHAI**): One health for sustainable development, 17 - 14 August 2013, Summerville Beach Resort, Porto de Galinhas, Brazil.
3. **Amimo, J.O**, E. Okoth, J.O Junga, W.O Ogara, M.N Njahira, A.N Vlasova, L.J Saif, A. Djikeng (2014) Detection and molecular characterization of selected swine enteric viruses in smallholder farms in Kenya and Uganda. 9th Biennial Conference and Exhibition of Faculty of Veterinary Medicine, University of Nairobi, 3 - 5 September 2014, Upper Kabete Campus, Nairobi, Kenya. Abstr

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LIST OF ABBREVIATIONS

RV - Rotavirus
RVA - Group A rotavirus
RVB - Group B rotavirus
RVC - Group C rotavirus
HRV - Human rotavirus
KoV - kobuvirus
PAstV - Porcine astrovirus
EM - Electron microscopy
PAGE - Polyacrylamide gel electrophoresis
EIA - Enzyme immunoassay
RT-PCR - Reverse transcription polymerase chain reaction
VP - Viral protein
NSP - Nonstructural protein
RdRP - RNA dependant RNA polymerase
dsRNA - double stranded RNA
DLP - Double layered particle
TGE - Transmissible gastroenteritis
PRRS - Porcine respiratory and reproductive syndrome
cDNA - complementary DNA
MEM - Minimum essential media
PBS - Phosphate buffered saline
PEDV - porcine epidemic diarrhea virus
PCV2 - Porcine circovirus type 2
GDP - Gross domestic product
BecA - Bioscences of east and central Africa
ILRI - International Livestock Research Institute
UoN - University of Nairobi
OSU - The Ohio State University
USA - United State of America
FAO - Food and Agricultural Organization of United Nation
OH - the state of Ohio

CHAPTER 1

1 INTRODUCTION

1.1 Background

Rotaviruses (RV) are common enteric pathogens of children (<5 years) and young animals (calves, piglets and foals). They are generally species-specific, but cross-species transmission is possible, as has been demonstrated experimentally. Rotavirus infections account for approximately 611,000 deaths in children each year, mainly in developing countries (Parashar *et al.*, 2006). RV-associated enteritis also has huge economic impact in livestock industry due to mortality and reduced growth on recovered animals. Comparison of genetic sequences of human and animal RVs often reveals close identity and has provided evidence for frequent intersections between the evolution of human and animal RVs; hence, the public health concern. Information on human and animal RV distribution and the dominant RV strains in developing countries is scarce. In the global context, information on the prevalence and epidemiology of genogroup B and C RVs is scarce for both humans and animals, with no vaccines available. In this study the aim was to provide detailed information on the prevalence of genogroups A and C RVs and also the distribution of G and P genotypes of group A RVs circulating in the USA and P genotypes circulating in Kenya and Uganda and ascertain their similarities and zoonotic potential. This study will generate a database that will serve as a platform for future RVs surveillance activities in humans and animals in the East African region. Continuous epidemiological surveillance is critical for vaccine development, for understanding the short- and long term effects of the vaccines on RV ecology, and for implementing future vaccine strategies especially for

developing countries where there is greater diversity of RV strains and RV vaccine efficacy is decreased or highly variable. The characterization of the circulating RV strains in the East African region is an important step towards understanding the genetic diversity of RV and will help to identify potential transmission routes for atypical human and animal infections.

1.2 Objectives of the study

1.2.1 Overall objective

The overall objective of this study was to determine the distribution of RVs and the distribution of G and P genotype combinations of RVAs circulating in swine populations raised under smallholder production system in Kenya and Uganda and in highly specialized commercial farms in the USA. This study also aimed to assess the similarity and zoonotic potential of RVs from domestic pigs compared to regional close contact human RV strains.

1.2.2 Specific objectives

1. To detect and estimate the prevalence of RV genogroups (A and C) and group A RV genotypes in fecal samples from domestic pigs of various ages collected in USA, Kenya and Uganda.
2. To assess genetic relatedness and zoonotic potential of selected RVA and RVC genotypes (especially re-emerging genotypes) detected in pigs based on partial and complete genomic sequence.
3. To detect and genetically characterize other enteric viruses (Kobuviruses and Astroviruses) affecting pigs in smallholder farms in Kenya and Uganda.

1.3 Justification of the study

The prevalence of RV genogroups and RVA genotypes changes over time and is affected by age, vaccination status, production systems and geographic location. This study hypothesizes that the prevalence of RVA and RVC and emerging RVA genotypes (G1, G6, G9, G10, G12) may be currently underestimated contributing to vaccine failures both in humans and livestock. Therefore, a study of circulating RVs is needed to monitor the effectiveness of current RV vaccines, where available, and to develop and implement new efficacious vaccines against dominant RVs and other control measures.

One reason for failure of RV vaccines in developing countries is the greater genetic diversity of RVs circulating in humans in the developing countries and the potential emergence of new strains from animal reservoirs. Therefore, documenting changes occurring in strains through partial or complete genome sequencing may help to elucidate RV vaccine failures if they occur, or to identify the dominant strains for vaccine development. Additionally, complete genome sequencing of genogroups and genotypes will lead to a better understanding of the genetic relationships among the various strains, the role of the various gene segments in host range restriction and the mechanism of RV evolution.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Introduction

Neonatal diarrhea is a major endemic health problem for most swine herds worldwide due to its economic impact. Diarrhea in pigs is a multifactorial and multi-etiological disease. Management failure might play an important role in the development of the disease, besides infection by microorganisms such as bacteria, protozoa and viruses (Katsuda *et al.*, 2006). Viral diseases of swine (e.g. TGE, PEDV, Circovirus, Rotavirus (RV), swine influenza, PRRS) represent the greatest challenges in the control and prevention of infectious diseases, hence, they further increase cost of treatment and prevention. Rotaviruses are a major cause of viral gastroenteritis in children and young animals worldwide, including nursing and weaned piglets.

Rotavirus infection cannot be diagnosed exclusively on the basis of clinical presentation, because the clinical features of RV gastroenteritis do not differ from those of gastroenteritis caused by other pathogens. Confirmation of RV infection by laboratory tests is necessary for reliable RV surveillance, and can be useful in clinical settings to avoid inappropriate use of antimicrobial therapy. Rotaviruses are enteric pathogens causing acute watery dehydrating diarrhea in the young of various host species, including birds and mammals. Rotaviruses account for approximately 611,000 deaths in children each year, mainly in developing countries (Parashar *et al.*, 2006). Likewise, rotavirus-associated enteritis is a major problem in young calves (Saif & Jiang, 1994), weaning and post-weaning piglets (Kapikian, 1996) and foals (Conner *et al.*, 1983; Dickson *et al.*, 1980). Rotaviruses are generally species specific, but cross-species transmission

is possible, as has been demonstrated experimentally. Several case studies have indicated infection of humans by animal RVs. Rotaviruses causing diarrhea in pigs were demonstrated by Woode and coworkers (Woode *et al.*, 1976). This virus is widespread in pig populations. It is present in most, if not all, pig herds, with a virtually 100% seroconversion in the adult stock. Antibodies to group A RV, whose distribution is worldwide, can be found in 90–100% pigs. Rotaviruses of groups B and C are also present in swine herds but more detailed data on the evidence of antibodies to these groups are scarce (Marthaler *et al.*, 2012).

2.2 Classification and transmission of rotaviruses

Rotavirus (RV) is a genus of the Reoviridae family of double-stranded RNA viruses. They have a genome of 11 segments of dsRNA encoding 6 structural viral proteins (VP1-VP4, VP6, VP7) and 6 nonstructural proteins (NSP1-NSP6) as described in **Figure 2.1**. With the exception of genome segment 11 which encodes 2 proteins (NSP5 and NSP6) the genome segments are monocistronic. Full gene-protein assignments have been achieved for several RV strains (Estes, 2001; Estes & Kapikian, 2010). The genome is enclosed in a triple layered particle (**Figure 2.1)**: the inner layer consisting of VP2 encloses the genome and two minor structural proteins, VP1 and VP3, thus forming the 'core'; the middle layer consisting of VP6 surrounds the core, forming a double layered particle (DLP); the outer layer, consisting of VP7 and spike-like projections of VP4, enwraps the DLP to form the triple-layered particle or infectious virion (Estes & Kapikian, 2010).

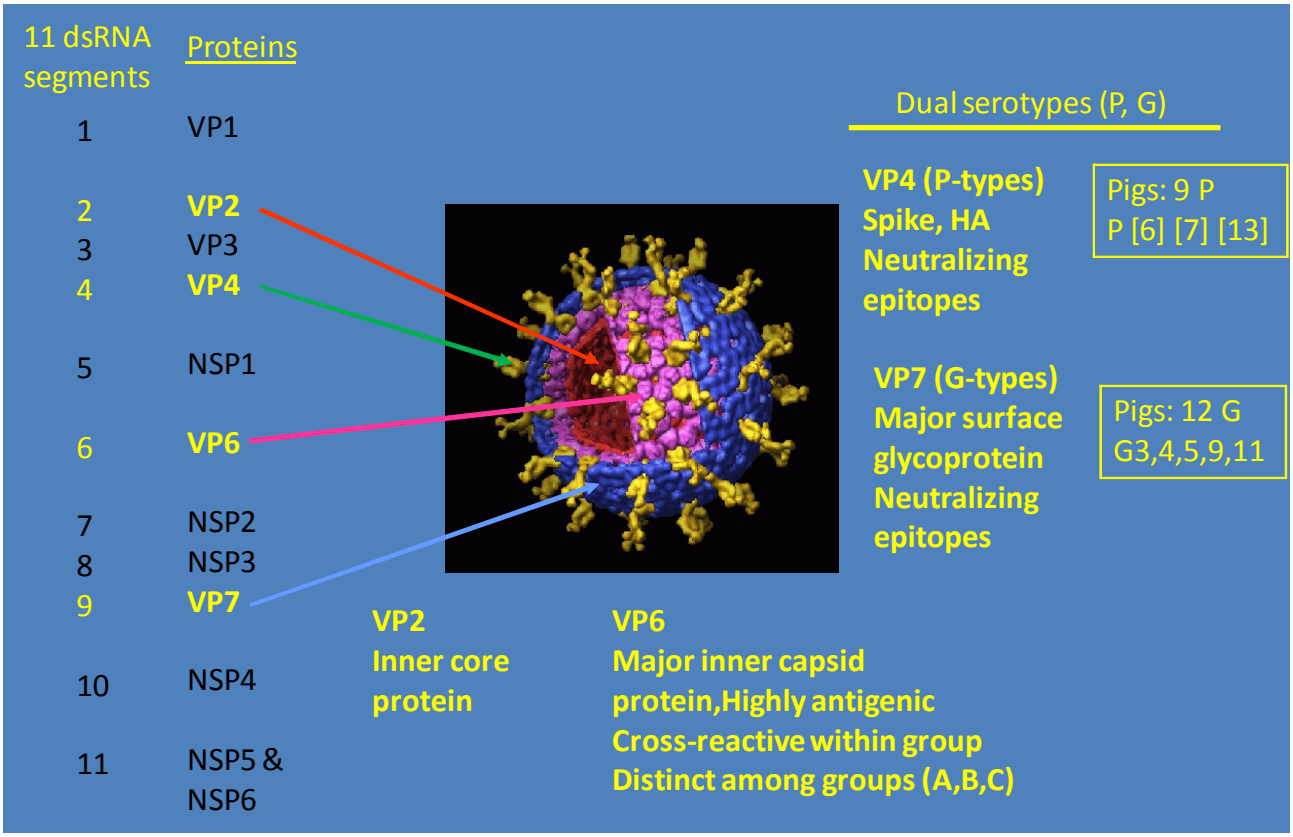


Figure 2.1 Structure and Classification of Rotaviruses (adapted from (Kapikian *et al.*, 2001))

Rotaviruses are classified into 8 groups (A–H) based on their antigenic relationships at VP6 gene and the pattern of migration of the dsRNA segments in polyacrylamide gel electrophoresis (Ciarlet & Estes, 2002; Estes *et al.*, 1989; Saif & Jiang, 1994). The most common groups which infect humans and animals are A, B and C, with high prevalence of RVAs. Groups D - G RVs are relatively more often detected in healthy animals but an association with disease has also been reported.

Group B and C RVs have been identified in some mammalian host species, including humans, while group E RV has been detected only in pigs. Until now, group D, F and G RVs have been identified exclusively in poultry (Saif & Jiang, 1994), while recently group H RV has been identified in pigs (Molinari *et al.*, 2014; Wakuda *et al.*, 2011). Representatives of a novel human non-group A-C RV have been described (Alam *et al.*, 2007; Yang *et al.*, 1998; Yang *et al.*, 2004). The question whether they represent a new serogroup or belong to one of the known non-group A, B, and C RVs is unclear, as no serologic data are available for this new strain and few sequence data are available for comparison of reference strains within serogroups D to H.

Molecular characterization

According to the VP4 (and VP4-gene) and VP7 (and VP7-gene) antigenic and molecular characterization, Gp A RVs are further classified into different G and P types. Rotavirus strains are characterized with a dual classification system with G and P serotype or G and P genotype. Whilst G serotype and genotype match, there are differences between P serotypes and genotypes. Currently within genogroup A, 26 G genotypes (structural viral protein [VP] 7 related) and 33 P genotypes (VP4 related) have been identified (De Grazia *et al.*, 2010).

Recent estimates show that serotypes G1, G3, G4, and G9 account for 90% of all human RV infections in North America and Europe; however, these serotypes are responsible for less than

70% of cases in Africa (Santos & Hoshino, 2005). Whereas P[8] and P[4] account for over 90% of human P types circulating worldwide, the relative frequency of these two serotypes seems to be lower in Africa, where P[6] accounts for almost a third of all P types detected (Santos & Hoshino, 2005). A review by Sanchez-Padilla (Sanchez-Padilla *et al.*, 2009) revealed that the most common human G type detected in sub-Saharan Africa was G1 (34·9%), followed by G2 (9·1%), and G3 (8·6%) while the most common P types detected in the region were P[8] (35·5%) and P[6] (27·5%). In the mid-nineties, a new emerging RV G9 in combination with P[6] or P[8] genotype was spreading across all the continents. The origin of this genotype was probably in animals (Hoshino *et al.*, 2005). It was speculated that animals are a reservoir for new RVs following the interspecies transmission between humans and animals (Cook *et al.*, 2004; Iturriza-Gomara *et al.*, 2003).

Vaccines against the most important serologic group of rotaviruses, RVAs, are available for the prevention of RV diarrhea in calves, piglets and foals, and, more recently, they have become available for the prevention of RV disease in infants and young children (Heaton & Ciarlet, 2007). The ability of a RV vaccine to protect against a wide panel of RV strains is important in developing countries, where there is greater diversity of RV strains (Gentsch *et al.*, 2005; Santos & Hoshino, 2005). Because the RV genome is segmented, reassortment events are common as has been demonstrated in previous studies (Iturriza-Gomara *et al.*, 2001). Reassortment events result in new RV strains with distinct antigenic properties (Gentsch *et al.*, 2005; Matthijnssens *et al.*, 2006).

Recent data based on sequencing of the full RV genome (11 segments) has revealed that the major existing human RV strains have lineage likely derived from animal strains e.g Wa-like, DS-1-like and AU-1-like, PA260-97, BA222-05-like strains (Ghosh *et al.*, 2011a; Matthijnssens

et al., 2008a; Matthijnssens *et al.*, 2008b; Matthijnssens *et al.*, 2011b; Matthijnssens *et al.*, 2008c). The emergence of a new human–animal reassortant or animal RV strains into the human population could have a huge impact on the spread of RV and also on prevention measures. The efficiency of licensed RV vaccines in the market and reasons for vaccine failures need to be analyzed with particular attention to new, emerging RV genotypes and strains with unusual genotype combinations (Esona *et al.*, 2010). Therefore, a study of animal RVs and their zoonotic potential is a key step to acquiring an in-depth understanding of the ecology and evolution of RVs.

Rotavirus is most commonly transmitted by the fecal-oral route and by person to person through close contact (Chin, 2000)**.** Other less common modes of transmission are contaminated fomites, food and water. The ubiquitous nature of RV infection indicates that improvement in sanitation and hygiene will not adequately prevent transmission, and thus, vaccines remain the cornerstone of RV infection prevention.

For animal RVAs, the prevalence of genotypes is documented to some extent for mainly bovine and avian species which tend to differ significantly from human RV (HRV) RVA (Abe *et al.*, 2009; Cao *et al.*, 2009; Caruzo *et al.*, 2010; Cashman *et al.*, 2010; Elschner *et al.*, 2005; Matthijnssens *et al.*, 2011a; Matthijnssens *et al.*, 2008b; Monini *et al.*, 2008; Ursu *et al.*, 2011). However, similarities between RV strains found in pigs and humans suggest that swine may serve as a reservoir for HRV or co-infections may result in reassortment between related human and pig strains much like the scenario for influenza viruses. The reason being that physiologically, pigs resemble humans in their digestive system and they are the only species susceptible to HRV diarrhea (Saif, 1996; Yuan & Saif, 2002).

2.3 Pathogenesis and clinical symptoms

Pathogenesis: Rotaviruses replicate exclusively in the cytoplasm of mature enterocytes covering the tips of the small intestine villi (Kapikian, 1996). These cells have enterokinase, an enzyme necessary for activating trypsin, which activates RVs. Virus replication induces degeneration and lysis of the enterocytes and, consequently, atrophy of the villi. The degree and extent of the atrophy is lower than that caused by other enteric viral infections in swine, such as transmissible gastroenteritis or porcine epidemic diarrhea. The atrophy of the villi is more severe and affect the surface intestinal epithelium of young piglets. The recovery of the intestinal epithelial cells starts shortly after infection and intestinal villi are restored. The main mechanism of RVs induced diarrhea is a low feed absorption, a consequence of the destruction of mature enterocytes (Woode & Crouch, 1978). It also causes a decrease in activity of digestive enzymes. Undigested feed increases osmotic pressure in the intestinal lumen, leading to water retention which results in diarrhea. The proliferation of immature enterocytes on the crypts, which replace the mature enterocytes that have been destroyed, contributes to diarrhea by means of their secretory activity (Paul *et al.*, 1999).

Symptoms of rotavirus infection: A sporadic, seasonal, often severe gastroenteritis of children and young animals, characterized by vomiting, fever and watery diarrhea. Although rotavirus diarrhea is generally more severe than acute diarrhea due to other agents, illness caused by rotavirus is not distinguishable from that caused by other enteric viruses for any individual patient.

2.4 Rotavirus Detection Methods

Techniques for RV detection include i) Electron microscopy (EM); ii) Antigen detection; and iii) Nucleic acid detection such as PAGE and nucleic acid amplification i.e. RT-PCR. Most of these methods are relatively efficient at detecting RVs, at least in part because of the large amount of intact RV present in stool specimens of human and animals with gastroenteritis.

2.4.1 Electron microscopy (EM)

Electron microscopy is highly specific for detection of RV and is as sensitive as some enzyme immunoassays (EIAs). However, the method is too labor intensive for routine detection of RV in large numbers of stool specimens. In addition, EM requires an expensive instrument and highly trained personnel and cannot distinguish between RVs of different groups (Nakata *et al.*, 1987; Theil *et al.*, 1986) unless immune electron microscopy is used (Saif *et al.*, 1977).

2.4.2 Antigen detection - Enzyme immunoassay (EIA) and Latex agglutination and lateral-flow immunoassays (immunochromatography)

The most widely used methods for RV diagnosis are based on detection of protein antigens on RV particles in stool specimens. The most appropriate antigen detection format for large-scale surveillance studies is an EIA that uses RV specific antibodies to capture antigen onto wells of plastic plates. The antigen is then detected in a colorimetric reaction using a second RV-specific antibody coupled to a detector enzyme. **Latex agglutination**, utilizing latex particles coated with anti-RV antibodies can be used as an alternative to EIA and rapid near patient tests using immunochromatographic methods are being used widely in consulting rooms (Pazdiora *et al.*, 2002).

2.4.3 Nucleic acid detection and nucleic acid amplification

Because of the large quantities of RV 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 RV 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 RVs of Groups A, B, and C (Steele *et al.*, 2004). For Group A RVs, most samples that are positive for RV by EIA will be positive for the characteristic pattern of RV 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 RV infections for surveillance studies. However, this method is very labor intensive and time consuming.

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

2.5 Porcine rotavirus distribution

The information on porcine RV distribution and prevalent strains in developing countries is limited. Globally, information on the prevalence and epidemiology of group B and C RVs is scarce for both humans and animals, with no vaccines available for them. Therefore, it is critical to study the distribution and the possibility of interspecies transmission of groups A, B and C RVs between humans and pigs, especially in developing countries where the rate of childhood diarrhea is highest, there are uncommon and more diverse HRV RVA genotypes and RV vaccine efficacy is decreased or highly variable (Armah *et al.*, 2010a; Bresee *et al.*, 1999; Gentsch *et al.*, 2005; Matthijnssens *et al.*, 2009; Parashar *et al.*, 2006). In addition the introduction of RV vaccines into this region may provide selective pressure for emergence of new human RV strains, including ones from animal reservoirs.

2.6 East Africa Pig Industry and rotavirus infections

The Livestock sub-sector in East Africa contributes about 10% of the Gross Domestic Product (GDP) and accounts for over 30% of farm gate value of agricultural commodities. The demand for proteins of animal origin in the region is on the increase due to high population growth and rural-urban migration. Pork contributes to protein demand because pigs have high reproductive rates, high feed conversion efficiency, they have capacity to use by-products from other primary industry and they require less space (FAO, 2012). Small-scale production (free range or small backyard herds) constitutes up to 70% of the total pig farmers in Kenya and Uganda. Pigs often live in close proximity to humans and hence cause public health concern. Therefore, there is a need for increasing health standards, including adequate veterinary services (FAO, 2012).

In the developing countries, including Kenya and Uganda, one of the major causes of mortality among children (<5 yrs) and young piglets is diarrheal disease (Othero *et al.*, 2008; Wabacha *et al.*, 2004). Tate and co-workers reported that the costs of care and treatment for RV gastroenteritis in children are high (Tate *et al.*, 2009). In the swine industry, piglets that suffer RV diarrheal infections have stunted growth and some may die, resulting in high economic losses. RV prevalence in animals in Kenya is largely unknown but suspected to be among the major causes of suckling and weaning piglet diarrheal disease and a concern for pork producers due to mortality, reduced growth and high cost of treatment and control. Vaccination against RV in developing countries is uncommon, ineffective or provides inconsistent results in both humans and animals (Armah *et al.*, 2010b; Bresee *et al.*, 1999; Patel *et al.*, 2009).

East Africa is also known for the large population of wild (bush) pigs and warthogs that are in close contact, share common water sources and virus pools with domestic pigs and can travel long distances creating favorable conditions for intermixing and spread of viral strains. Other specific features affecting agriculture and way of life in this region are: i) frequent floods; ii)absence of developed sewage systems leading to fecal contamination of human and animal water supplies; and iii) lack of security and sanitation measures and secure housing for animals. All these features could contribute to RV circulation and spread. Additionally, farm workers handling livestock or children in direct contact with animals, especially young animals that are frequently shedding RV may be directly exposed to RV contaminated feces. The frequent handling of piglets, lambs and calves during the first few weeks of life could lead to transmission of RV infections from animals to humans and *vice versa*.

The systematic molecular characterization of animal (especially swine) and human RV diversity from developing countries such as Kenya and Uganda could provide a comprehensive analysis of

the current situation in the region, documenting the effects of specific features of the regional agriculture, healthcare and veterinary services. Earlier complete genome sequence data has revealed the animal lineages of many existing human RV strains (Ghosh *et al.*, 2011a; Ghosh *et al.*, 2011b; Matthijnssens *et al.*, 2011a). Considering the high potential of RV strains to reassort and the newly accepted classification system based on sequencing of all 11 genes (Matthijnssens *et al.*, 2011a), ideally complete genomic sequencing should be performed to fully characterize historic and new animal and human strains in the region and to assess their similarities and interrelatedness among species and finally to ascertain their zoonotic potential. Besides, in sub-Saharan African countries, features listed above including low socioeconomic status, crowded and unhygienic living conditions, offer a favorable environment for complex reassortment and/or interspecies transmission events.

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Study areas

3.1.1 United State of America - the state of Ohio

Pig production in this study area is characterized by highly specialized commercial systems where each farm keeps more than 10,000 pigs (**Figure 3.1**). The pig fecal samples from nursing and weaned pigs of less than 3 months old, with or without diarrhea, were collected with the assistance of farm veterinarians and farm managers. Seven farms from northwest, northeast and central parts of the state of Ohio, USA were identified for this study. This study used the fecal samples that were collected from seven swine farms in 2004, 2011 and 2012 in a wider study of porcine enteric viruses including RVs in Dr. Linda Saif's laboratory in The Ohio State University, USA. Although rotavirus gastroenteritis is predominantly seasonal, every effort was made to collect samples throughout the year. An increase in RVs infections outside of the normal seasonal pattern may indicate the emergence of a new strain into an immunologically naive population.

3.1.2 Kenya and Uganda

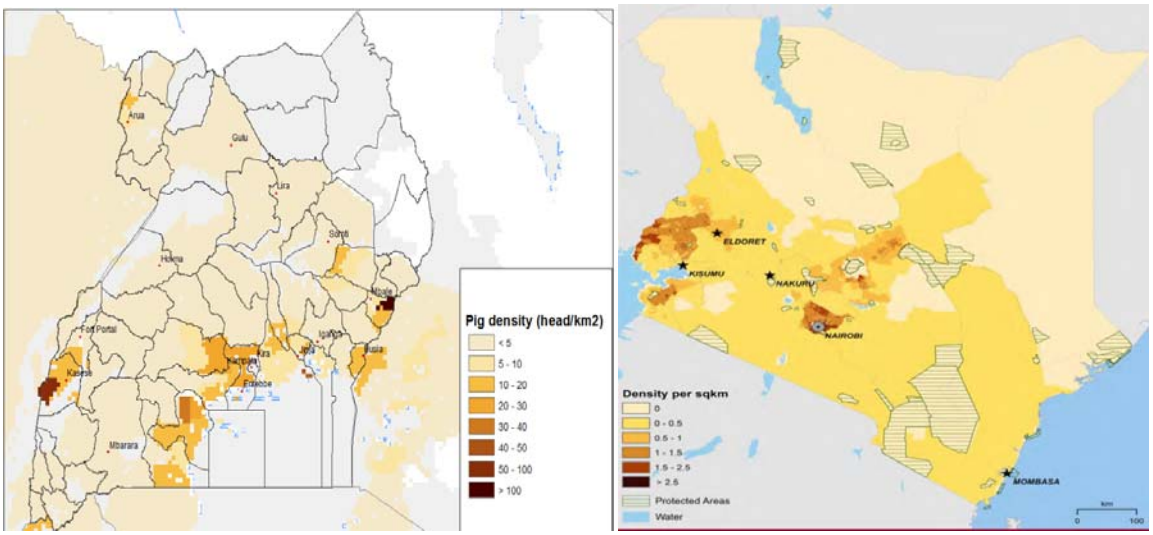
This study was conducted in the western parts of Kenya and eastern parts of Uganda. The study is part of a wider project designed to study African swine fever dynamics in East African region. These sites were selected based on unique swine production systems; predominated with free

range and backyard system (**Figure 3.2**); pig interaction with humans and wildlife; and also based on the pig density in the region as shown in **Figure 3.3**.

In East Africa the study was conducted in Kenya in two administrative districts that is Teso and Busia while in Uganda the study site was in Busia and Tororo districts (**Figure 3.4**). The pig production system in the study area is mainly a smallholder system with farmers having between 1 and 25 pigs per household. Pigs in the study area are kept under different management systems including free-range; tethered; housed; or a mix of free-range and housed; tethered and housed; and free-range and tethered. Additionally, the study areas are characterized by high prevalence of different pig genotypes (exotic, crossbred and indigenous pigs) and close interaction between domestic pigs, wild pigs and human. Also, rural pig populations and urban pig populations were identified in order to allow for the detection of zoonotic RV infections and reassortant strains resulting from dual infection with animal and human RVs.

Figure 3.1. Pigs in a highly commercial pig production system in a farm in the USA

Figure 3.2. Pigs in smallholder production system in western Kenya



(a) (b)

Figure 3.3. Average pig densities in Uganda (a) and Kenya (b) (FAO, 2007)

3.2 Sampling design and sample size determination

3.2.1 Sampling design

Pig keeping households were selected randomly from the four districts (Busia and Teso in Kenya and Tororo and Busia in Uganda). Selection was done using a multistage sampling method from the districts to the village level: At the district level two sub-locations were selected randomly, in the sub-location two villages were selected randomly, from which the households were selected with the assistance of the local administration and veterinary field staff. In each of the villages, 10 pig keeping households were selected for questionnaire administration with assistance of the local leaders. Randomly, on average 3 (range 1 to 5) piglets (6 months of age or less) were sampled during scheduled visits or in response to calls from farmers, hence, a total of 480 pig fecal samples were collected. The sampled animals were matched, where possible, for age, sex and breed during the sampling. The study involved veterinarians for collection of samples.

3.2.2 Sample size determination

The study assumed a 95% confidence level, 50% prevalence, and a margin of error (confidence interval) of +/- 5%. The confidence level corresponds to a Z-score (95% = 1.96)

$n = t^2 \times p(1-p) / m^2 = (1.96^2 * 0.5(1-0.5))/0.05^2 = 384.16 =$ (estimated at 384 pigs)

Where:

n= Sample Size; **t^2** = a constant value for confidence level at 95% (standard value of 1.96); **p**=estimated prevalence of RV and **m**= margin of error

This gives an average of 2.4 pigs per household (estimated to 3 pigs per household) totaling to 480 samples

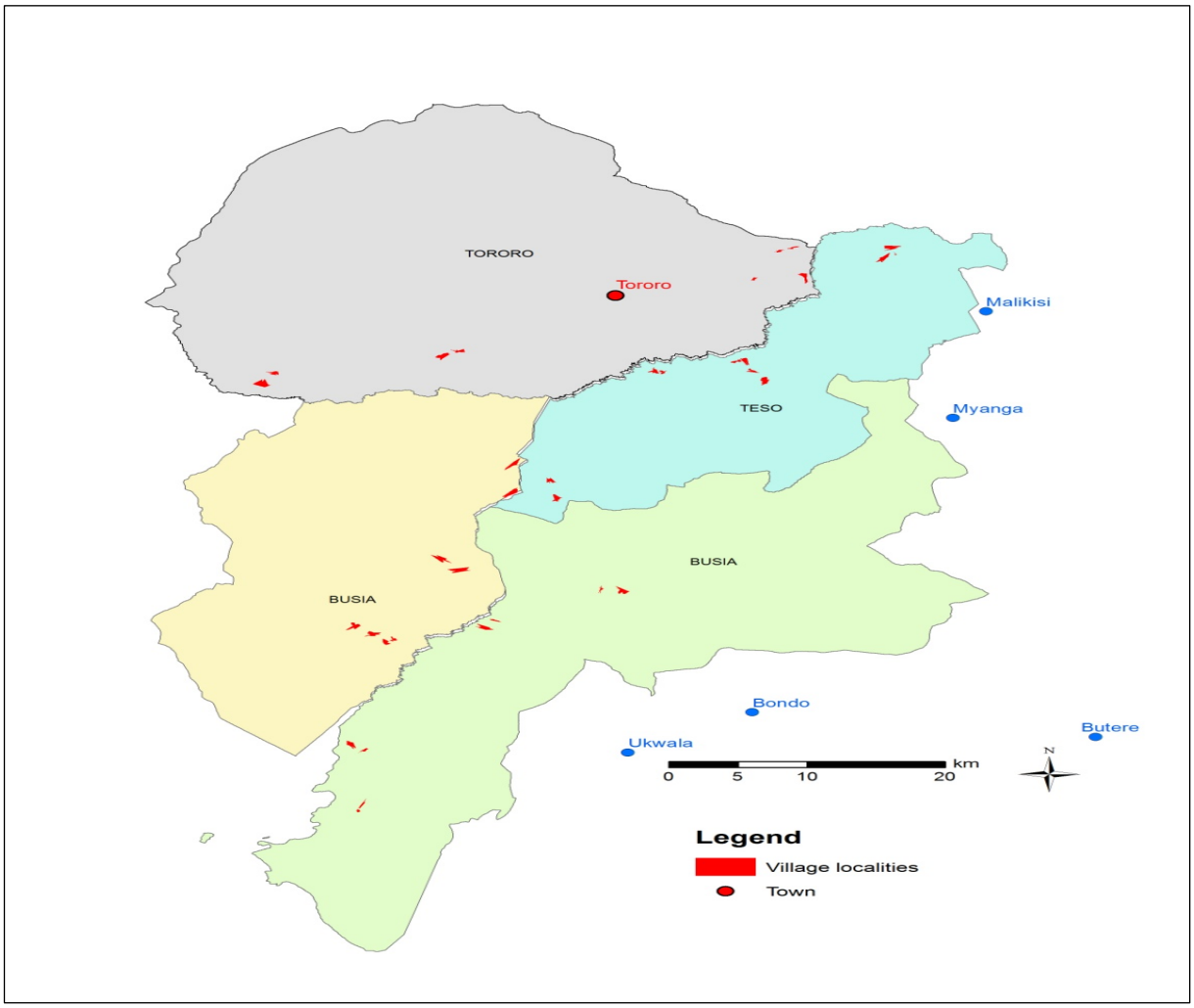


Figure 3.4. The map showing the districts in Kenya and Uganda where study was carried out (red points)

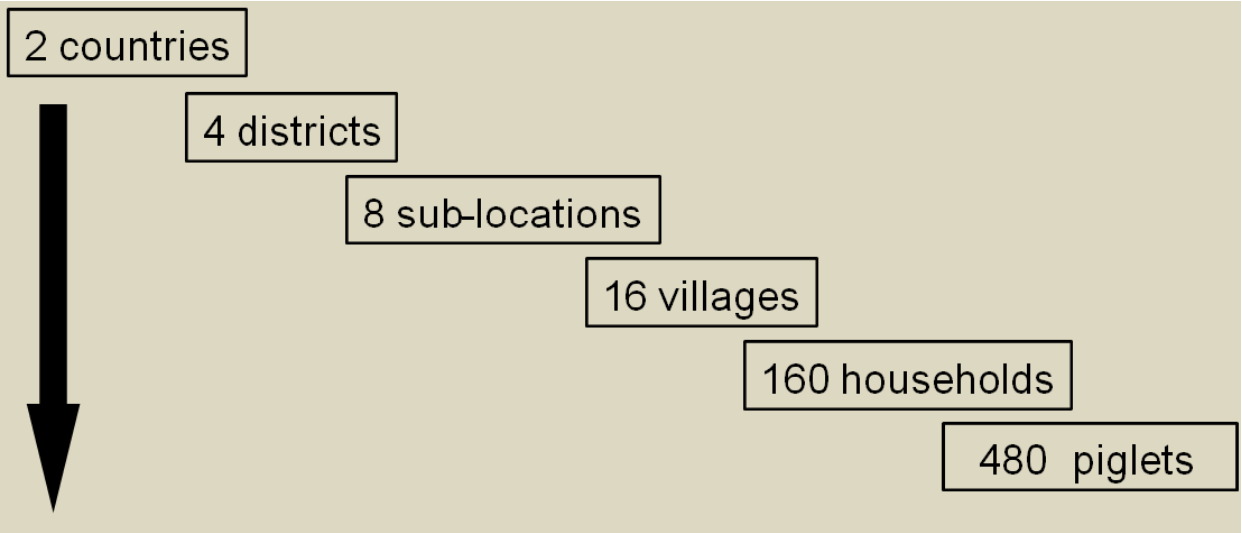


Figure 3.5. The flow chart showing the multistage sampling procedure used

3.3 Epidemiological data collection

A prepared questionnaire form was used to collect epidemiological data such as animal's ID, age, breed and sex; date and place of collection of specimens, vaccination status; diarrheal status and the husbandry system used on the farm (**Appendix 1**).

3.4 Fecal sample collection procedure

Fecal samples were collected into labeled (label included - pig's ID, age, breed and sex, diarrheal status, date and place of collection of specimens) sterile plastic fecal cups or 15ml centrifuge tubes following stimulation of the anal sphincter with a sterile swab. Fecal material was not collected from the floor and new gloves were used for each animal to avoid mixing of samples. When no defecation occurs, rectal swabs were taken and placed into 15 ml eppendorf tubes, ice-cooled and transported to the laboratory within 2 hours of collection. On reaching the laboratory, approximately 5 grams of feces from each sample were transferred into a plastic bag that was labeled and kept at -20°C until required for testing. The remaining part was stored at -70°C in original sampling container.

3.5 Sample preparation and RNA Extraction Procedure

3.5.1 Preparation of 10% Fecal Suspension and storage

Upon receipt, the fecal samples were stored at -20°C. Then, 10% fecal suspensions were prepared in Minimum Essential Medium (MEM) or phosphate buffered saline (PBS), and centrifuged at 1800xg for 30 min at 4^0C. The supernatants were stored at -70^0C until use. **Figure 3.6** shows a flow chart of how the fecal samples were processed in the laboratory.

3.5.2 RNA extraction

This was done using RNeasy mini kit protocol from Qiagen company (CA, USA) according to the manufacturer's instructions. Briefly, 10 µl B-mercaptoethanol was mixed with 1 ml RLT lysis buffer, thereafter, 350 µl of mixture from step 1 was added to 250 µl of each sample in eppendorf tube, then, 350 µl of 75% ethanol was added and mixed. The mixed sample was transferred to an RNeasy mini column placed in a 2 ml collection tube, then centrifuged for 15 sec, at 8000xg at 23^0C. The flow-through was discarded. Seven hundred (700) µl of washing buffer (RW1) was added to the RNeasy mini column, centrifuged for 15 sec, 8000xg at 23^0C and discard flow-through. Then 500 µl RPE buffer was added to the RNeasy column and centrifuged for 15 sec, 8000xg at 23^0C and flow-through discarded. To completely dry the mini column, the remaining residual ethanol was removed by centrifugation at 13000xg for 1 minute. Mini columns were transferred to the 1.5 ml tubes and the RNA was eluted by adding 40 µl of RNase-free water directly onto the RNeasy silia-gel membrane, centrifuged for 1 min at 8000xg at 23^0C. The eluted RNA was placed on ice immediately and then store at -70^0C or -20^0C until used.

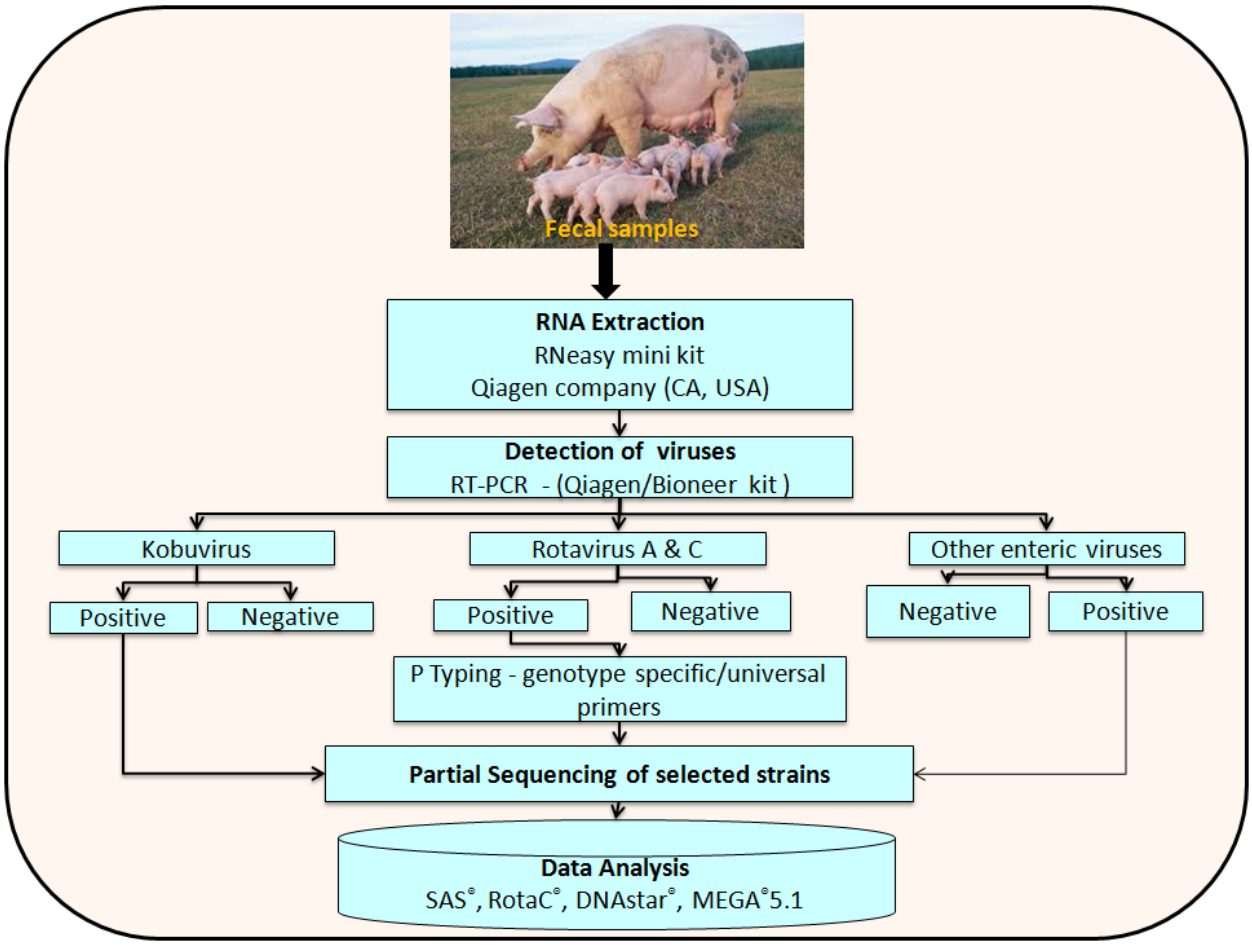


Figure 3.6. Flow chart on how the fecal samples were processed while in the Laboratory

3.6 Detection and characterization of rotavirus genogroup A and C and partial sequencing

Conventional RT-PCR was used for detection of the RV genogroups with validated primer sets for each genogroup (Table 3.1) using Qiagen kit according to the manufacturer's instructions. Briefly, PCR mix for N + 2 (N = No. of tubes in test) was prepared with one sample having the following; 10X Reaction buffer (5µl), $MgCl_2$ (2.5µl), dNTPs (1.0µl), F-primer (0.5µl), R-primer (0.5µl), RNAsin (0.25µl), Go Taq-Flexi (0.25µl), AMV RT (0.25µl) and RNase-free H_2O (12.25µl). Then, 2µl of RNA sample was added to 23µl of PCR master mix to have a total of 25µl of the final PCR reaction volume. The tubes were then put into the thermal cycler (ABI 9700) for PCR reaction for amplification.

The PCR products from the above reaction were viewed using a 3% Agarose gel stained with gel red®, picture taken under UV light and the data recorded as positive and negative samples. The specific band from PCR products of positive samples for each genogroup was extracted using Qiaquick gel extraction kit according to manufacturer's instructions for partial sequencing. DNA sequencing was carried out by using BigDye Terminator Cycle chemistry and 3730 DNA Analyzer (Applied Biosystems, Foster, CA). The positive samples selected for sequencing was based on the intensity of the band in the gel, the enough quantity available and quality of the PCR product. The origin of the sample and the age of the animal from where the sample was taken was also taken into consideration.

Table 3.1 Generic Primers for detection of Group A and C Porcine RV

RV group	Name	Sequence	Product (bp)	Reference
Group A	NSP3-F	ACC ATC TAC ACA TGA CCC TC	87	(Pang et al., 2004)
	NSP3-R	GGT CAC ATA ACG CCC C		
Group C	VP6L-F	ACA GTA TTT CAG CCA GGD TTT C	260	(Amimo *et al.*, 2013b)
	VP6L-R	AGC CAC ATA GTT CAC ATT TCA TC		

3.7 Data analysis

Summary statistics were performed using SAS® (SAS, 2002) data analysis program. The prevalence of RVs in the study area was calculated as number of pigs positive divide by total number of pigs sampled. A chi-square test or Fisher's exact test when at least one of the expected frequency in the cells was less than five, was used to assess the relationship between several factors (country, districts, farm, management system, age group and diarrheal status) and the RV detection using procedure frequency in the SAS computer program (SAS, 2002). The level of significance was at 5%.

For nucleotide and predicted amino acid sequence alignment as well as phylogenetic analysis, the CLUSTAL_O (http://www.ebi.ac.uk/Tools/msa/clustalo/) and MEGA 6.0 software packages was used (Tamura et al., 2013). Genetic distances were calculated using the Kimura two-parameter algorithm and phylogenetic trees constructed based on the neighbor-joining method. Phylogenetic trees were statistically supported by bootstrapping over 1000 replicates.

CHAPTER 4

4 PREVALENCE AND GENETIC HETEROGENEITY OF PORCINE GROUP C ROTAVIRUSES IN NURSING AND WEANED PIGLETS IN OHIO, USA AND IDENTIFICATION OF A POTENTIAL NEW VP4 GENOTYPE

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http://www.ncbi.nlm.nih.gov/pubmed/23428382)

4.1 Abstract

Swine fecal samples collected from seven farms were screened for group C rotaviruses (RVCs) using a reverse transcription-polymerase chain reaction assay. A total of 380 samples were tested and 19.5% were positive. Of the 128 samples collected in 2012, 23.5% from nursing piglets and 8.5% from weaned piglets were RVC positive, with a higher RVC frequency in diarrheic (28.4%) than in non-diarrheic (6.6%) piglets. Two strains (RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6Px) from two different farms were characterized genetically to gain information on virus diversity based on full length sequences of the inner capsid VP6, enterotoxin NSP4 and the outer capsid VP7 and VP4 genes. The VP6 gene of the two strains showed high (99%) nucleotide identity to one another, 84-91% identity to other porcine strains and 81-82% identity to human and bovine strains. The NSP4 gene analysis revealed that RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6Px strains were not closely related to each other (87% identity), but shared higher identity with prototype RVC/Pig-wt/USA/Cowden/1980/G1Px strain (93% and

89%, respectively) and were more distantly related to human strains (72-76% identity). The VP7 gene analysis indicated that the two strains were distantly related to one another (72% identity). RVC/Pig-wt/USA/RV0143/2012/G6Px was most closely related to porcine RVC G6 strains (82-86% identity), whereas RVC/Pig-wt/USA/RV0104/2011/G3PX was most closely related to porcine HF (G3) strain (94% identity). Analysis of the full length nucleotide sequence of the VP4 gene revealed that RVC/Pig-wt/USA/RV0143/2012/G6Px was distantly related to porcine (75%), bovine (74%) and human (70%) strains. The deduced amino acid identity (69.5-75.6%) of VP4 between RVC/Pig-wt/USA/RV0143/2012/G6Px and other RVC were low; hence, the study proposes that this strain comprises a new VP4 genotype. These results indicate high genetic heterogeneity in RVCs genes and the concurrent co-circulation of different genotypes at the same time. The findings of this studyare useful for the development of more accurate diagnostic tools, for basic research to understand gene function and to provide information for vaccine development.

Key words: group C rotavirus, prevalence, genetic diversity, swine

4.2 Introduction

Neonatal diarrhea is a major endemic health problem for most swine herds worldwide due to its economic impacts. Diarrhea in piglets is a multi-factorial and multi-etiological disease. Rotaviruses (RVs) are a major cause of viral gastroenteritis in children and young animals worldwide, including nursing and weaned piglets (Chang *et al.*, 2012). RVs comprise a genus in the *Reoviridae* family of dsRNA viruses, with a genome of 11 segments of dsRNA encoding 6 structural viral proteins (VP1-VP4, VP6 and VP7) and 5 nonstructural proteins (NSP1-NSP5/6). RVs are classified into 7 groups (A–G) based on antigenic relationships of their VP6 proteins

(Kapikian, 2001). The most common groups that infect humans and animals are groups A, B and C, with the highest prevalence for Group A RV. The outer capsid proteins, VP4 and VP7, induce neutralizing antibodies and form the basis for G and P genotype/serotype assignment (Estes & Kapikian, 2010). A dual typing system based on the genome segments encoding VP4 (P genotypes) and VP7 (G genotypes) is used. To date, 27 different G- and 35 P-genotypes have been described in both humans and animals (Matthijnssens *et al.*, 2011a) for group A RVs; however, a formal classification system for RVCs based on VP4 and VP7 genes has not yet been established, although 6 G-types (G1-G6) have been identified and proposed by researchers using sequence analysis of multiple human and animal RVC strains (Rahman *et al.*, 2005). Porcine RVCs exhibit G1, G3, G5 and G6 genotypes, bovine RVCs exhibit G2 genotype and human RVCs exhibit G4 genotype (Collins *et al.*, 2008; Martella *et al.*, 2007b; Rahman *et al.*, 2005)

Group C RVs were first identified in swine in 1980 (Bohl *et al.*, 1982; Saif *et al.*, 1980a). Subsequently, RVCs have been detected in humans, cows, ferrets and dogs (Mawatari *et al.*, 2004; Otto *et al.*, 1999; Saif & Jiang, 1994). In humans, RVC infection has been associated with gastroenteritis worldwide, leading to the recognition that RVCs are important enteric pathogens (Bányai *et al.*, 2006). Previous studies have reported 28% to 70% antibody prevalence against RVCs in pigs by 8 weeks of age. The antibody prevalence increases with age and reaches 79% to 100% in adult pigs (Saif & Jiang, 1994; Terrett & Saif, 1987). Diarrhea outbreaks associated with RVCs have been documented in nursing, weaning and post-weaning pigs (Kim *et al.*, 1999; Saif *et al.*, 1980a; Saif & Jiang, 1994) either alone or in mixed infection with other enteric pathogens (Chang *et al.*, 2012). A possible zoonotic role of animal RVCs has also been hypothesized based on increased seroprevalence rates to RVCs in human populations (Iturriza-Gómara *et al.*, 2004). Furthermore, RVC surveillance has revealed transmission of RVCs

between animal species: bovine strain WD534tc was shown to be of porcine origin (Chang *et al.*, 1999b). Detection of animal-like RVCs in humans necessitates further detailed studies of the epidemiology and genetic diversity of animal RVCs, especially in regions where humans and animals, or different animal species often live in close contact making mixed infections more common.

The limited information on porcine RVC prevalence and diversity and lack of a commercial diagnostic assay for the detection of RVCs has hindered detailed knowledge of RVCs with regard to their epidemiology, their genetic heterogeneity and their pathogenic potential in pigs of different ages. To date the vaccine (ProSystem Rota, Intervet Inc., Merck Animal Health) that has been developed against RVs in swine contains only G4 and G5 genotypes of group A RV. Since there is no heterologous protection between RVs belonging to different groups or genotypes/serotypes, understanding the genetic heterogeneity of RVCs is critical to design effective prophylactic tools against RV infections, including development, optimization and improvement of effective RV vaccines. Therefore, the aim of this study was to investigate the prevalence of porcine RVC strains and to genetically characterize them from historical (2004) and recent (2011 and 2012) fecal samples collected from swine herds located in the state of Ohio, US.

The GenBank accession numbers of RVC/Pig-wt/USA/RV0104/2011 are KC164673 (NSP4), KC164674 (VP6), KC164675 (VP7) and RVC/Pig-wt/USA/RV0143/2012 are KC164676 (NSP4), KC164677 (VP6), KC164678 (VP7), and KC164679 (VP4). The accession numbers of all representative known RVC strains for each gene mentioned in this study are listed in **Appendix 2**

4.3 Materials and methods

4.3.1 Samples

Two sets of porcine fecal samples collected from diarrheic and non-diarrheic nursing and post weaning piglets from 7 swine herds located in Ohio, US were screened for RVCs (Table 4.1). One set included historical fecal samples collected in 2004 (118) and the second set included recent samples collected in 2011 (134) and 2012 (128). Samples collected in 2012 were from both nursing (81) and weaned (47) piglets and they were collected from piglets with diarrhea (67) and without diarrhea (61) (Table 4.1). All samples were screened by RT-PCR, using validated primers designed from multiple alignment of the VP6 gene of representative human and porcine RVC reference strains (Martella *et al.*, 2007a).

4.3.2 RNA extraction and RT-PCR

The RNA was extracted from 250µl of 10% (wt/vol) fecal suspensions of rectal swabs or stool samples in Minimum Essential Media (MEM) using RNeasy mini kit (Qiagen, CA, USA) according to the manufacturer's instructions. The total RNA recovered was suspended in 40µl of nuclease free water and stored at -70^0C until used. PCR inhibitors in the samples were determined to be negligible based upon results obtained by making 10-fold and 100-fold dilutions of RNA. Conventional RT-PCR was used for detection of the RVCs with validated primer sets (available from the authors per request) using Promega reagents according to the manufacturers' instructions (Martella *et al.*, 2007a). The amplicons were analyzed in 1.5-3% agarose gel. The tissue cultured porcine rotavirus C (Cowden) and nuclease free water was used as positive and negative controls, respectively.

4.3.3 Sequencing and molecular analysis

To confirm the PCR results and obtain genetic information on virus diversity, the VP6 amplicons of six positive samples were sequenced. The DNA was purified by using a QIAquick gel extraction kit (QIAGEN, Inc.) according to the manufacturer's protocol. The amplicons were sequenced directly using forward and reverse RT-PCR primers. DNA sequencing was carried out by using BigDye Terminator Cycle chemistry and 3730 DNA Analyzer (Applied Biosystems, Foster, CA). The resulting partial (260bp) VP6 gene sequences of the RVCs were aligned with corresponding fragments of selected reference strains of human, porcine and bovine RVC strains. Distance analysis and phylogenetic inference were conducted using the Mega 5.0 software package (Tamura *et al.*, 2011).

Based on results of the partial VP6 gene sequences, two of the RVC strains (RVC/Pig-wt/USA/RV0104/2011/G3PX & RVC/Pig-wt/USA/RV0143/2012) were selected from two different clusters and further characterised using full-length sequences of their NSP4, VP6 and VP7 and VP4 genes (Partial VP4 gene for RVC/Pig-wt/USA/RV0104/2011/G3Px strain) using specific primers for each gene (available from the authors per request). These two samples were collected from nursing piglets (RVC/Pig-wt/USA/RV0104/2011/G3PX from piglet from Farm 2, 2011, diarrhea status unknown, and RVC/PIG-WT/USA/RV0143/2012 from piglet from Farm 6, 2012, with diarrhea) and were only RVC positive. cDNA was synthesised using 3µl of the total RNA using SuperScript III^{TM} first-strand synthesis system for RT-PCR according to manufacturer's protocol (Invitrogen) using gene specific primers for each gene. Two µl of cDNA was used as a template for the PCR reaction, PrimeSTAR GXL DNA Polymerase (Takara, Japan) was used for high fidelity synthesis and PCR was conducted under the following

conditions: 35 cycles at 98^0C for 10s, 50^0C for 20s and 68^0C for 2 min. The amplicons were analyzed in 1.5% agarose gel, the DNA was purified using QIAquick gel extraction kit (QIAGEN, Inc.) and sequenced in both directions using the same primers. The nucleotide sequences obtained were compared with those of similar sequences from reference strains available from GenBank using BLAST software (http://www.ncbi,nlm.nih.gov/BLAST/). The DNA sequences were aligned using the ClustalW method and phylogenetic dendrograms were constructed for each gene using the neighbor-joining method supported with a bootstrap test of 1000 replicates in MEGA 5 software (Tamura *et al.*, 2011).

4.4 Results

4.4.1 Incidence of RVCs in nursing piglets

A total of 380 samples were tested and 19.5% (74/380) were positive for RVCs. When these data were compiled with surveillance results for groups A and B RVs, the study found that 10.8% of RVC positive samples also contained either group A (6.7%) or B (4.1%) RVs. The prevalence of RVCs varied among the farms and between different years within the same farm ranging from 15.6% to 100% (Figure 4.1).

Table 4.1 The year and number of sample collections, pig ages, diarrhea status and RVC infection incidence

Year of collection, total # of samples	Farm ID*	Month samples collected in	Number of samples	Diarrhea status	Age**	RVC positive samples (%)
2004, n=118	Farm 1	April	n=32	N/A***	<3 weeks	5 (15.6%)
	Farm 2	July	n=31	N/A	<3 weeks	6 (19.4%)
	Farm 2	Dec	n=40	N/A	<3 weeks	3 (7.5%)
	Farm 3	May	n=15	N/A	<3 weeks	0 (0%)
2011, n=134	Farm 2	July	n=39	N/A	<3 weeks	14 (36%)
	Farm 2	Dec	n=20	N/A	<3 weeks	11 (55%)
	Farm 4	June	n=37	N/A	<3 weeks	12 (32.4%)
	Farm 5	April	n=34	5 loose, others no diarrhea	<3 weeks	0 (0%)
	Farm 6	Nov	n=4	diarrhea	<3 weeks	0 (0%)
2012, n=128	Farm 2	Jan	n=8	diarrhea	<3 weeks (n=4), gilts (n=4)	0 (0%)
	Farm 2	March	n=60	most no diarrhea (only 6 with diarrhea)	<3 weeks (n=17), >3 weeks (n=43)	4 (6.8%)
	Farm 2	May	n=55	most diarrhea (only 5 with no diarrhea)	<3 weeks	14 (25.5%)
	Farm 6	Jan	n=1	diarrhea	<3 weeks	1 (100%)
	Farm 7	May	n=4	diarrhea	<3 weeks	4 (100%)

* within same year, each farm # appears as many times as it was sampled; ** <3weeks - nursing piglets, >3 weeks - weaned piglets; *** N/A - not available

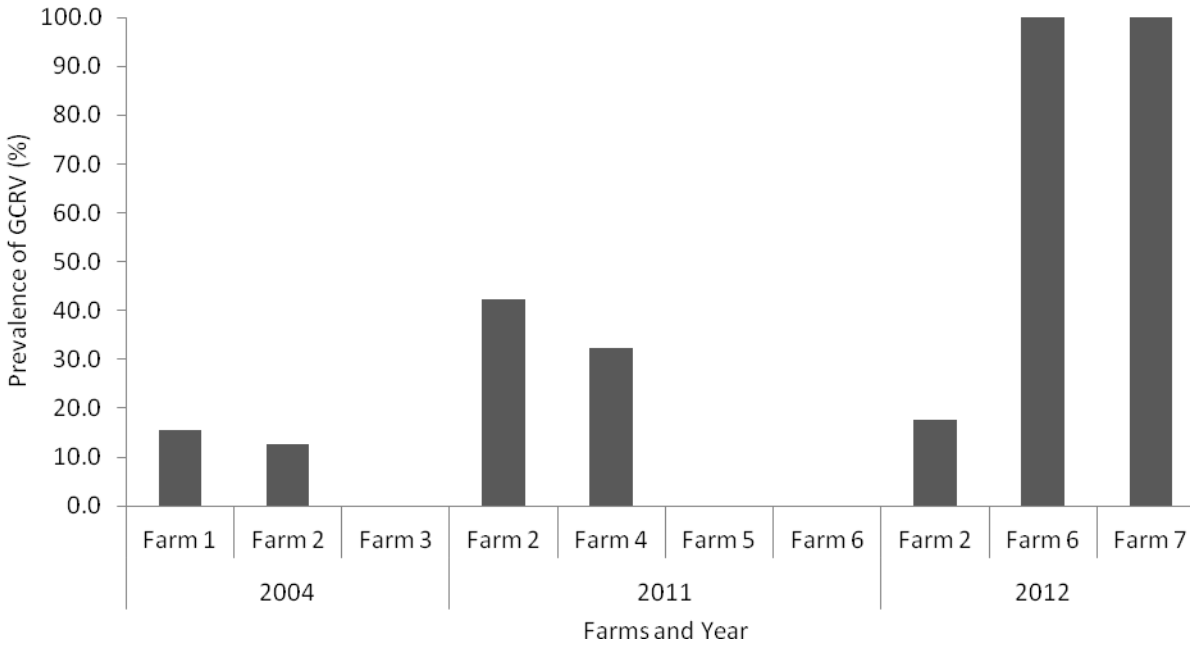

Figure 4.1. Prevalence of RVCs between and within surveyed farms and years. In 2004, farms 1, 2 and 3 were each sampled once; in 2011, farms 4, 5 and 6 were each sampled once and farm 2 was sampled twice; in 2012, farms 6 and 7 were sampled once and farm 2 was sampled 3 times

RVC were detected in most farms (except Farm 3 and 5) but not for every sampling: for example, no RVC were detected on Farm 3 in 2004 and Farms 5 and 6 in 2011. It is noteworthy that samples from Farms 6 and 7 were from diarrheic piglets submitted to the laboratory for diagnosis. Yearly differences ($P<0.05$) in the prevalence of RVCs were observed among the 3 years: 2004 (11.9%), 2011 (27.6%) and 2012 (18%). Overall, the RVC incidence was highest in summer (26%) followed by winter (19.5%) and then spring (16.4%). The RVC prevalence differed within each year: in 2004, the highest prevalence occurred in summer, while in 2011, winter had the highest prevalence and in 2012, the detection rate was highest in spring. However, seasonal influences on the prevalence of RVC infections were not evident across the years studied even for the Farm 2 that was sampled at least twice a year in different seasons (Figure 4.2, Table 4.1).

Based on 128 samples collected in 2012, the RVC prevalence was higher in the nursing (23.5%, 19/81) compared to the weaned (8.5%, 4/47) piglets. Interestingly, the entire weaned pigs positive for RVC infection were asymptomatic, whereas all nursing piglets positive for RVC infection were symptomatic. The relationship between RV detection and diarrhea was evaluated in samples collected in 2012. The detection rate of RV was significantly ($P<0.01$) higher in piglets with diarrhea (50.7%, 34/67) than in non-diarrheic piglets (16.4%, 10/61). Three (2.3%) samples had mixed RV infections (2 RVA+RVB and 1 RVB+RVC) and all were from diarrheic piglets. Regardless of the age of piglets, RVC was detected more frequently (28.4%) in diarrheic piglets than in piglets without diarrhea (6.6%). The relationship between RVC detection and diarrheal and age status of the piglets could not be evaluated for samples collected in 2004 and 2011 because the information about the diarrheal status was not always available.

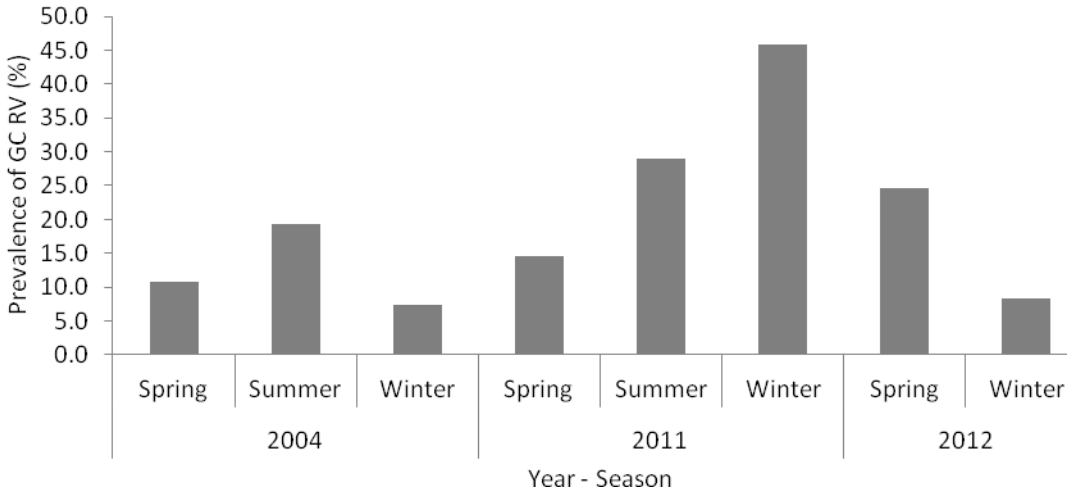

Figure 4.2 Annual and seasonal variability of RVC prevalence within and among the 3 years. Only farms 2 and 6 were sampled repeatedly in different years: farm 2 (July and December, 2004; July and December, 2011; January, March and May, 2012); farm 6 (November, 2011; January, 2012). Farms 1, 3, 4 and 5 were each sampled just once in April, 2004, May-June, 2011, April, 2011, and May, 2012, respectively

4.4.2 Molecular analysis of NSP4, VP6, VP7 and VP4 genes RVC strains

Molecular analysis of the partial (260bp) VP6 gene segment of six selected positive samples confirmed them as porcine RVCs, although an unexpected heterogeneity in a relatively short fragment was revealed. The partial VP6 gene segment of the six strains had high nucleotide sequence identity with reference sequences of human (82.5-86%) and porcine (86.2-97.2%) RVCs. Most of the field strains shared high nucleotide identity between one another (97.2-99.5%) and clustered (>92% identity) with recent Korean and Brazilian strains (data not shown). To gain more information on genetic heterogeneity of RVC strains circulating in swine in the surveyed farms, two likely divergent strains (**RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX**) from two different swine herds collected in 2011 and 2012 were selected and their full-length sequences for NSP4, VP6, VP7 and VP4 genes were determined and analysed.

VP6 Gene: Full length sequences of the VP6 gene of the RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX strains showed 99% nucleotide identity to each other and 84-91% nucleotide identity to other porcine strains. Phylogenetic analysis revealed that RVC/Pig-wt/USA/RV0104/2011/G3PX was clustered with the RVC/Pig-wt/USA/RV0143/2012/G6PX in a monophyletic branch, closely related to other porcine strains, specifically Brazilian strains (90-91% nt identity); however, they were distantly related to the human and bovine branches (data not shown).

Table 4.2 Full length nucleotide sequence identity between the VP7 gene of RVC/Pig-wt/USA/RV0104/2011/G3Px and RVC/Pig-wt/USA/RV0143/2012/G6Px RVC strains and the available published RVC strains in GenBank

			% nucleotide Identity	
Strains	**Host**	**G-type**	**RVC/Pig-wt/USA/RV0104/2011/G3Px**	**RVC/Pig-wt/USA/RV0143/2012/G6Px**
RVC/Pig-wt/ITA/118/05-1/2005/G1Px	Porcine	G1	77	75
RVC/Pig-wt/IRL/45/06/Cork/2006/G1Px	Porcine	G1	71	75
RVC/Pig-wt/USA/Cowden/1980/G1Px	Porcine	G1	77	76
RVC/Pig-wt/KOR/CUK-6/2011/G1Px	Porcine	G1	77	75
RVC/Pig-wt/USA/WH/xxxx/G1Px	Porcine	G1	75	76
RVC/cow-wt/USA/Shintoku/xxxx/G2Px	Bovine	G2	74	77
RVC/Cow-wt/JPN/Yamagata/2002/G2Px	Bovine	G2	74	76
RVC/Pig-wt/USA/HF/xxxx/G3Px	Porcine	G3	**94**	73
RVC/Human-wt/BRA/Belem/xxxx/G4Px	Human	G4	75	77
RVC/Human-wt/KOR/CAU-10-312/2010/G4Px	Human	G4	75	76
RVC/Human-wt/NGA/Jajeri/1999-00/G4Px	Human	G4	76	77
RVC/Human-wt/NGA/Moduganari/1999-00/G4Px	Human	G4	76	77
RVC/Human-wt/RUS/Nsk09-B43/2009/G4Px	Human	G4	74	77
RVC/Human-wt/JPN/OK450/1989/G4Px	Human	G4	75	77
RVC/Human-wt/GBR/Preston/xxxx/G4Px	Human	G4	75	77
RVC/Pig-wt/ITA/134/04-18/2004/G5Px	Porcine	G5	74	77
RVC/Pig-wt/KOR/CUK-5/2011/G5Px	Porcine	G5	74	76
RVC/Pig-wt/ITA/134/04-2/2004/G6Px	Porcine	G6	76	83
RVC/Pig-wt/IRL/1GA/05/Cork/2005/G6Px	Porcine	G6	74	81
RVC/Pig-wt/IRL/281/07/Dublin/2007/G6Px	Porcine	G6	72	81
RVC/Pig-wt/ITA/344-04-7/2004/G6Px	Porcine	G6	75	85
RVC/Pig-wt/ITA/43/06-16/2006/G6Px	Porcine	G6	77	**86**
RVC/Pig-wt/ITA/43/06-22/2006/G6Px	Porcine	G6	77	84
RVC/Pig-wt/USA/RV0104/2011/G3Px	Porcine	G3?		72
RVC/Pig-wt/USA/RV0143/2012/G6Px	Porcine	G6?	72	

For each strain the highest identity to the reference strain is shown in bold

NSP4 Gene: The sequence analysis of the full length NSP4 gene showed that the two field strains (RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX) were distantly related to each other (87% nucleotide identity). They were closely related to prototype RVC/Pig-wt/USA/Cowden/1980/G1Px strain with sequence identity of 93% and 89% for RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX, respectively, while distantly related to human strains (72-76%). NSP4 based phylogenetic analysis revealed that RVC/Pig-wt/USA/RV0104/2011/G3PX and the prototype RVC/Pig-wt/USA/Cowden/1980/G1Px strain belonged to the same cluster while RVC/Pig-wt/USA/RV0143/2012/G6PX formed a different branch indicating increased diversity in porcine RVCs in the NSP4 gene.

VP7 gene: The complete coding nucleotide sequence of the VP7 gene of the RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX strains was determined and compared with VP7 gene sequences of other RVC strains available in the GenBank database. Sequence comparison indicated that one strain (RVC/Pig-wt/USA/RV0143/2012/G6PX) was most closely related to RVC G6 strains with sequence identities ranging from 82-86% while the other strain (RVC/Pig-wt/USA/RV0104/2011/G3PX) was closely related to RVC/Pig-wt/USA/HF/xxxx/G3Px strain (G3) with sequence identity of 94%. The two strains were distantly related to one another with sequence identity of 72% and they were also distantly related to other porcine RVC G-types, human and bovine strains with sequence identities ranging from 72 to 77% as shown in Table 4.2. Phylogenetic analysis (Figure 4.3) that included available VP7 gene sequences of RVCs confirmed that RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX strains belonged to the two distinct porcine RVC genotypes, G3 and G6 strains, respectively and were distantly related to the human (74-77%) and bovine strains (74-77%).

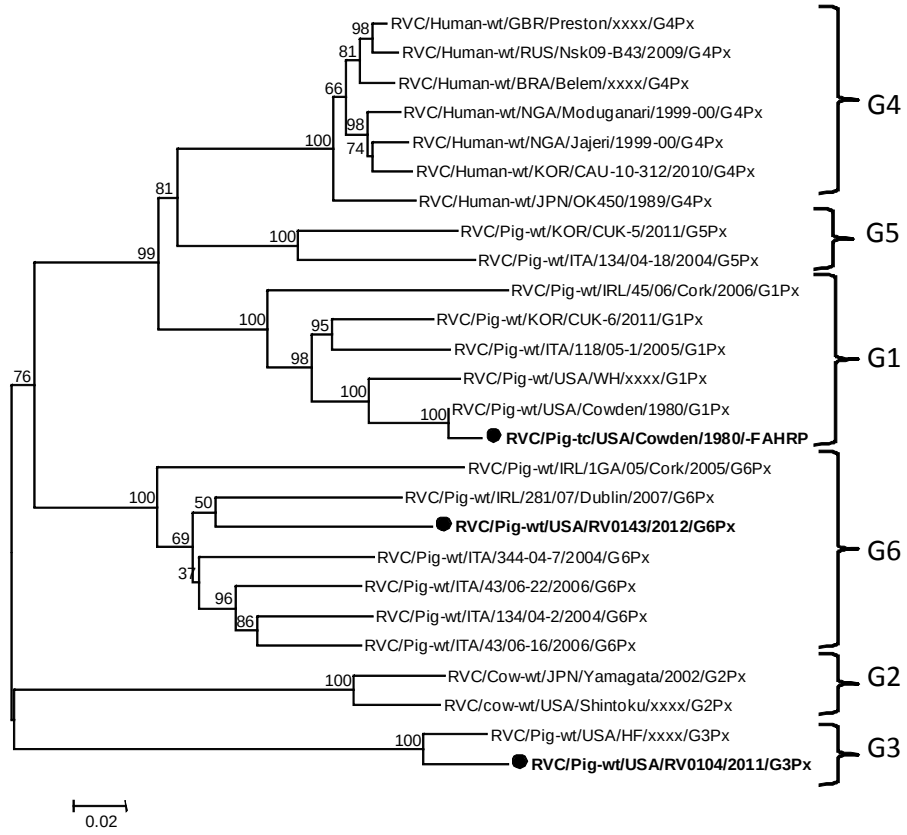

Figure 4.3 Phylogenetic dendrogram of full-length VP7 gene sequences of RVC/Pig-wt/USA/RV0104/2011/G3Px, RVC/Pig-wt/USA/RV0143/2012/G6Px strains and Lab adapted RVC/Pig-wt/USA/Cowden/1980/G1Px strain (bold, dots) compared with VP7 gene sequences of known human, bovine and porcine RVCs available in GenBank

VP4 gene: Partial (602nt) sequence of the VP4 gene (VP8* segment) of the RVC/Pig-wt/USA/RV0143/2012/G6PX strain revealed higher diversity of RVCs, with low sequence identity with porcine (76.3%), bovine (76%) and human (77.4-78.4%) RVC strains (Table 4.3). The human RVC/Human-wt/BGD/BS347/2005/G4Px strain showed higher (78.4%) identity with the RVC/Pig-wt/USA/RV0143/2012/G6PX strain than with bovine and other porcine strains. The partial (311nt) VP4 (VP8*) gene sequence of the RVC/Pig-wt/USA/RV0104/2011/G3PX strain also showed higher diversity, with lower sequence identity to RVC/Pig-wt/USA/RV0143/2012/G6PX strain (71.4%) and bovine RVC/cow-wt/USA/Shintoku/xxxx/G2Px strain (79.1%); however, it was closely related to porcine strain RVC/Pig-wt/USA/Cowden/1980/G1PX(84.2%) and human strains (79.4-83.3%).

A phylogenetic tree was constructed for the RVC reference strains (human and porcine) and the two identified strains (RVC/Pig-wt/USA/RV0143/2012/G6PX and RVC/Pig-wt/USA/RV0104/2011/G3PX). Based on the partial VP4 (VP8*) gene all human strains clustered in a monophyletic branch while the RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX strains were in separate branches in the phylogenetic tree (Figure 4.4). The two strains were distantly related to each other and were in different branches as was also observed for the VP8* segment of the VP4 gene. After sequencing the full length VP4 gene of the RVC/Pig-wt/USA/RV0143/2012/G6PX strain the study observed that it was more closely related to both RVC/Cow-wt/USA/Shintoku/xxxx/G2Px (74.1%) and RVC/Pig-wt/USA/Cowden/1980/G1PX (74.6%) RVC strains than to human strains (70%).

Table 4.3 Sequence identity of partial VP4 (VP8*) gene of selected divergent field strains (RVC/Pig-wt/USA/RV0104/2011/G3Px and RVC/Pig-wt/USA/RV0143/2012/G6Px) compared with VP4 gene sequences for known human, bovine and porcine RVCs available in GenBank

Name	RVC/Pig-wt/USA/RV0104/2011/G3Px (311nt)	RVC/Pig-wt/USA/RV0143/2012/G6Px (602nt)
RVC/Human-wt/KOR/CAU-10-312/2010/G4Px	83.3	78.2
RVC/Human-wt/JPN/Y11-1/2011/G4Px	82.3	78.4
RVC/Human-wt/BGD/BS347/2005/G4Px	79.4	**78.6**
RVC/Human-wt/CHN/208/xxxx/G4Px	82.3	77.4
RVC/Human-wt/NGA/Moduganari/1999-00/G4Px	82.0	77.7
RVC/cow-wt/USA/Shintoku/xxxx/G2Px	79.1	75.9
RVC/Pig-wt/USA/Cowden/1980/G1Px	**84.2**	76.3
RVC/Pig-wt/USA/RV0104/2011/G3Px	-	71.4

For each strain the highest identity to the reference strain is shown in bold

Similarly, the deduced amino acid sequence of VP4 of the RVC/Pig-wt/USA/RV0143/2012/G6PX strain also showed lower identity to known human (69.5-69.9%), bovine RVC/cow-wt/USA/Shintoku/xxxx/G2Px (74.4%) and porcine RVC/Pig-wt/USA/Cowden/1980/G1PX (75.6%) strains (data not shown). Phylogenetic analysis of the full length VP4 gene of the RVC/Pig-wt/USA/RV0143/2012/G6PX strain and reference strains in GenBank confirmed this relationship with the RVC/Pig-wt/USA/RV0143/2012/G6PX, RVC/cow-wt/USA/Shintoku/xxxx/G2Px and RVC/Pig-wt/USA/Cowden/1980/G1PX strains clustering in one branch (Figure 4.5). The fulllength VP4 gene for RVC/Pig-wt/USA/RV0104/2011/G3PX was not obtained due to polymorphic positions in some primer binding sites.

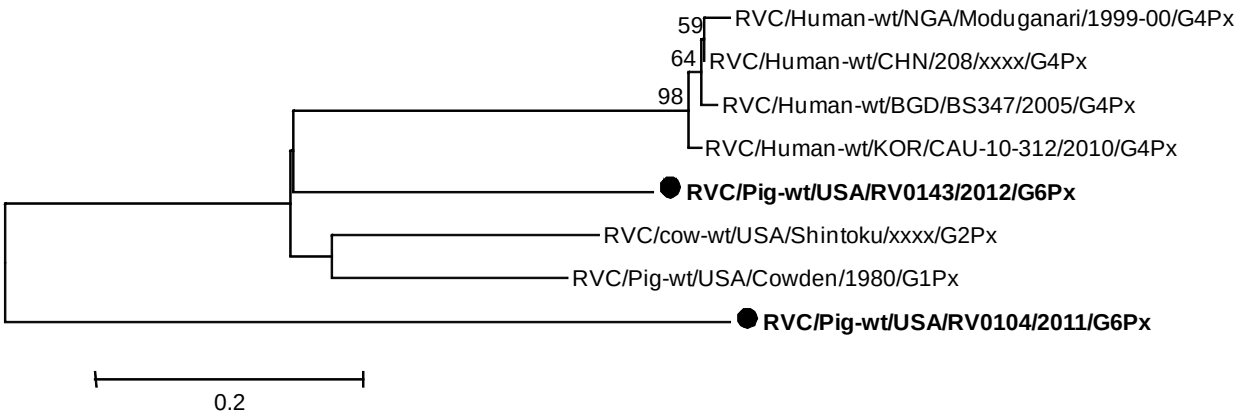

Figure 4.4. Phylogenetic dendrogram of partial VP4 gene (VP8*) of selected field strains (bold, dots) compared with similar segment of VP4 gene sequences for known human, bovine and porcine RVCs available in GenBank

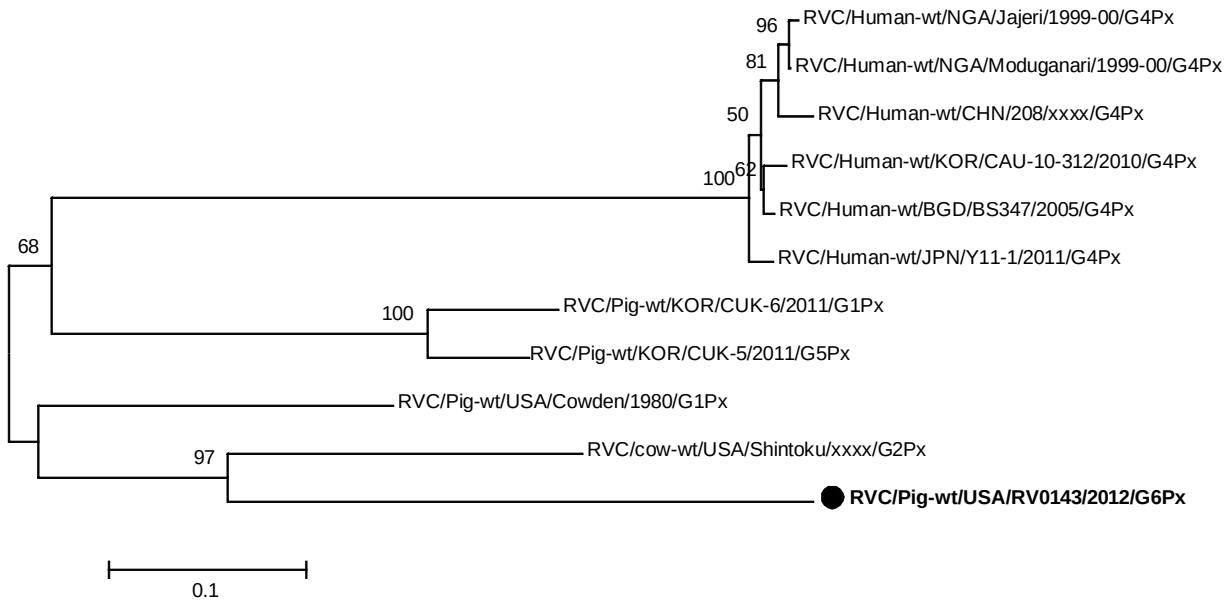

Figure 4.5. Phylogenetic dendrogram of full length VP4 gene of the RVC/Pig-wt/USA/RV0143/2012/G6Px strain compared with VP4 gene sequences of known human, bovine and porcine RVCs available in GenBank. *strains with partial (VP8*) sequences

4.5 Discussion

RVC are a cause of diarrheal disease outbreaks either in nursing or older pigs, leading to high mortality (Kim *et al.*, 1999). Seroprevalence studies of RVCs have also revealed high rates of infection and indicated that the virus is widespread (Terrett & Saif, 1987; Tsunemitsu *et al.*, 1992). However, the information on prevalence and genetic characterization of porcine RVCs is very limited, especially in the US hampering the implementation of control measures specific to RV infections. This study report an overall prevalence of RVCs of 19.5% in fecal samples from 7 swine herds in Ohio, which was slightly lower than the 26.3% reported in S. Korea (Jeong *et al.*, 2009) and 28.7% in Italy (Martella *et al.*, 2007a). However, these prior studies analyzed samples from only diarrheic pigs, whereas this study used samples from both diarrheic and non-diarrheic pigs. Of the 128 samples collected in 2012 the prevalence of RVC among nursing piglets was 23.5%, but only 8.5% among weaned piglets. Interestingly, all the weaned piglets positive for RVC were non-diarrheic, whereas, all nursing piglets with RVC infection were diarrheic. These results indicate that RVC is an important enteric pathogen causing diarrhea more frequently in nursing piglets than weaned piglets as has also been described in other studies (Chang *et al.*, 2012; Kim *et al.*, 1999; Saif & Jiang, 1994). Overall, the high prevalence and distribution of porcine RVCs suggests that they are widespread in nursing piglets from Ohio swine farms, similar to that documented in S. Korea and Italy (Jeong *et al.*, 2009; Martella *et al.*, 2007a). Further studies are required to ascertain the pathogenic potential of RVC in different age groups in swine to be able to institute effective prophylactic measures for prevention and control of RVC infection in swine.

RVCs were detected in most farms sampled (excluding Farm 3 and 5), and the prevalence varied among the farms and between different years within the same farm, indicating that differences in

swine herd management might influence RV prevalence. Overall, porcine RVCs were detected more commonly in summer (26%) followed by winter (19.5%) and then spring (16.4%). Seasonal distribution of RVC infections among the years was not evident from these results because in each year the seasonal prevalence of porcine RVCs differed (Figure 4.2). To date, there is lack of information showing a clear seasonal distribution of porcine RVCs in the US; hence, further epidemiological studies with repetitive sampling throughout the year are required to fully understand the seasonal distribution of this virus.

Porcine RVCs have been detected in diarrheic and non-diarrheic fecal samples from nursing, weaning and post-weaning pigs, either alone or in combination with other enteric pathogens (Collins *et al.*, 2008; Kim *et al.*, 1999; Martella *et al.*, 2007a; Morin *et al.*, 1991; Saif & Jiang, 1994). In this study, 28.4% (19/67) of diarrheic fecal samples collected in 2012 tested positive for RVCs while 6.6% (4/61) of the non-diarrheic fecal samples collected at the same time were positive of RVCs. Consistent with findings of other studies, this study observed that 11% (8/74) of RVC positive samples had mixed infections with groups A and B RVs, suggesting that a number of enteric pathogens, either singly or in combination can augment the clinical presentation of porcine RVC infections (Jeong *et al.*, 2009; Martella *et al.*, 2007a). This argument is supported by the observation that experimental co-infection of calves with group A RVs enhances fecal shedding of a bovine RVC and the extent of histopathological lesions in the small intestine (Chang *et al.*, 1999b).

This study analyzed the fulllength sequences of NSP4, VP4 (VP8*/VP5*) for RVC/Pig-wt/USA/RV0104/2011/G3PX strain), VP6 and VP7 genes of two strains (RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX) to evaluate the genetic relatedness of these cotemporary RVC strains. Initial analysis of the partial (260bp) VP6

gene of six porcine RVCs showed that they share lower nucleotide sequence identity (82.5-86%) with human RVCs which is consistent with previous reports (Collins *et al.*, 2008; Jeong *et al.*, 2009; Martella *et al.*, 2007a).

The intermediate-layer capsid VP6 protein mediates RV group and subgroup specificity. Analysis of the full length VP6 gene of the two RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX strains revealed that they were closely related to each other with nucleotide sequence identity of 99%: however, the genetic distance was variable between these and other known RVCs, ranging from 81% to 91% and they were most closely related to recent RVCs identified in Brazil (91%). Similar genetic variability in the same gene of porcine RVC has been reported in Italy (Martella *et al.*, 2007a), Brazil (Medici *et al.*, 2010) and S. Korea (Jeong *et al.*, 2009; Lee *et al.*, 2011).

The full length sequence analysis of the NSP4 gene of the two field strains showed that they were not closely related to each other (87%): however, they were more closely related to the porcine prototype RVC/Pig-wt/USA/Cowden/1980/G1Px strain (89-93%), a further indication of genetic heterogeneity of RVCs. The two strains were distantly related to human strains which is consistent with a previous report by Chang and co-workers (Chang *et al.*, 1999a) who showed a higher divergence between porcine RVC/Pig-wt/USA/Cowden/1980/G1Px strain and human Bristol strain. The NSP4 protein has an important function as the viral enterotoxin, which causes secretory diarrhea in mice (Ball *et al.*, 1996).

Major serotype-specific neutralization sites have been associated with the VP7 protein of RVs. The VP7 gene sequences of different serotypes revealed extensive sequence conservation among serotypes, which was suggested to reflect structural and functional constraints necessary to preserve the architecture of the VP7 protein (Gunn *et al.*, 1985). To date 27 G genotypes have

been identified for RVAs (Matthijnssens *et al.*, 2011a); however, a formal classification system of RVCs based on the VP7 gene has not yet been established, although 6 G-types (G1-G6) strains have been identified and proposed by researchers using sequence analysis of multiple human and animal RVC strains (Rahman *et al.*, 2005). For RVAs, it was observed that strains having ≥80% nucleotide identity in VP7 gene sequences belong to the same G-genotype (Matthijnssens *et al.*, 2008a). The identity between two field strains (RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX) complete VP7 gene sequences and RVC strains available in GenBank was examined (Table 4.2). The RVC/Pig-wt/USA/RV0143/2012/G6PX strain was most closely related to porcine RVC G6 strains with nucleotide sequence identities ranging from 82% to 86% while RVC/Pig-wt/USA/RV0104/2011/G3PX was closely related to RVC/Pig-wt/USA/HF/xxxx/G3Px strain with nucleotide sequence identity of 94%. Lineages were confirmed by the VP7 gene phylogenetic tree (Figure 4.3). The two strains were distantly related to one another with sequence identity of 72% and also to other porcine G-types, human and bovine strains with sequence identities ranging from 72 to 77%, an indication of greater genetic heterogeneity. Furthermore, the two strains were detected in two different swine herds suggesting high variability among wild-type RVC strains and that divergent strains are widely spread in US swine farms.

The VP4 gene is a multifunctional spike protein with hemagglutinating, neutralizing and fusion activities and antigenic properties (Chang *et al.*, 2012; Estes *et al.*, 1983). To date based on cutoff value of ≥80% nucleotide identity for VP4 gene there are 35 P genotypes of RVAs identitied (Matthijnssens *et al.*, 2011a); however, there is no information on formal classification of RVCs based on differences in the VP4 gene. To date only one porcine RVC (RVC/Pig-

wt/USA/Cowden/1980/G1Px, accession no. M74218) has full length sequence available in GenBank. This study also charaterized the RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX strains based on on their partial VP4 gene (VP8* and VP5* segments) sequences. The VP8* segment of the VP4 gene of the RVC/Pig-wt/USA/RV0143/2012 strains showed higher identity (77.4-78.4%) to human than to bovine (75.9%) and porcine (76.3%) strains, whereas the VP8* segment of the RVC/Pig-wt/USA/RV0104/2011/G3PX strain showed high identity with both porcine (84.2%) and human strains (79.4-83.3%). Although both RVC/Pig-wt/USA/RV0104/2011/G3PX and RVC/Pig-wt/USA/RV0143/2012/G6PX strains showed a closer relationship to human strains, they were distantly related to one another (71.3%). The analysis of the VP5* segment of the VP4 gene showed similar results with nucleotide sequence identity between the two strains (78%) and the known human (71-72%), bovine (78%) and porcine (76%) strains below the recommended cutoff value (80%) (data not shown). In the phylogenetic tree of the partial VP4 gene (VP8* and VP5* segments), all human RVCs clustered in one branch, an indication that they originate from a common ancestor. These results suggest that there might be interspecies transmission of RVCs; however, this is unclear and requires further studies to acertain the interspecies transmission of porcine RVCs. Other RVCs are thought to be associated with interspecies transmission events (Chang *et al.*, 1999b; Gabbay *et al.*, 2008; Jeong *et al.*, 2009; Martella *et al.*, 2007b; Martella *et al.*, 2008). For example, Iturriza-Gomara *et al*, 2004 reported increasing seroprevalence rates to RVCs in human populations living in rural areas.

Analysis of the fulllength nucleotide sequence of the VP4 gene of the RVC/Pig-wt/USA/RV0143/2012 and other RVC strains revealed that the RVC/Pig-wt/USA/RV0143/2012 VP4 gene is ~~more~~ closely related to that of bovine RVC/cow-wt/USA/Shintoku/xxxx/G2Px

(74.1%) and porcine RVC/Pig-wt/USA/Cowden/1980/G1PX(74.6%) strains than to human (70-70.4%) strains. Similarly, analysis of the deduced amino acid sequence identity between the full length VP4 of the RVC/Pig-wt/USA/RV0143/2012/G6Px strain and VP4 of the known human, bovine and porcine RVC strains revealed that the RVC/Pig-wt/USA/RV0143/2012/G6Px strain shares less than 76% amino acid sequence identity with VP4 of the known human, bovine and porcine RVC strains (69.5-69.9%, 74.4% and 75.6%, respectively). Based on the cutoff values for RVAs of ≥80% nucleotide identity (Matthijnssens *et al.*, 2008b) and ≥89% deduced amino acid identity (Ciarlet *et al.*, 1997; Estes, 2001) for new VP4 genotypes, the nucleotide and deduced amino acid identity between strain RVC/Pig-wt/USA/RV0143/2012/G6Px and the known RVC strains was below these cutoff values. Therefore, the study propose that the RVC/Pig-wt/USA/RV0143/2012/G6Px strain should represent a new RVC VP4 genotype.

In summary, this study demonstrates that porcine RVC are widespread and genetically diverse in swine herds in Ohio, US, which poses challenges for development of diagnostic and prophylactic tools for prevention of RV infections. Therefore, detailed molecular epidemiological surveillance is needed for a better understanding of the diversity of RVCs and their role in diarrhea in swine of various ages. Information acquired from full length sequences of NSP4, VP6 and VP7 and VP4 genes in this study will be useful in the development of accurate diagnostic tools and also in basic research for understanding the functions of these genes. Additionally, the data generated in this study will be helpful in understanding the pathogenic potential and diversity of the current porcine RVCs.

CHAPTER 5

5 DETECTION AND GENETIC DIVERSITY OF PORCINE GROUP A ROTAVIRUSES IN HISTORIC (2004) AND RECENT (2011/12) SWINE FECAL SAMPLES IN OHIO, USA: PREDOMINANCE OF G9P[13] GENOTYPE IN NURSING PIGLETS

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5.1 Abstract

Epidemiological surveillance of porcine group A rotavirus (RVA) strains was conducted in 5 swine herds in Ohio, USA using historical (2004) and recent (2011-2012) fecal samples. Of the 371 samples examined, 9.4% (35/371) were positive for RVA. The RVA detection rates increased from 5.9% in 2004, 8.5% in 2011 to 13.8% in 2012. A total of 23 positive samples were analyzed for RVA G and P genotypes. The dominant G-P combination was G9P[13] found in 60.9% of positive samples. The other combinations were G9P[7] (8.7%), G4P[13] (8.7%), G11P[13] (4.3%) and G11P[7] (4.3%). Sequence analysis of partial VP7 genes of selected strains revealed that the G4 strains were closely related to one another (95%) and to a lower extent to human (82-84%) and porcine (84-86%) G4 strains. The G11 strains detected shared identical VP7 gene sequence (100%) and were closely related to human (85-86%) and other porcine (83%) G11 strains. The G9 strains identified were closely related to one another, human and other porcine strains (96-97%, 89-91% and 89-91% nucleotide identities, respectively). The VP4 gene analysis revealed that P[7] strains were closely related to each other and to P[7] strains

isolated from porcine, bovine and panda (91-99%, 92-99% and 92-99%, respectively). The P[13] strains showed a higher diversity among themselves and with other porcine P[13] strains ranging from 83% to 99% and 82-97%, respectively. The results obtained in this study demonstrate broad genetic heterogeneity of the RVA strains and suggest the possibility of genetic reassortment between different RVA genotypes within these farms.

5.2 Introduction

Gastroenteritis in pigs is a multifactorial and multi-etiological disease. Management differences may play an important role in development of the disease, in addition to coinfection by microorganisms such as bacteria, protozoa and viruses (Katsuda *et al.*, 2006; Wittum *et al.*, 1995). Viral diseases of swine, such as coronavirus [Transmissible Gastoenteirits virus (TGEV)/Porcine Epidemic Diarrhea virus (PEDV)], circovirus type 2 (PCV2), rotavirus (RV), swine influenza and porcine respiratory and reproductive syndrome virus (PRRSV) infections represent continuing challenges for the swine industry, increasing costs of treatment and prevention.

Rotaviruses are a major cause of viral gastroenteritis in children and young animals worldwide (Parashar *et al.*, 2006). RV infections cannot be diagnosed solely based on clinical signs, because they do not differ from those caused by other intestinal pathogens. Laboratory tests are necessary for reliable RV diagnosis and surveillance, and can be useful in clinical settings to avoid inappropriate use of antibiotics that cannot prevent viral diarrheas. Large numbers of excreted viral particles during RV infection, a wide range of susceptible species, resistance to environmental conditions and disinfectants, and the possibility of concurrent infections with different RV strains leading to new virus strains, all contribute to RV prevalence and genetic diversity (Chang *et al.*, 2012; Collins *et al.*, 2010; Estes & Kapikian, 2010).

Rotavirus is a genus of the *Reoviridae* family of dsRNA viruses, having a genome of 11 segments of dsRNA encoding 6 structural (VP1-VP4, VP6 and VP7) and 6 nonstructural viral proteins (NSP1-NSP5/6). The RVs are classified into 7 groups (Gp) (A–G) based on antigenic relationships of their VP6 protein (Kapikian, 2001). Groups A, B and C RVs infect humans and animals with RVA usually in highest prevalence (Chang *et al.*, 2012). Based on differences in the outer capsid VP7 and VP4 proteins that contain epitopes that independently induce neutralizing antibodies, RVs are classified into G and P types, respectively. Within RVAs there are 27 G genotypes and 35 P genotypes identified to date (Matthijnssens *et al.*, 2011). Twelve G genotypes (G1 to G6, G8 to G12 and G26) and 13 P genotypes (P[1], P[5] to P[8], P[11], P[13], P[19], P[23], P[26], P[27], P[32] and P[34] have been associated with pigs (Collins *et al.*, 2010; Martella *et al.*, 2007a; Matthijnssens *et al.*, 2011; Steyer *et al.*, 2007). However, G3, G4, G5 and G11 are the most common G genotypes identified in swine and are usually associated with P[5], P[6] and P[28]; the others are considered less common genotypes in swine (Matthijnssens *et al.*, 2008; Okitsu *et al.*, 2011; Wang *et al.*, 2010).

Porcine RVs are not only an important enteric pathogen because of their economic impact on swine production in terms of mortality and morbidity, but they are a potential source of heterologous RV infections in humans and cattle (Ghosh *et al.*, 2007b; Martella *et al.*, 2010). Knowledge of the molecular epidemiology of RVAs is crucial for preventing or reducing the incidence of porcine RVA diarrhea as well as transmission of porcine RVA to humans. Since protection is lower to heterologous strains, understanding the genetic diversity of RVAs is critical for design of effective prophylactic tools including vaccine development, optimization and improvement. This paper reports the prevalence as well as genetic diversity of RVAs in

historical (2004) and recent (2011 & 2012) fecal samples from 5 swine farms located in the state of Ohio, USA.

Table 5.1 The year and number of samples collected, pig ages and diarrhea status

Year of collection, total # of samples	Farm ID*/location	Month samples collected in	Number of samples	Diarrhea status	Age**
2004, n=118	Farm 1 (Central OH)	April	n=32	N/A***	<3 weeks
	Farm 2 (NW OH)	July	n=31	N/A	<3 weeks
	Farm 2 (NW OH)	December	n=40	N/A	<3 weeks
	Farm 3 (Central OH)	May	n=15	N/A	<3 weeks
2011, n=130	Farm 2 (NW OH)	July	n=39	N/A	N/A
	Farm 2 (NW OH)	December	n=20	N/A	<3 weeks
	Farm 4 (NE OH)	June	n=37	N/A	<3 weeks
	Farm 5 (NE OH)	April	n=34	5 diarrhea; others no diarrhea	<3 weeks
2012, n=123	Farm 2 (NW OH)	January	n=8	diarrhea	<3 weeks (n=4), gilts (n=4)
	Farm 2 (NW OH)	March	n=60	most no diarrhea (only 6 with diarrhea)	<3 weeks (n=17), >3 weeks (n=43)
	Farm 2 (NW OH)	May	n=55	most diarrhea (only 5 with no diarrhea)	<3 weeks

* within same year, each farm # appears as many times as it was sampled; ** <3weeks - nursing piglets, >3 weeks - weaned piglets; *** N/A - not available;
Central OH – Central Ohio, NW OH – North-western Ohio, NE OH – North-eastern OH.

5.3 Materials and Methods

5.3.1 Sample

A total of 371 fecal samples were collected in 2004 (n=118), 2011 (n=130) and 2012 (n=123) from 5 selected swine farms in Ohio, USA for detection of RVAs (Table 5.1). Samples collected in 2004 were all from nursing piglets with no information about diarrheal status (Wang *et al.*, 2006), whereas for the 2011, samples age information was missing for 39 samples from Farm 2 and diarrhea status was not available for 96 samples. However, samples collected in 2012 were from both nursing (n=76) and weaned (n=47) piglets and they were collected from piglets with diarrhea (n=62) and without diarrhea (n=61). Fecal samples (feces or swabs) were collected from individual piglets and placed in labeled sterile plastic tubes and transported to the Laboratory on ice. Ten percent fecal suspensions (wt/vol) were prepared in Minimum Essential Medium (Life Technologies, NY, USA), vortexed and centrifuged at 1,800 x g for 30 min at 4^0C. The clarified supernatants were used for RNA extraction. None of the farms surveyed used the RVA vaccine (ProSystem Rota, Intervet Inc., Merck Animal Health) licensed for swine in the US.

5.3.2 RNA Extraction

RNA was extracted from 250µl starting volume of centrifuged 10% fecal suspensions using RNeasy mini kit from Qiagen Company (CA, USA) according to the manufacturer's instructions. The total RNA recovered was suspended in 40µl of nuclease-free water and stored at -70 ^{0}C until used.

Table 5.2. Primer sets used for RVA detection and genotyping

Genotypes	Name	Sequence	Region	Amplicon (bp)	Reference
Detection primer set	NSP3F	ACCATCTACACATGACCCTC	963–982	87	(Pang *et al.*, 2004)
	NSP3R	GGTCACATAACGCCCC			
Common VP7 gene	5'comVP7-F	GGCTTTAAAAGAGAGAATTTC	1-1062	1062	(Winiarczyk *et al.*, 2002)
	3'comVP7-R	GGTCACATCATACAATTCTAA			
G3	G3Fdeg	GAYAATTCRTGGAARGATACACTT	331-782	452	This study
	G3Rdeg	GTACAAGTRTTTGTYGTCACRYTCA			
G4*	JA-AV-G4-Fdeg	ACD TTA GGA RTY GGA TGT CA	655-1062	407	This study
G5 *	JA-AV-G5-Rdeg	YTT WKY RTC YGC RAT TTC	1-339	339	This study
G9	G9-VP7-4F	ACA GCA TAT GCA AAT TCG TCA CAG	241-388	148	This study
	G9-VP7-4R	CAG TTG GCC ACC CCT TAG TCA			
G11	JA-G11Fdeg	GAYGAYAAGTGGARAGATACTCTC	331-589	259	This study
	JA-G11Rdeg	CYTCATYWGTYTGTTGATAATARTAAAGA			
Universal VP4 gene	793F	TGG AAA GAA ATG CAR TAY AA	793-1583	812	This study
	1583R	CCH GAR AAC ATN GAR AAC ATA TC			
Universal VP7 gene	VP7-39F	GCTCYTTTTRATGTATGGTATTGAATATA CCAC	39-406	368	(Distefano *et al.*, 2005)
	VP7-406R	CTT TAA AAT ANA DGA DCC WRT YGG CCA			

*For G4 genotyping, common primer 3'comVP7-R was used as reverse primer, while for G5, common primer 5'comVP7-F was used as forward primer.

5.3.3 Detection of RVA

Conventional reverse transcription-polymerase chain reaction (RT-PCR) was used for detection of the RVAs with validated primer sets, NSP3F (963–982) and NSP3R (1,049-1,034), (Table 5.2) using Promega reagents according to the manufacturer's instructions (Promega, CA, USA). Prior to reverse transcription, 2µl of RNA sample was mixed with 0.5µl of Dimethyl sulfoxide (Sigma-Aldrich, MO, USA), heated at 97^0C for 5 min, then snap chilled on ice, to denature the double stranded segments of the RV genomic RNA. For the RT-PCR reaction the following conditions were applied: incubation for 30 min at 50^0C for RT reaction, preheating the sample at 94^0C for 5 min for initial denaturation followed by 40 PCR cycles at 94^0C for 15 sec, 56^0C for 1 min and 72^0C for 30 sec, then a final extension for 7 minutes at 72^0C and then 4^0C for storage until removed from the machine. The PCR products from the above reaction were analyzed in a 3% Agarose gel after staining with EZ-Vision dye (Amresco) and UV-light transillumination. The amount of PCR inhibitors in the samples were determined to be negligible based upon results obtained by making 10-fold and 100-fold dilutions of each RNA sample.

A chi-square test was used assess the relationship between several factors (farm, year, season, age group and diarrheal status) and the RVA detection using procedure frequency in the SAS computer program (SAS, 2002).

5.3.4 Genotyping and genetic analysis of RVA strains

Using the known human, porcine and bovine RVA VP7 gene sequences available in GenBank, genotype specific primers was designed in this study (Table 5.2) for common G genotypes found in swine (G3, G4, G5, G9 and G11) to genotype the RVA positive samples directly without sequencing. The specificity of these G-typing primers was evaluated using a panel of well

characterized laboratory reference strains consisting of human RVA strains Wa (G1), DS-1 (G2), M-strain (G3), 69M (G8) and a characterized human G9P[6] strain obtained from the CDC and the porcine RVA strains Gottfried (G4) and OSU (G5). All genotype specific one-step RT-PCR reactions were conducted individually with each primer pair, similarly to the above RVA detection RT-PCR protocol but with varying annealing temperatures and extension times. Selected genotyped samples and the samples that could not be genotyped using the above primers were subjected to RT-PCR and partial sequencing using a universal primer set designed previously for the VP7 gene (Distefano *et al.*, 2005) to determine the G types for all strains and perform phylogenetic analysis. P types were determined using a universal primer pair (793F/1583R) for RT-PCR and partial sequencing of the RVA VP4 gene (793-1604nt region, this study).

The nucleotide sequences obtained from the selected strains were compared with the same segment of similar sequences of known strains available in GenBank using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The DNA sequences were aligned using the ClustalW method. The dendrograms were constructed using the neighbor-joining method supported with a bootstrap test of 1000 replicates for each gene in MEGA 5 software (Tamura *et al.*, 2011). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [2] and are in the units of the number of base substitutions per site. The P genotypes were assigned to each sample after sequence analysis using either BLAST (blastn) search (http://www.ncbi.nlm.nih.gov/) and/or **RotaC**$^{\mathbf{2.0}}$ automated genotyping tool for Group A RVs (Maes *et al.*, 2009).

The GenBank accession numbers of all field strains and representative known RVA strains for each gene mentioned in this study are listed in **Appendix 3.**

5.4 Results

5.4.1 Incidence of RVA

A total of 371 samples from five swine herds were tested and 9.4% (35/371) were positive for RVAs (Table 3). In comparison with screening results for groups B (RVB), C (RVC) RVs and porcine sapoviruses (SaV), this study found that 25.7% (9/35) of RVA positive samples also contained either RVB or RVC or SaV. There were significant defferences in RVA detection among the farms ($p<0.01$) and the years ($p<0.05$) studied. The prevalence of RVAs varied among farms (0-18.9%) and between different years within the same farm, such as seen in Farm 2, ranging from 1.4% to 13.8% (Table 5.3). The RVA were detected in four out of the five farms sampled. An increase in RVA detection was observed from 5.9% in 2004, 8.5% in 2011 to 13.8% in 2012. There were no significant differences ($p>0.05$) in RVA detection rates between the seasons during the years surveyed. Overall, the prevalence of RVAs was higher in spring (11.1%), followed by summer (8%) and then winter (6.9%). However, RVA detection rates in seasons differed within each year: in 2004, the highest prevalence occurred in spring (13%), while in 2011, summer (11.6%) had the highest prevalence and in 2012, the detection rate was highest in winter (18.2%) as shown in Table 5.3.

The relationship between RV detection and diarrhea was evaluated in 123 samples collected in 2012 since information about the diarrheal status was not available for samples collected in 2004 and 2011. Although the diarrhea rate was higher in the RVA positive pigs, diarrheal status had no significant influence ($p>0.05$) on RVA incidence in the surveyed farms. RVA was detected in

17.7% (11/62) and 9.8% (6/61) of the diarrheic and non-diarrheic piglets, respectively (Table 5.3). Additionally, of the 123 samples collected in 2012, RVA was detected significantly ($p<0.01$) more often in nursing piglets (21.1%, 16/76) than in weaned piglets (2.1%, 1/47) as shown in Table 5.3. Within the nursing piglets, the study showed that RVA detection was higher in piglets without diarrhea (27.8%) than in piglets with diarrhea (19%), whereas in weaned piglets, RVA was detected in only one sample from an asymptomatic piglet.

5.4.2 Assessment of the specificity of gene specific primers for G genotyping

The specificity of the G-typing primers designed in this study (for G3, G4, G5, G9 and G11) was evaluated. There were no cross-reactions among the genotypes tested; hence, these primers were used for the G typing of RVAs. However, it is important to note that these primers were used separately in a single reaction for the detection of specific genotypes.

5.4.3 Distribution of G and P genotypes

The results indicated that several genotypes of RVA are currently circulating in OH swine. Of the 35 RVA positive samples, 23 were further characterized by G and P typing and 12 samples were of insufficient quantity/quality for further molecular characterization. The G types were determined for 95.7% (22/23) of the samples using genotype specific primers designed in this study (Table 5.2), whereas one sample was G genotype untypeable. The P types were determined for 91.3% (21/23) of the samples, whereas 2 samples were untypeable (Table 5.4). The study used a combination of BLAST (blastn) search, phylogenetic tree analyses (MEGA 5) and RotaC$^{2.0}$ web based tool (Maes *et al.*, 2009) analysis of the partial sequence of VP7 and VP4 genes of the 23 samples with those of established RVA genotypes.

Table 5.3 Summary of RVA prevalence in the Ohio swine herds during the study period

Details		N	Prevalence (%)	Details		N	Prevalence (%)
	Overall	371	9.4				
Farms**	Farm 1	32	15.6	2004	Spring	46	13.0
	Farm 2	253	8.7		Summer	31	0.0
	Farm 3	15	6.7		Winter	41	2.4
	Farm 4	37	18.9	2011	Spring	41	2.4
	Farm 5	34	0.0		Summer	69	11.6
Year*	2004	118	5.9		Winter	20	10.0
	2011	130	8.5	2012	Spring	112	13.4
	2012	123	13.8		Winter	11	18.2
Seasons[ns]	Spring	199	11.1	Farm 1	2004	32	15.6
	Summer	100	8.0	Farm 2	2004	71	1.4
	Winter	72	6.9		2011	59	6.8
Diarrheal status[a,ns]	Diarrhea	62	17.7		2012	123	13.8
	Non-diarrhea	61	9.8	Farm 3	2004	15	6.7
Age group[a**]	Nursing	76	21.1	Farm 4	2011	37	18.9
	Weaned	47	2.1	Farm 5	2011	34	0.0

[a] Information on diarrheal status and age group was only available for 123 samples collected in 2012;
*prevalence rate differed significantly $p<0.05$; **=$p<0.01$; ns= did not differ significantly.

Table 5.4 Combinations of G and P genotypes of 23 porcine RVAs identified in USA farms

Genotypes	G4	G9	G11	Unknown	Total
P[7]	0	2 (2012)	1 (2004)	0	**3**
P[13]	2 (2011)	14 (n=11, 2011; n=3, 2012)	1 (2004)	1	**18**
Unknown	0	2 (n=1, 2011; n=1, 2012)	0	0	**2**
Total	**2**	**18**	**2**	**1**	**23**

Using G type specific primers or an established nucleotide cut-off value of 80% for delineation of VP7 and VP4 genotypes (if universal primers followed by direct sequencing was used) (Matthijnssens *et al.*, 2008), the 23 strains were classified into 3 G-genotypes (G4, G9 and G11) in combination with two P-genotypes (P[7] and P[13]) that are either commonly or exclusively associated with porcine RVAs. Among the G types, G9 was predominant in 78% of the RVA positive samples, while G4 and G11 each were detected in 9%. The P[13] genotype was dominant among the P types, constituting 78% of the RVA positive samples, followed by P[7] (13%). Five G-P type combinations were identified, with G9P[13] being the dominant combination (61%) followed by G9P[7] and G4P[13] at 9% each, while G11P[7] and G11[13] were identified in one sample each (4%). Two samples were untypeable for P type and one sample was untypeable for G type (Table 5.4). The G9 genotype was detected in all the 3 years while G4 and G11 genotypes were only detected in 2011 and 2004 samples, respectively. The P[13] genotype was detected in all the years studied while P[7] was detected only in 2004 and 2012.

5.4.4 Sequence analysis of the partial VP7 gene of G4, G9 and G11 porcine RVAs

From the 22 strains that were G typed, 7 randomly selected strains (two G4, three G9 and two G11, from Farms 1, 2 and 4, from 2004 and 2011) were sequenced for further characterization of the partial VP7 gene (316nt) and the assessment of the genetic identity within each G type. The two G4 strains [RV0120 and RV0132 (Farm 2)] detected in 2011 were closely related to one another (95%) and they formed a separate branch in the phylogenetic tree (Figure 5.1). The RV0120 and RV0132 strains displayed 79-85% and 82-87% nt identity with G4 human, and 77-82% and 77-85% with porcine RVAs, respectively, with the highest nt identity (85%-87%) to human RV D151 identified in Mexico. Interestingly, the two strains showed the lowest nt

identity (77%) to porcine G4 strain 1/07/Ire identified in Ireland. Three G9 strains [RV0036 (Farm 4), RV0084 and RV0146 (Farm 2)] detected in 2011 and 2012 were closely related to each other with 90-97% nt identity. They were also closely related (89-91%) to both human and porcine G9 RVAs. However, they formed a separate branch in the phylogenetic tree (Figure 5.1). Two strains [QW359 and QW362 (Farm 1)] from historical samples (2004) that were characterized as G11 strains were identical to each other, with 100% nt identity. They displayed 85-86% nt identity with human G11 and 83% nt identity with other G11 porcine RVAs. Both the QW359 and QW362 strains clustered in a monophyletic branch in the phylogenetic tree (Figure 5.1).

5.4.5 Sequence analysis of the partial VP4 gene of P[7] and P[13] porcine RVAs

The VP4 type was determined for 21 of the 23 RVA samples. A BLAST search for the 21 strains identified them as P[7] or P[13] types. A phylogenetic tree was constructed using the partial VP4 gene sequences (635bp) of the 14 selected strains identified in this study and those of the existing 35 P genotypes in GenBank (Figure 5.2). The quality of 7 samples was insufficient for sequence analysis. The three P[7] strains shared high nt identity (91-100%) with one another and with other P7 porcine strains. Two P[7] strains (QW362 and RV0161) clustered in a monophyletic branch together with the OSU (X13190) strain which was identified in the US, while the RV0263 strain clustered with the PRG9121 (JF796737) strain detected in South Korea (Figure 5.2). Additionally, BLAST (Blastn) search and ClustalW analysis of the VP4 segment of the P[7] field strains demonstrated that they were closely related (92-99%) to bovine RVA strains (KJ25-1_HM988968, KV0407_EU873009) isolated in South Korea and to porcine-like RVA strain CH-1 (HQ641296) detected in a panda in China.

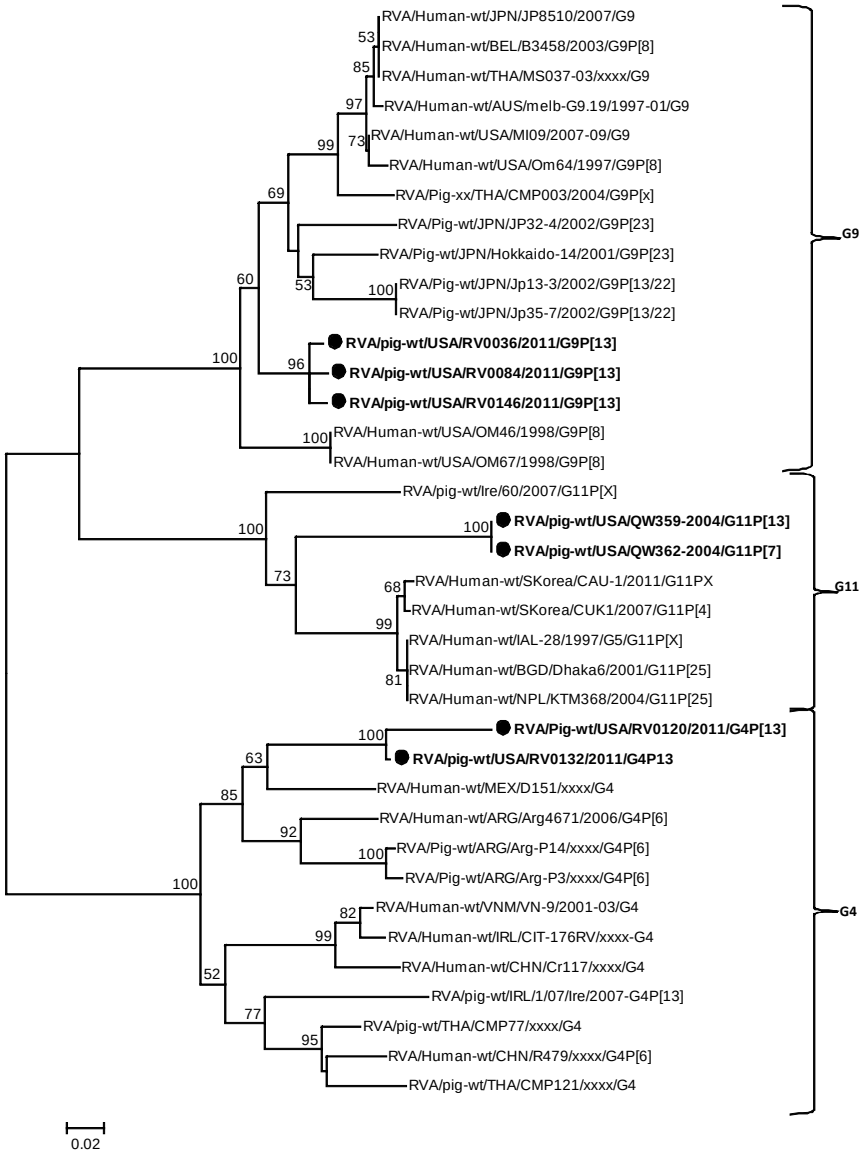

Figure 5.1 Phylogenetic dendrogram of the partial (73-388nt) sequence of RVA VP7 gene of field strains (bold) compared with available VP7 gene sequences for human and porcine RVA G genotypes. Boostrap values less than 50% are not shown.

The P[13] genotypes appeared to be genetically diverse in the surveyed farms. The phylogenetic analysis of the partial VP4 gene showed 5 genetic sublineages of the P[13] genotype: one sublineage formed by strains identified in Japan; a second sublineage by strains from Thailand and India; a third sublineage by a strain from S. America; a fourth sublineage branch was formed by an Australian strain; and the fifth new sublineage was formed by some of strains detected on this study (Figure 5.2). Analysis of P[13] strains identified in this study and VP4 gene sequences available in GenBank, showed that they were from porcine RVAs sharing variable nt identities amongst themselves of 81%-99%. On the phylogenetic tree, they clustered together with other porcine P[13] strains, forming 3 distinct lineages: i) India/Thailand Lineage (HP113 and CMP213); ii) Japanese Lineage (FGP28); and iii) a new lineage- USA strains (Figure 5.2). Upon analysis using the RotaC$^{2.0}$ tool, a close relationship was also observed between the P[13] strains detected in this study and a number of P[13] porcine strains of which strain A46 (AY050274) shared the highest nucleotide identity (83.9-86.1%). On the other hand, another close relationship was found between P[7] strains of which strain OSU (X13190) shared the highest nt identity (92-99.6%) .

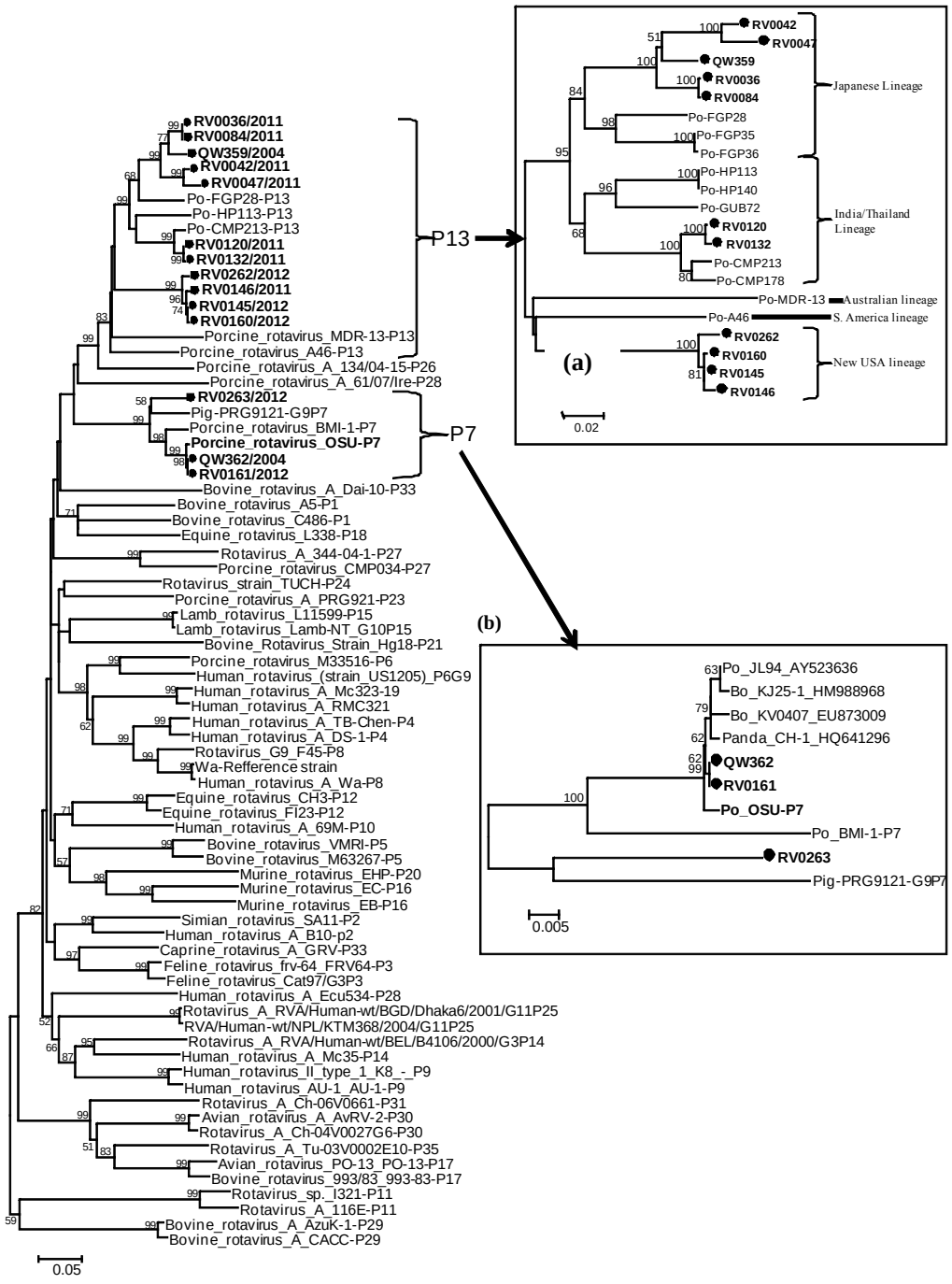

Figure 5.2 Phylogenetic dendrogram of the partial (859-1493nt, 635bp) nt sequence of RVA VP4 gene of field strains (bold) compared with available VP4 gene sequences for RVA P genotypes. Inset Figures a and b show the tree for P[13] and P[7] strains, respectively. Porcine OSU strain (**bold**) is the prototype for P[7] genotype (Bo=bovine; Po=porcine)

5.5 Discussion

Group A RVs are an important cause of acute gastroenteritis in children and young animals. Epidemiological information related to the prevalence and genotype specificities of porcine RVAs are beneficial for the development of accurate diagnostic tools and effective prophylactic measures against RVA infections in swine and to survey RVA strains closely related to human strains with potential for zoonotic transmission. In this study, evidence was collected for the occurrence of RVAs in nursing and weaned piglets in US pig farms, and the genetic heterogeneity among the strains identified was examined. The RVAs were detected in 9.4% of the fecal samples from four of the five swine herds over the 3 years of the study, suggesting the continual circulation of RVA in swine farms. Surprisingly, no RVA strains were detected from Farm 5. The prevalence of RVAs (9.4%) in this study was low compared to previous reports: 63.6% in USA (Marthaler *et al.*, 2012) 26% in Spain (Halaihel *et al.*, 2010), 38.3% in S. Korea (Kim *et al.*, 2010), 67.3% in Japan (Katsuda *et al.*, 2006), 17.2% (2009-2010) and 22.3% (2000-2001) in Thailand (Khamrin *et al.*, 2007; Saikruang *et al.*, 2012), 17% in Canada (Lamhoujeb *et al.*, 2010), 35.3% in Brazil (Racz *et al.*, 2000) and 25.7% in India (Kusumakar *et al.*, 2010). However, the RVAs prevalence reported here was higher than that reported in pre-weaned piglets in Argentina, 3.3% (Parra *et al.*, 2008), in suckling and weaned piglets in Germany, 4%, (Wieler *et al.*, 2001) and in weaned asymptomatic piglets in Ireland, 6.5% (Collins *et al.*, 2010). These results were similar to the prevalence reported in Denmark, 10% (Midgley *et al.*, 2012), and between 2006-2008 in Thailand, 10.7% (Okitsu *et al.*, 2011) and in Canada, 9.2% (Morin *et al.*, 1983). It is noteworthy that the above reports affirm variable RVA prevalence rates in the same geographic regions in different years. Consistent with findings by others, this study observed that 25.7% (9/35) of RVA positive samples, all from symptomatic nursing piglets, were

mixed infections with RVB, RVC or SaV (Wang *et al.*, 2006), suggesting that a number of enteric pathogens, either singly or in combination may have synergistic effects on the clinical course of porcine RVA infections (Jeong *et al.*, 2009; Martella *et al.*, 2007b; Marthaler *et al.*, 2012; Médici *et al.*, 2011). In contrast, RVA infections alone in nursing pigs receiving sow colostral and milk antibodies to RVA, may cause only transient diarrhea that may have subsided at the time of sample collection in this study. Recently, Marthaler et al (Marthaler *et al.*, 2012) reported higher mixed infection rates (86.4%) of RVA, RVB and RVC in piglets in the US; however, they examined only diagnostic diarrheic pig samples.

There was significant difference in the RVAs detection rate among the 5 farms ($p<0.01$) and 3 years ($p<0.05$) studied. An increase in RVA detection was observed from 5.9% in 2004 to 13.8% in 2012; however, the seasonal influences ($p>0.05$) on the prevalence of RVA infections were unclear among the years sampled (Table 3). To date there is a lack of information showing a clear seasonal distribution of porcine RVAs in the US and also globally; hence, further epidemiological studies are required to fully understand the seasonal pattern of RVA to implement effective intervention or management strategies for the porcine RVA infections.

Porcine RVAs have been detected in diarrheic and non-diarrheic fecal samples from nursing and post-weaning pigs either alone or in combination with other enteric pathogens (Collins *et al.*, 2008; Kim *et al.*, 1999; Martella *et al.*, 2007a; Morin *et al.*, 1991). In this study RVAs was detected in 17.7% and 9.8% of the diarrheic and non-diarrheic fecal samples, respectively, in 2012. Asymptomatic infections with RVA in swine have been reported in previous studies (Collins *et al.*, 2010; Midgley *et al.*, 2012; Parra *et al.*, 2008). The prevalence of RVAs in asymptomatic piglets observed in this study (9.8%) was slightly higher than that reported in Spain (7%) (Halaihel *et al.*, 2010) and Ireland (6.5%) (Collins *et al.*, 2010). However, a higher

prevalence of RVA in asymptomatic pigs was reported in Slovenia, 20% (Midgley *et al.*, 2012) and Italy, 71.5% (Martella *et al.*, 2007b). When the data obtained were compared with diagnostic results for RVB and RVC, RVA were detected more frequently (9.8%) in asymptomatic pigs than RVC (6.6%) and RVB (0%) RVs (unpublished data, 2013). This study also revealed that RVA are more frequently detected in nursing piglets (21.1%, 16/76) than in weaned piglets (2.1%, 1/47), which is consitent with prior reports (Chang *et al.*, 2012; Collins *et al.*, 2010). Among the nursing piglets, RVA was more often detected in asymptomatic (27.7%, 5/18) than in symptomatic (19%, 11/58) piglets, although due to low sample numbers the differences were not statistically significant. Higher maternal immunity levels (Hodgins *et al.*, 1999), the circulation of naturally attenuated RVA strains (Iosef *et al.*, 2002; Nguyen *et al.*, 2003) or RV shedding before the onset or after the resolution of diarrhea could account for the occurrence of RVA infections in asymptomatic animals.

The VP4 and VP7 encoding genes have different levels of nucleotide conservation since they are targets of host neutralization antibodies. Thus evaluating the differences in terms of pathogenicity, virulence and the nucleotide diversity in these genes is important in understanding RV infections and the prediction of efficiency of vaccines (Gregori *et al.*, 2012). This study also investigated the genetic heterogeneity of RVA circulating in swine farms in Ohio, USA. The G9 genotypes were detected at higher levels (78%) which was consistent with the recent study in Thailand (64.3%) and Spain (33%) where it was found to be the most common genotype (Midgley *et al.*, 2012; Okitsu *et al.*, 2011). However, this was in contrast with a recent study in Denmark and Slovenia (Midgley *et al.*, 2012) and Korea (Kim *et al.*, 2010) where G9 was detected at low levels (4% and 9.2%, respectively) in swine. The P[13] genotype was the most common P type (78%) in the US swine farms surveyed. The P[13] genotypes showed higher

diversity among themselves, forming 3 different sublineages, one sublineage clustering with the India/Thailand strains, and another sublineage clustering with the Japanese strains while the third group clustered separately on their own branch. To my knowledge this is the first time RVA P[13] has been reported in US swine at this high level. The RVA strains bearing P[13] VP4 specificity are host restricted, commonly detected among pigs and have not been identified from other animal sources or humans (Chan-It *et al.*, 2008; Estes, 2001; Ghosh *et al.*, 2007; Teodoroff *et al.*, 2005). Recent studies have reported P[6] genotype as the most common P type in Slovenia (41%) and Spain (56%) in Europe (Midgley *et al.*, 2012). Three samples analyzed was P[7] genotypes, two associated with G9 and one with G11 genotypes. Interestingly, regardless of the G genotypes, P[7] genotypes were phylogenetically closer to each other and to P[7] genotypes detected in swine, cattle and pandas as compared to P[13] genotypes. The G11P[13] and G11P[7] strains contained almost identical G11 sequences, whereas the G11P[7] and G9P[7] strains had nearly identical P[7] sequences. This further supports the possibility of genetic reassortment between RVA strains within these farms.

This study reported fewer distinct G-P genotype combinations (5) which was in contrast to recent studies in Europe (Collins *et al.*, 2010; Martella *et al.*, 2010; Midgley *et al.*, 2012) where they reported more genotype combinations. However, recent studies in Asia also reported fewer G-P genotype combinations in swine (Kim *et al.*, 2010; Okitsu *et al.*, 2011), while in Canada a single genotype combination (G4P[6]) was most commonly detected (Lamhoujeb *et al.*, 2010). Although the detected G and P genotypes are common in pig populations worldwide (Collins *et al.*, 2010; Martella *et al.*, 2007a; Matthijnssens *et al.*, 2011; Steyer *et al.*, 2007), the strains identified in this study displayed marked genetic variation, especially G9 and P[13] strains, suggesting genetic variability in RVA strains circulating in the US pig population. The genetic

diversity, such as reported in this study, in the same region (and sometimes the same farm) may have resulted from the introduction of new RVA strains.

Since RV infections cannot be diagnosed solely based on clinical signs, laboratory tests are necessary for reliable RV surveillance, and can be useful in clinical settings to avoid inappropriate use of antibiotics which cannot prevent viral diarrheas. However, since it was not possible to characterize G and P genotypes for some RVA positive samples, which may be attributed to either low viral load in the fecal sample or variability in the primer binding sites due to genetic diversity at the nucleotide level (Collins *et al.*, 2010; Gregori *et al.*, 2012; Kerin *et al.*, 2007), it is necessary to constantly review and update genotyping primers as more sequencing data becomes available as was done in this study.

To date, the only RVA vaccine (ProSystem Rota, Intervet Inc., Merck Animal Health) licensed for swine in the US contains only G4 and G5 genotypes of RVA, according to the manufacturer, with no information about P genotype. However, none of the farms surveyed used the RVA vaccine; hence, it is unclear whether the diversity reported in this study may pose a challenge for future prophylactic strategies for prevention and control of RV associated gastroenteritis in nursing and weaned piglets. Therefore, given the low frequency of RVA detected and the low number of farms surveyed within only OH, large-scale epidemiological investigations are needed to better evaluate both the ecology and genetic diversity of porcine RVAs circulating in US swine farms. Acquiring information on the epidemiology of RVAs, non-group A RVs and other viral enteric pathogens will be important to design effective prophylactic measures, to decrease the impact of enteric infections in swine and to minimize the financial loss for swine producers.

CHAPTER 6

6 DETECTION AND GENETIC CHARACTERIZATION OF PORCINE GROUP A ROTAVIRUSES IN ASYMPTOMATIC PIGS IN SMALLHOLDER FARMS IN EAST AFRICA: PREDOMINANCE OF P[8] GENOTYPE RESSEMBLING HUMAN STRAINS

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6.1 Abstract

Viral enteritis is a serious problem accounting for deaths in neonatal animals and humans worldwide. The absence of surveillance programs and diagnostic laboratory facilities have resulted in a lack of data on RV associated diarrheas in pigs in East Africa. This chapter describes the incidence of group A rotavirus (RVA) infections in asymptomatic young pigs in East Africa was described. Of the 446 samples examined, 35.6% (117/446) were positive for RVA. More nursing piglets (78.7%) shed RVA than weaned (32.9%) and grower (5.8%) pigs. RVA incidence was higher in pigs that were either housed_free-range (77.8%) or tethered_free-range (29.0%) than those that were free-range or housed or housed-tethered pigs. The farms with larger herd size (>10 pigs) had higher RVA prevalence (56.5%) than farms with smaller herd size (24.1-29.7%). This study revealed that age, management system and pig density significantly ($p<0.01$) influenced the incidence of RVA infections, with housed_free-range management system and larger herd size showing higher risks for RVA infection. Partial (811-1604nt region) sequence of the VP4 gene of selected positive samples revealed that different

genotypes (P[6], P[8] and P[13]) are circulating in the study area with P[8] being predominant. The P[6] strain shared nucleotide (nt) and amino acid (aa) sequence identity of 84.4-91.3% and 95.1-96.9%, respectively, with known porcine and human P[6] strains. The P[8] strains shared high nt and aa sequence identity with known human P[8] strains ranging from 95.6-100% and 92-100%, respectively. The P[13] strains shared nt and aa sequence identity of 83.6-91.7% and 89.3-96.4%, respectively, only with known porcine P[13] strains. In conclusion, this study demonstrates that infection with RVA is frequent in East African piggeries. The P[6] and P[8] genotypes detected were genetically closely related to human strains suggesting the possibility of interspecies transmission. Further studies are required to determine the role of RVA in swine enteric disease burden and to determine the genetic/antigenic heterogeneity of the circulating strains for development of accurate diagnostic tools and to implement appropriate prophylaxis programs.

Key words (Group A rotaviruses, Prevalence, asymptomatic pigs, East Africa)

6.2 Introduction

Smallholder pig production systems (free range or small backyard herds) constitute >70% of the total pig farming in Kenya and Uganda (FAO, 2012). Traditional/backyard systems are the main systems in Western Kenya and Eastern Uganda where this study was conducted. Pigs in this region often live in close proximity to humans, causing public health concerns, especially when there are intermittent floods or torrential rains facilitating human and livestock exposure to many infectious pathogens including RVs. The East Africa region is also known for the large population of wild (bush) pigs and warthogs that are in close contact with domestic pigs creating favorable conditions for intermixing and spread of viral strains. Additionally, pig farming

systems practiced in this study region may lead to contamination of water bodies and food crops with animal viruses including RVs, via animal excreta.

Rotavirus (RV) is a genus of the *Reoviridae* family of dsRNA viruses, having a genome of 11 segments of dsRNA encoding 6 structural (VP1-VP4, VP6 and VP7) and 5 nonstructural (NSP1-NSP5/6) viral proteins. RVs are classified into 8 groups (Gp) (A–H) based on antigenic relationships of their VP6 protein (Matthijnssens *et al.*, 2012). The most common groups which infect humans and animals are group A, B and C. Group A rotavirus (RVA) is one of the most frequent viral agents detected in diarrheic piglets from 1-8 weeks of age (Saif *et al.*, 1994). However, asymptomatic RV infections also occur in pigs of all ages (Collins *et al.*, 2010; Steyer *et al.*, 2007). Based on the differences in the outer capsid VP7 and VP4 proteins carrying independent epitopes for neutralizing antibodies, RVAs are classified into G and P types respectively. There are 27 G genotypes and 37 P genotypes of RVA identified to date (Matthijnssens *et al.*, 2011a), 12 G genotypes (G1 to G6, G8 to G12 and G26) and 13 P genotypes (P[1], P[5] to P[8], P[11], P[13], P[19], P[23], P[26], P[27], P[32] and P[34] have been associated with pigs (Collins *et al.*, 2010; Matthijnssens *et al.*, 2011a). However, G3, G4, G5 and G11 are the most common G genotypes identified in swine and are usually associated with P[5], P[6] and P[28], the rest are considered uncommon genotypes in swine (Matthijnssens *et al.*, 2008a; Okitsu *et al.*, 2011; Wang *et al.*, 2010).

Rotavirus-associated enteritis is a major problem in young calves (Saif & Jiang, 1994), nursing and post-weaning piglets (Kapikian, 1996) and foals (Conner *et al.*, 1983) causing economic losses to farmers because of retarded growth for recovered animals and costs of treatments as well as high morbidity and variable mortality rates. RV infection causes diarrheal disease in suckling and weaned pigs that is usually resolved in 2-3 days if not complicated by secondary

microbial infections (Chang *et al.*, 2012). The prevalence of porcine RVA in the East Africa region is unknown but is suspected to play an important role in diarrhea in suckling and weaned piglets. Knowledge of the molecular epidemiology of RVA is crucial for preventing or reducing the incidence of porcine RVA diarrhea as well as transmission of porcine RVA to humans, as pigs may play a role as reservoirs for human RV strains (Palombo, 2002). Epidemiological studies are also critical for development of accurate RV diagnostic tools and potential RV vaccines based on the dominant RV genotypes. Therefore, the objective of this study was to investigate the prevalence and genetic heterogeneity of RVA in asymptomatic pigs of various ages under variety management practices.

6.3 Materials and methods

6.3.1 Sample

A total of 446 fecal samples were obtained from nursing and post-weaned piglets raised in small-scale farms (n=1 to 25 pigs per farm) with different management systems in Western Kenya (n=239) and Eastern Uganda (n=207). The management system consisted of pigs being, i) housed and tethered, ii) housed and free-range, iii) tethered, iv) tethered and free-range, v) housed and vi) free-range. Pigs in the study area were mostly fed from swirl from local hotels and household leftover food. Sample collection consisted of 47 samples from nursing pigs (less than 4 weeks of age), 210 samples from post-weaning pigs (3-4 months of age) and 189 samples from growers (5-6 months old). All pigs appeared clinically healthy at the time of sampling except for a few pigs which had loose stool. Fresh samples were collected from individual pigs, placed into a sterile 15ml centrifuge tube and stored on dry ice before being transported to the International Livestock Research Institute (ILRI) local laboratory in Busia where the samples

were stored at -70°C until used. The samples were then shipped to BecA-ILRI laboratories in Nairobi, Kenya for the analysis.

6.3.2 RNA Extraction

Fecal samples were prepared as 10% (v/v) fecal suspensions in either minimum essential media (MEM) or phosphate buffered saline (PBS). The suspensions were vortexed and centrifuged for 30 min at 1,800xg, 4°C. Total genomic RNA was extracted from 250µl starting volume of centrifuged 10% fecal suspensions using RNeasy mini kit from Qiagen Company (Qiagen Valencia, CA, USA) according to the manufacturer's instructions. Recovered RNA was suspended in 40µl of nuclease-free water and stored at -70°C until used.

6.3.3 Detection of RVA

Conventional reverse transcription-polymerase chain reaction (RT-PCR) was used for detection of the RVAs with validated primer sets, NSP3F (963–982) ACCATCTACACATGACCCTC and NSP3R (1,049-1,034) - GGTCACATAACGCCCC (Pang *et al.*, 2004) using Bioneer Accupower® PCR premix (Bioneer Co, S. Korea) according to manufacturer's instructions, with minor modifications. Accupower® PCR premix is a ready to use lyophilized premix of dNTPs, Top DNA polymerase, reaction buffer, a tracking dye and a stabilizer. Briefly, in each 20µl reaction premix tube, 2µl (5-50ng) of cDNA template was mixed with 1µl (10pmol) each of forward and reverse primers, 16µl of nuclease free water was added to top it to 20µl. The PCR was performed at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 56°C of 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. The temperature was then held at 4°C. The PCR products were analyzed by 3% agarose gel electrophoresis and visualized by ultraviolet illumination after staining with gel red™ nucleic acid gel stain (Biotium, Hayward, CA). Cell

cultured porcine RVA prototype OSU (X13190) was used as positive control in all PCR reactions. Statistical analysis was performed with SAS® (SAS, 2002) to compare the proportions of RVA positive samples among the countries, districts, age groups, herd sizes and the management systems evaluated. The analysis was performed using the chi-square (χ^2) test or Fisher's exact test. The confidence limit for the statistical tests was set at 95 % ($P<0.05$).

6.3.4 Genetic analysis of RVA strains using VP4 gene

Complementary DNA (cDNA) was synthesized from selected positive samples using Maxima first strand cDNA synthesis kit (Thermo scientific®) according to manufacturer's instructions. Thereafter, the cDNA was either immediately amplified or stored at -20°C. P types were determined using a universal primer pair [793F/1583R (Amimo *et al.*, 2013a)] for PCR and partial sequencing of the RVA VP4 gene (793-1604nt region). The nucleotide sequences obtained from the selected strains were compared with the same segment of similar sequences of known strains available in GenBank using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The DNA sequences were aligned using a multiple sequence alignment program, Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and the sequence identity calculated using the same program. The dendrograms were constructed using the neighbor-joining method supported with a bootstrap test of 1000 replicates in MEGA 6 software (Tamura et al., 2013). The P genotypes were assigned to each sample after nucleotide sequence analysis using either BLAST (blastn) search and/or **RotaC$^{2.0}$** automated genotyping tool for RVA (Maes *et al.*, 2009).

The GenBank accession numbers of all field strains and representative known RVA strains mentioned in this study are listed in **Appendix 4.**

6.4 Results and discussion

6.4.1 Incidence of group A rotavirus

This study investigated the prevalence of porcine RVA infections as well as the genetic heterogeneity among the identified strains based on their VP4 gene in asymptomatic pigs of various ages raised under different management systems. Of the 446 porcine fecal samples screened, 117 (26.2 %) tested positive for RVA (Table 6.1). The incidence of RVA reported in this study (26.2%) was higher compared to the prevalence reported in Ireland, 6.5% (Collins *et al.*, 2010), Spain, 7% (Halaihel *et al.*, 2010), USA, 9.4% (Amimo *et al.*, 2013a), Thailand, 10.7% (Okitsu *et al.*, 2011), Denmark and Slovenia, 10-20%% (Midgley *et al.*, 2012) and India, 25.7% (Malik *et al.*, 2013). However, these results were similar to the results reported in Vietnam, 32.7% (Anh *et al*., 2014), in Brazil, 35.3% (Racz *et al.*, 2000) and in Korea, 38.3% (Kim *et al.*, 2010). Higher prevalence (71.5%) was reported in asymptomatic pigs in Italy (Martella *et al.*, 2007a).

This study provides evidence that porcine RVA infections are widespread in East Africa suggesting that they are likely endemic in East Africa piggeries. The association of RVA with enteric disease in pigs in the study area remains unclear since only samples from asymptomatic pigs was analyzed and no data was available that tested for RVA in pigs with gastroenteritis from the same farms; however, some of the positive samples came from farms with a history of diarrhea.

The highest rate of RVA positive samples was determined in nursing (37/47 samples, 78.7 %) compared to weanling (32.9%) and grower (5.8%) pigs (Figure 6.1). The prevalence of RVAs in nursing piglets (78.7%) observed in this study was very high when compared to those reported in other studies in different regions, 21.1% in USA (Amimo *et al.*, 2013a), 26% in Spain (Halaihel

et al., 2010), 38.3% in S. Korea (Kim *et al.*, 2010), 67.3% in Japan (Katsuda *et al.*, 2006), 17.2-22.3% in Thailand (Khamrin *et al.*, 2007; Saikruang *et al.*, 2012), 35.3% in Brazil (Racz *et al.*, 2000) and 25.7% in India (Kusumakar *et al.*, 2010). A recent report in the USA revealed that RVA was more prevalent in asymptomatic (27.8%) than symptomatic (19%) nursing piglets (Amimo *et al.*, 2013a).

Table 6.1 Summary of RVA prevalence in the smallholder swine farms in East Africa during the study period

Details	Groups	N	Positive	Prevalence (%)
	Overall	**446**	**117**	**26.2**
Country[ns]	Kenya	239	65	27.2
	Uganda	207	52	25.1
District*	Busia_Ke	113	40	35.4
	Teso	126	25	19.8
	Busia_Ug	120	27	22.5
	Tororo	87	25	28.7
Age group***	nursing	47	37	78.7
	Postweaned (3-4mo)	210	69	32.9
	Growers (5-6mo)	189	11	5.8
Management system**	Free range	9	1	11.1
	Housed	7	2	28.6
	Housed;Free range	18	14	77.8
	Tethered	238	51	21.4
	Tethered;Free range	169	49	29.0
	Housed;Tethered	3		0
	Housed;Tethered;Free range	2		0
Herd size**	1-5 pigs	386	93	24.1
	6-10 pigs	37	11	29.7
	>10 pigs	23	13	56.5

Prevalence rate differed significantly *=p<0.05; **=p<0.01; ***=p<0.001; ns= not significant

RVA was detected in both Kenya (27.2%) and Uganda (25.1%) in nearly equal proportions, and in all the four districts sampled (Table 6.1), an indication of general circulation and endemicity of the virus on the tested farms. Maternally-derived antibodies (Hodgins *et al.*, 1999) or the circulation of naturally attenuated RV strains (Nguyen et al., 2003) could account for the occurrence of asymptomatic infections observed in this study. There was a significant variation ($p<0.05$) in the detection of RVA in the 4 districts studied (Figure 6.1), with proximity to major towns presenting higher risks of infection (Tororo in Uganda, 28.7% and Busia in Kenya, 35.4%), leading to the conclusion that proximity to major towns may play a role in RVA infection due to high pig density and increased human population.

Based on management systems and herd sizes in the study area, confinement of pigs and large herd size presented higher risk of RVA infection, with housed_tethered pigs shedding more virus than free ranging pigs (Table 6.1, Figure 6.1). This could be a result of viral accumulation in the pig housing facilities or where pigs are tethered. Additionally, RVA was more prevalent in the farms with larger herd size (56.5%) than farms with small herds (24.1-29.7%) as shown in Figure 6.1. Although, RV infection is endemic in pig herds worldwide, there is a limited number of comparable studies throughout Africa focusing on porcine RV infection and risk factors associated with RV disease in pigs (Steele *et al.*, 2004). To my knowledge this is the first study of RVs in pigs in East African region.

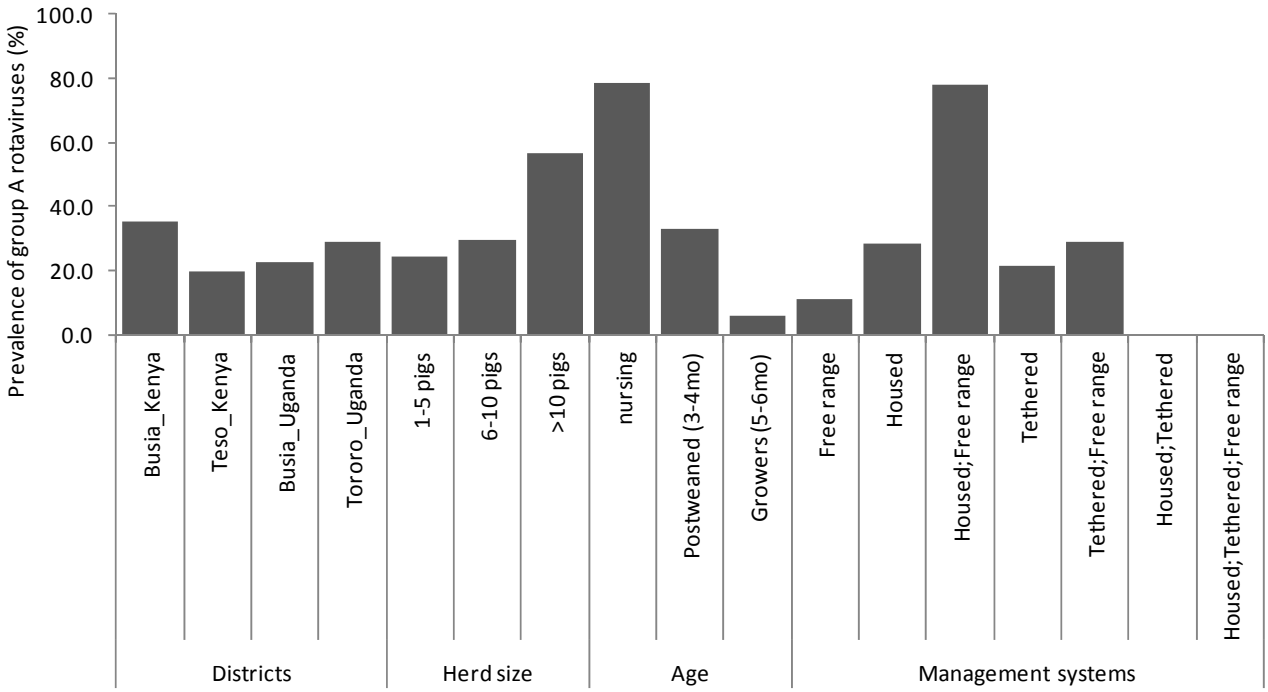

Figure 6.1 Prevalence (%) of porcine RVA in East Africa among districts, herd sizes, age groups and management systems

6.4.2 Molecular analysis of the partial VP4 gene

The RV VP4 and VP7 encoding genes have different levels of nucleotide conservation since they are targets of host neutralization antibodies. To gain more information on the genetic heterogeneity of porcine RVA strains circulating in swine in the East African farms, 30 RVA positive samples were selected based on the intensity of the PCR product band, sample origin and age of the sampled pig, and their partial VP4 gene region was sequenced. Eighteen (18) samples yielded sufficient product for further molecular characterization, while 12 samples yielded product of insufficient quantity/quality which could be attributed to inhibitory substances that reduces PCR sensitivity and/or a higher diversity of viral genomes. In addition, the samples examined in this study were from asymptomatic pigs which may have lower viral load. Based on sequence analysis of VP4 gene using the 80% nucleotide cutoff value established to distinguish 35 P-types (Matthijnssens *et al.*, 2011a), the 18 strains from this study were classified into three different P-types including P[6], P[8] and P[13].

A single P[6]-type strain (5.6 %) was detected in a nursing piglet that shared 84.4-91.3% and 95.1-96.9% nucleotide (nt) and amino acid (aa) sequence identities, respectively, with known porcine and human P[6] strains available in the GenBank. Interestingly, at the nucleotide level the detected P[6] strain (K-003-5) was more closely related to human Hu/BEL/BE2001/2009/G9P6 strain (91.3%) detected in an infant in Belgium than the porcine Gottfried (M33516) P[6] strain (84.4%). At the amino acid sequence level, the K-003-5 strain clustered with porcine Gottfried strain in the same branch, however, this was supported with low (58%) bootstrap value (Figure 6.3). Using RotaC® online tool for assigning genotypes, K-003-5 strain was most similar (85.8%) to P6/Hu/DRC86 (DQ005122) strain detected in a human in the Democratic Republic of the Congo in Africa (Matthijnssens *et al.*, 2006). These findings

revealed that RV remains a potential zoonotic infection and it is likely that interspecies transmission of RVs may occur in this region; hence, further research is needed to ascertain this fact in the study region.

A total of 15/18 (83.3%) strains were of the P[8] genotype. The field P[8] strains showed some diversity among them as shown in Figures 6.2 & 6.3. They shared nt and aa sequence identities ranging from 97.8 to100% and 94.1 to 100%, respectively. These strains were genetically similar to human P[8] strains available in GenBank with nt and aa identity of 95.8-100% and 93.4-100% respectively. Based on RotaC® online tool these P[8] field strains were most similar to human P[8] prototype Wa strain with nucleotide sequence identities ranging from 99.2-99.7%. This finding again confirms that RV remains a potential zoonotic infection.

Two out of 18 (11%) strains detected were P[13]. These two strains were more diverse sharing 87.6% and 94.2% nucleotide and amino acid sequence identity with each other, respectively, each falling in a different branch in the phylogenetic tree (Figure 6.2 & 6.3). This finding is similar to recent report in the USA where P[13] strains detected in young pigs were found to be more diverse compared to other P types (Amimo *et al.*, 2013a). The strains detected were genetically closest to a group of swine P[13] strains detected in Japan and India with nucleotide and amino acid sequence identities of 83.6-91.7% and 89.3-96.4%, respectively. However, using RotaC® online tool these strains were most similar to P13/Po/A46 (AY050274) strain with 84.0-85.2% nt identity. The P[13] genotypes appear to be host restricted, and are commonly detected among pigs but have not been identified from other animal sources or humans (Chan-It *et al.*, 2008; Ghosh *et al.*, 2007a). Recent studies have reported that the P[6] genotype as the most common P type in Slovenia and Spain (Midgley *et al.*, 2012) and the P[13] genotype in the USA (Amimo *et al.*, 2013a).

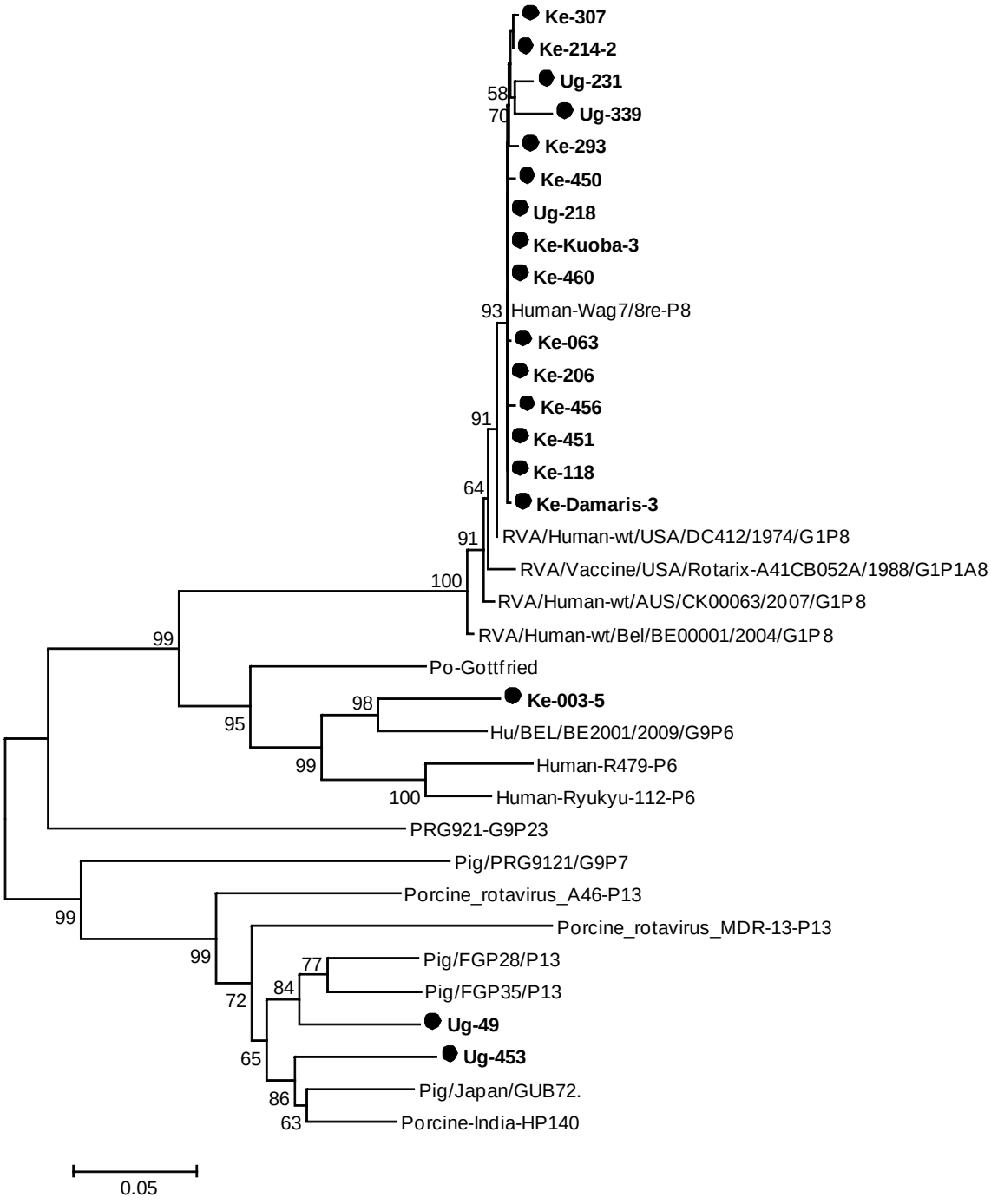

Figure 6.2 Phylogenetic dendrogram of the partial (811-1604nt) nucleotide sequence of RVA VP4 gene of field strains (dot, bold) compared with available VP4 gene sequences for RVA P genotypes in GenBank.

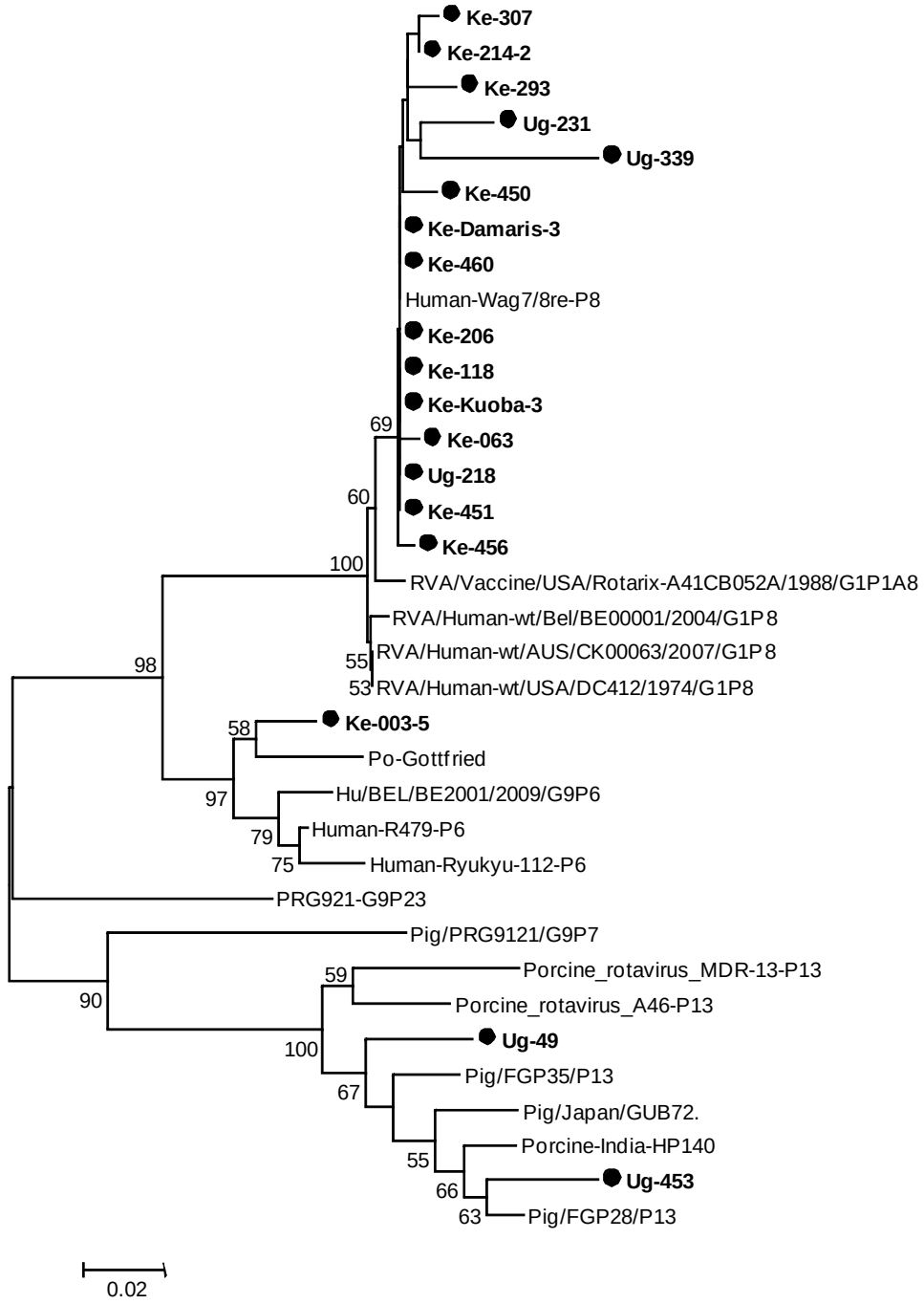

Figure 6.3 Phylogenetic dendrogram of the partial (288-512nt) amino acid sequence of RVA VP4 gene of field strains (bold, dot) compared with available VP4 gene sequences for RVA P genotypes in GenBank.

In conclusion, this is the first epidemiological study on the prevalence of porcine RVA infections in pigs in Africa and it provides evidence that porcine RVA is circulating in asymptomatic piglets at high rates. Further studies on risk factors for RVA infections and the association of this virus with gastroenteritis are necessary, especially in developing countries where a majority of pigs are raised in smallholder production system with varied management. The evidence collected also revealed a striking genetic heterogeneity of the RVAs circulating in East African herds, with multiple strains being detected in animals, however, P[8] strains were most dominant. A striking finding was the close genetic similarities between the pig P[6] and P[8] strains detected and the corresponding human strains. Thus, it is important to screen for the presence of RV in asymptomatic animals and to characterize these RVs. Asymptomatic animals could serve as natural reservoirs/sources of new emerging genotypes in both animals and humans, especially in view of the close proximity between them in some geographic regions.

CHAPTER 7

7 MOLECULAR DETECTION AND GENETIC CHARACTERIZATION OF KOBUVIRUSES AND ASTROVIRUSES IN ASYMPTOMAT LOCAL PIGS IN EAST AFRICA

(This chapter has been published in Archives of Virology, 2014: 159 (6): 1313-1319

http://www.ncbi.nlm.nih.gov/pubmed/24327095)

7.1 Abstract

In this study, swine fecal specimens (n=251) collected from nursing and weaned piglets raised under smallholder production systems were screened for the presence of kobuviruses by RT-PCR. Porcine kobuviruses were detected in 13.1% (33/251) of the samples. This study demonstrated that porcine kobuvirus infections exist in indigenous pigs in Kenya and Uganda and that the prevalence was higher in young piglets than older pigs: nursing piglets (15%), post weaned (3 months old) pigs (17%), 4-month-old pigs (10%). Genetic analysis of the partial RNA dependent RNA polymerase (RdRp) region (690nt) revealed that kobuviruses circulating in East Africa are diverse, sharing nucleotide sequence identities ranging from 89.7-99.1% and 88-92.3% among them and with known porcine kobuviruses, respectively. The nucleotide sequence identities between kobuvirus strains and those of human, bovine and canine kobuviruses were 69.4-70.7%, 73.1-74.4% and 67-70.7%, respectively. Additionally, upon sequencing selected samples that showed consistent 720bp RT-PCR bands while using the same primer set, porcine astroviruses were detected in these samples belonging to type 2 and type 3 *Mamastroviruses*. To

my knowledge this study reports the first detection and molecular analysis of both porcine kobuviruses and astroviruses in African region. Further studies are required to determine the role of these viruses in gastrointestinal infections of pigs in this region and to determine the genetic diversity of the circulating strains to develop accurate diagnostic tools and implement appropriate control strategies.

(Key words: Astroviruses, kobuvirus, local pigs, genetic analysis, East Africa)

7.2 Introduction

Small-scale pig production (free range or small backyard herds) constitutes >70% of the total pig farms in East Africa. Pigs in this region often live in close proximity to humans, causing public health concerns and indicating a need for increased health standards. East Africa is also known for the large population of wild (bush) pigs and warthogs that are in close contact with domestic pigs creating favorable conditions for intermixing and spread of viral strains. Emerging viruses represent a threat to human and food animal health, as evidenced by sporadic outbreaks of influenza and coronaviruses. Hence, knowledge about the diversity of viruses present in reservoir animals can lead to a better understanding of the origin of emerging pathogens. Kobuviruses are members of the *Picornaviridae* family, which are small, non-enveloped viruses with single-stranded, positive-sense genomic RNA. The genus kobuvirus is comprised of three officially recognized species, Aichi virus, bovine kobuvirus, and porcine kobuvirus (Reuter *et al.*, 2008). The *kobuvirus* genome is approximately 8.3 kb, which is organized into three structural (VP0, VP3 and VP1) and seven non-structural (2A-2C and 3A- 3D) regions with a leader protein (L). The 3D gene region encodes a viral RNA-dependent RNA polymerase (RdRp) and represents a conserved region among kobuviruses (Reuter *et al.*, 2011). Nucleotide and amino acid identities

of the 3D RdRp coding region among porcine kobuviruses, bovine kobuviruses and aichi viruses varied from 74.0% to 81.0% (Reuter *et al.*, 2009a). Aichi virus was first detected in Japan in 1989 from a human patient with acute gastroenteritis (Yamashita *et al.*, 1991). Thereafter, Aichi viruses have been detected in Asia, Europe, South America and Tunisia (Oh *et al.*, 2006; Pham *et al.*, 2007; Reuter *et al.*, 2009b; Sdiri-Loulizi *et al.*, 2010). Bovine kobuvirus was first recognized in 2003 as a cytopathic contaminant in cell culture medium derived from bovine sera in Japan. Later, it was found in fecal samples of clinically healthy cattle (Yamashita *et al.*, 2003). Bovine kobuvirus has also been detected in domestic sheep in Hungary (Reuter *et al.*, 2010a; Reuter *et al.*, 2010b). Porcine kobuvirus (S-1-HUN/2007: EU787450) was first identified from fecal samples of domestic pigs in 2008 in Hungary (Reuter *et al.*, 2008). Thereafter, porcine kobuviruses have been reported in additional countries including Asian countries (Khamrin *et al.*, 2010; Park *et al.*, 2010), the Netherlands and Brazil (Barry *et al.*, 2011) and the USA (Sisay *et al.*, 2013). The prevalence of kobuvirus infection in pigs ranges from 30% to 99%. This large variation could be as a result of different ages within the populations evaluated, diarrheal status, regions and other factors. Studies showing association of porcine kobuvirus infection with clinical disease are limited; however, a recent study in Korea reported an association between porcine kobuvirus detection and diarrhea in pigs (Park *et al.*, 2010). An association between kobuvirus infection and age has been reported in cattle and in pigs (Jeoung *et al.*, 2011).

Astroviruses belong to the family *Astroviridae* which consists of two genera, *Avastrovirus and Mamastrovirus*, whose members are associated with gastroenteritis in avian and mammalian hosts, respectively. Astroviruses are generally associated with either mild or severe enteric disease symptoms such as diarrhea and vomiting in a number of mammalian species (Jonassen *et al.*, 2001). Bridger in the UK and Saif et al in the USA reported the first porcine astroviruses

(PAstV) detected in pigs by electron microscopy (Bridger, 1980; Saif *et al.*, 1980b). Later in 1990, PAstV was isolated in Japan (Shimizu *et al.*, 1990). Recently, Mor and co-workers also reported PAstV in pigs in the USA with a prevalence of 62% (Mor *et al.*, 2012). To date, five PAstV types (PAstV1– PAstV5) have been identified from different countries, including Czech Republic, Colombia, Canada, the USA, China and Hungary (Indik *et al.*, 2006; Lan *et al.*, 2011; Laurin *et al.*, 2011; Luo *et al.*, 2011; Reuter *et al.*, 2012; Shan *et al.*, 2011). Among the five types, PAstV1, PAstV2 and PAstV3 were identified in fecal samples from healthy pigs.

Epidemiological information on the geographical distribution, incidence and genetic diversity of kobuviruses in African swine populations is unknown. This study report the first detection of porcine kobuviruses in samples collected from nursing and weaned pigs in East Africa, and analyze the phylogenetic relationships between the East African porcine *kobuvirus* strains and known *kobuvirus* strains as well as other representative picornaviruses. This study also reports the first detection of porcine *astrovirus* in this region.

Accession numbers of nucleotide sequences strains described in this study were deposited in GenBank under; kobuviruses - KF494340 - KF494343 and KF597279 and astroviruses - KF597280- KF597282

7.3 Material and methods

7.3.1 Sample collection

A total of 251 fecal samples were obtained from nursing and post-weaned piglets raised in small-scale farms (n=1 to 25 pigs per farm) with different management systems in Western Kenya (n=140) and Eastern Uganda (n=111). The management system consisted of pigs being housed, housed & tethered, housed & free-range, tethered, tethered & free-range and free-range. The pigs

were mostly fed swirl from hotels and household leftover food. The sample collection consisted of 41 samples from nursing pigs (less than 4 weeks of age), 90 samples from post-weaning pigs (12 weeks of age) and 120 samples from growers (16 weeks of age). All pigs appeared clinically healthy at the time of sampling. Fresh samples were collected from individual pigs, placed into a sterile specimen container and stored on dry ice before being transported to the International Livestock Research Institute (ILRI) laboratory in Busia where the samples were stored at -70^{0}C until use. The samples were then shipped to BecA-ILRI laboratories in Nairobi for analysis.

7.3.2 RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Fecal samples were prepared as 10% (v/v) fecal suspensions in minimum essential media (MEM). The prepared sample suspensions were vortexed and centrifuged for 30 min at 1,800xg, 4^{0}C. RNA was extracted from 250µl starting volume of centrifuged 10% fecal suspensions using RNeasy mini kit from Qiagen Company (Qiagen Valencia, CA, USA) according to the manufacturer's instructions. The total RNA recovered was suspended in 40µl of nuclease-free water and stored at -70 ^{0}C until used. cDNA was synthesised using Maxima first strand cDNA synthesis kit (Thermo scientific®) according to manufacturers' instructions. Then the cDNA was either amplified immediately or stored at -20^{0}C.

Previous studies using the *Calicivirus* primer pair p110/p290 (Jiang *et al.*, 1999; Le Guyader *et al.*, 1996) targeting the calicivirus RdRp gene (317-nt for norovirus and 329-nt for sapovirus) also amplified the 3D RdRp regions of porcine kobuvirus (~1100-nt) (Sisay *et al.*, 2013), which was confirmed by sequence analysis of 5 representative suspect samples in the present report. This same primer pair was used for the detection of porcine kobuviruses from East African pigs. RT-PCR was carried out using Accupower® PCR premix (Bioneer Co, S. Korea) according to

manufacturer's instructions. Accupower® PCR premix is a ready to use lyophilized premix of dNTPs, Top DNA polymerase, reaction buffer, a tracking dye and a stabilizer. Briefly, in each 20µl reaction premix tube, 2µl (5-50ng) of cDNA template was mixed with 1µl (10pMol) each of forward and reverse primers, 16µl of nuclease free water was added to top it to 20µl. The PCR was performed at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 48°C of 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. The temperature was then held at 4°C. The RT-PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by ultraviolet illumination after staining with gel red™ nucleic acid gel stain (Biotium, Hayward, CA). When bands were found in the region of 1,100 bp on the agarose gel, the study tentatively considered the results kobuvirus positive. The samples with the correct band size were purified using QIAquick PCR purification kit (Qiagen Valencia, CA USA), according to the manufacturer's instructions for sequencing. In other fecal samples, an approximately 720-nt-long weak, non-specific PCR product was also observed. The nucleotide (nt) sequence of PCR product of 3 selected samples from 3 different farms were determined by direct sequencing.

7.3.3 Sequencing and molecular analysis

To confirm the RT-PCR results and to obtain genetic information on virus diversity, the partial 3D RdRp amplicons of 5 *kobuvirus* positive samples were sequenced directly using forward (p110) and reverse (p290) primers using BigDye Terminator cycle chemistry (Applied Biosystems, Foster City, CA, USA). The resulting RdRP gene sequences were edited using Bioedit version 7.1.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and compared with sequences of *kobuvirus* reference strains in the GenBank database by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence similarity analysis was performed for the

aligned nucleotide and amino acid sequences using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic analysis of the East African porcine kobuviruses with *kobuvirus* reference strains based on the nucleotide and amino acid alignments was conducted using the neighbor-joining method supported with a bootstrap test of 1000 replicates in MEGA 6 software (Tamura *et al.*, 2013). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3 parameter method and are in the units of the number of base substitutions per site.

To analyze porcine kobuvirus infection in pigs of the different ages with different management systems, the piglet fecal samples were divided according to the age of the animals into 3 groups of nursing, ≤1-month-old (n=47), weaned, (3-month-old, n=90) and growers (>4-month-old, n=120) animals. Statistical analysis was performed with SAS® to compare the proportions of kobuvirus positive samples among the 4 districts, the 2 countries, the 3 age groups evaluated and the 6 different management systems. The analysis was performed using the chi-square (χ^2) test or Fisher's exact test. The confidence limit for the statistical tests was set at 95 % ($P<0.05$).

7.4 Results and discussion

7.4.1 Incidence of kobuvirus

Kobuvirus RNA was detected in 13.1% (33/251) of the pig fecal samples analyzed using primer pair p110/p290 (Jiang *et al.*, 1999; Le Guyader *et al.*, 1996). Kobuvirus was detected in both Kenya (14.9%) and Uganda (15.5%) in equal proportions, and in all the four districts sampled (Table 7.1), an indication of a general circulation and endemicity of the virus on the tested farms. Among the age groups, younger piglets shed more virus than older pigs, a higher incidence (15

of 90, 16.7%) of kobuvirus was reported in post weaned (3-month-old) compared to nursing piglets (6 of 41, 14.6%) and grower pigs (12 of 120, 10%). This could possibly be due to an inefficient immune response, diminishing maternal immunity post weaning or other intrinsic age-related factors. Higher frequency rates in young piglets has also been reported in other studies (Barry *et al.*, 2011; Park *et al.*, 2010; Reuter *et al.*, 2009a). Based on management systems in the study area, confinement of pigs presented higher risk to kobuvirus infection, with housed pigs shedding more virus than free ranging pigs (Table 7.1). This could be as a result of viral accumulation in the pig houses or where pigs are tethered. Additionally, kobuvirus was more prevalent in the farms with larger herd size (>10 pigs, 20%) than farms with small herds (<10 pigs, 12.6%).

Table 7.1 Porcine kobuvirus infection status in pigs of different ages and raised under different management systems in East Africa

Details	groups	N	Positive (%)
	Overall	**251**	**33(13.1)**
Country	Kenya	139	18 (14.9)
	Uganda	112	15 (15.5)
Districts	Busia_Ke	69	9 (13.0)
	Teso	70	9 (12.9)
	Busia_Ug	64	10 (15.6)
	Tororo	48	5 (10.4)
Age group	Nursing-<1mo	41	6 (14.6)
	Weaner-3mo	90	15 (16.7)
	grower-4mo	120	12 (10.0)
Management systems	Free range	8	1 (12.5)
	Housed	6	2 (33.3)
	Housed & Free range	17	3 (17.6)
	Housed & Tethered	3	0 (0)
	Tethered	115	14 (12.2)
	Tethered & Free range	102	13 (12.7
No. of pigs per household	1 to 5	215	27 (12.6)
	6 to 10	16	2 (12.5)
	>10	20	4 (20.0)

The incidence of porcine *kobuvirus* infection reported in this study (13.1%) was lower than that reported in other countries which ranged between 30.1% in China to 99% in Thailand (Khamrin *et al.*, 2009; Reuter *et al.*, 2009a; Reuter *et al.*, 2010c; Yu *et al.*, 2011). A recent study in Korea detected porcine *kobuvirus* in 84.5% of diarrheic pigs and 19.3% in healthy pigs (Park *et al.*, 2010). Similarly, a study in Japan using samples from healthy pigs reported 45.4% prevalence of porcine kobuvirus (Khamrin *et al.*, 2010). This difference may be attributable to variations in the samples due to factors like sampling time, sampling place (distribution of sampling farms), sample size, age of pigs, and clinical background of the tested animal population (diarrhea or clinically healthy). The association of this virus with enteric diseases in pigs in the study area remains unclear since only samples from asymptomatic pigs were analyzed and no data was available that tested for kobuvirus in pigs with gastroenteritis from the same farms; however, some of the positive samples came from farms with a history of diarrhea. This study confirmed that infection with kobuvirus in asymptomatic pigs is common, as has been reported in other studies (Barry *et al.*, 2011; Dufkova *et al.*, 2013; Reuter *et al.*, 2010c). Further studies on risk factors for this infection are necessary and the association of this virus with gastroenteritis, especially in the developing countries where a majority of pigs are raised in smallholder production system with varied management.

Table 7.2 Nucleotide and amino acid sequence identities (%) of partial 3D RdRP gene of selected field strains compared with similar gene sequences for known kobuviruses and other picornaviruses in the GenBank

	Nucleotide sequence identity (%)					Amino acid sequence identity (%)				
Strains	**Damaris-3**	**K-118**	**Kuoba-3**	**K-460**	**K-1033**	**Damaris-3**	**K-118**	**Kuoba-3**	**K-460**	**K-1033**
swine/S-1-HUN/2007-EU787450	92.0	91.9	92.0	89.9	92.3	97.5	96.5	97.2	92.7	95.3
swine/K-30-HUN/2008-GQ249161	91.9	91.7	91.9	89.7	92.2	97.5	96.5	97.2	92.7	95.3
Swine/CH/HNXX-4/2012-JX401523	91.1	90.2	91.7	88.5	91.1	97.2	95.1	96.8	92.7	94.5
Swine/CH/HZ/2011-JX827598	90.9	90.2	91.4	88.0	90.9	97.2	96.5	96.8	93.6	95.3
swine/MF8047/2009/KOR-GU723956	91.2	91.2	91.2	88.9	90.9	96.8	95.8	96.5	92.7	95.3
Swine-WUH1/2011-CHN-JQ692069	91.1	92.0	91.4	89.4	91.1	97.2	96.8	96.8	94.1	95.3
Swine/XX/CHN/2011-KC204684	90.5	92.2	90.8	88.6	90.5	97.2	96.8	96.8	93.2	94.9
swine/KOR/2010-94DA1-HQ400962	90.6	91.6	91.6	91.8	91.0	92.6	91.4	92.6	92.0	96.9
swine/KOR/2010-95DA2-HQ400963	91.0	91.6	92.0	92.2	91.4	92.6	91.4	92.6	92.0	96.9
Swine/P224/11/CZ-JX232622	89.8	89.4	90.4	89.2	90.2	92.2	90.4	92.2	91.6	97.0
Swine/P46/11/CZ-JX232619	90.6	90.0	91.2	89.6	91.0	92.2	90.4	92.2	91.6	97.0
Swine/P5/11/CZ-JX232616	90.6	90.6	91.2	89.6	91.0	92.2	90.4	92.2	91.6	97.0
Aichivirus-AB040749	70.7	70.4	70.5	69.7	70.5	71.4	70.7	71.0	70.8	71.9
Aichivirus-1-NC_001918	70.4	70.1	70.2	69.4	70.2	71.4	70.7	71.0	70.8	71.9
Bo-U-1-AB084788	74.0	73.9	74.0	73.1	74.4	80.2	78.8	79.9	79.9	80.5
sheep/TB3/HUN/2009-GU245693	71.3	71.9	71.4	70.8	71.3	79.5	78.5	79.2	78.5	79.3
dog/AN211D/USA/2009-JN387133	69.7	69.0	69.6	67.7	70.1	68.6	67.8	68.2	69.0	69.1
Canine-US-PC0082-JN088541	70.4	69.4	70.2	67.0	70.7	68.6	67.8	68.2	69.0	69.1
Ljungan-87-012-AF327920-Parechovirus	39.3	39.5	39.2	40.0	39.2	25.6	26.3	25.3	24.0	25.2
Eq-Rhinovirus-X96871-Erbovirus	49.4	49.2	48.3	48.6	48.9	37.3	37.3	37.3	37.5	36.5
FMDV-O1K-X00871-Aphthovirus	49.8	49.1	50.2	49.4	50.0	34.5	34.2	34.2	35.5	35.0
HRV14-K02121-Enterovirus	41.4	41.1	41.6	41.6	41.1	34.6	34.6	34.6	32.7	33.1
Po-UKG/410/73-AF363453-enterovirus	43.4	43.6	43.9	44.2	43.2	34.1	34.1	34.1	33.2	32.5
Damaris-3 - KF494340	100.0	92.0	98.2	89.9	99.1	100.0	96.8	99.7	95.4	96.1
K-118 - KF494342		100.0	91.7	89.7	92.0		100.0	96.5	92.2	94.9
Kuoba-3 - KF494341			100.0	90.9	98.5			100.0	95.4	95.7
K-460 - KF494343				100.0	89.9				100.0	91.7
K-1033 - KF597279					100.0					100.0

7.4.2 Genetic and phylogenetic analysis of East African porcine kobuviruses

To gain more information on the genetic heterogeneity of porcine kobuvirus strains circulating in swine in the east African farms, 5 kobuvirus positive samples were selected based on the PCR product quality in the gel i.e. intensity of the band) and their partial 3D RdRP region sequenced. Genetic analysis of the partial sequence revealed that the kobuviruses circulating in the East African region are more variable, sharing nucleotide sequence identity ranging from 89.7-99.1% among them. At the nucleotide and amino acid level, they were 69.4–70.7% and 70.7–71.9% identical, respectively, to those of *Aichi* viruses, 73.1–74.4% and 78.8–80.5% to bovine kobuvirus, 70.8-71.9% and 78.5-79.5% to kobuvirus in sheep, 67-70.7% and 67.8-69.1% to canine kobuviruses and 88-92.3% and 90.4-97.5% to other porcine kobuviruses (Table 7.2).

Phylogenetic analysis of partial 3D RdRp nucleotide and amino acid sequences of porcine kobuvirus strains detected in this study, together with published sequences of kobuvirus reference strains (porcine kobuviruses, Aichi viruses and bovine kobuvirus) and representatives of other *picornaviruses* revealed that three of the detected strains (Damaris-3-KF494340, Kuoba-3-KF494341and K-1033- KF597279) grouped together and formed their own cluster while one of the strains (K-118-KF494342) clustered with Chinese strains and another strain (K-460-KF494343) clustered with Korean strains (Figure 7.1). This indicates that porcine kobuviruses circulating in the East African region are genetically diverse. Damaris-3-KF494340, Kuoba-3-KF494341and K-1033- KF597279 samples were collected in the same geographical location indicating that porcine kobuviruses in the east African region may be geographically restricted; however, more strains from different parts of east Africa needed to be sequenced to ascertain the geographical distribution of this virus. Genetic diversity among geographically separated porcine kobuviruses have been reported in other studies (Park *et al.*, 2010).

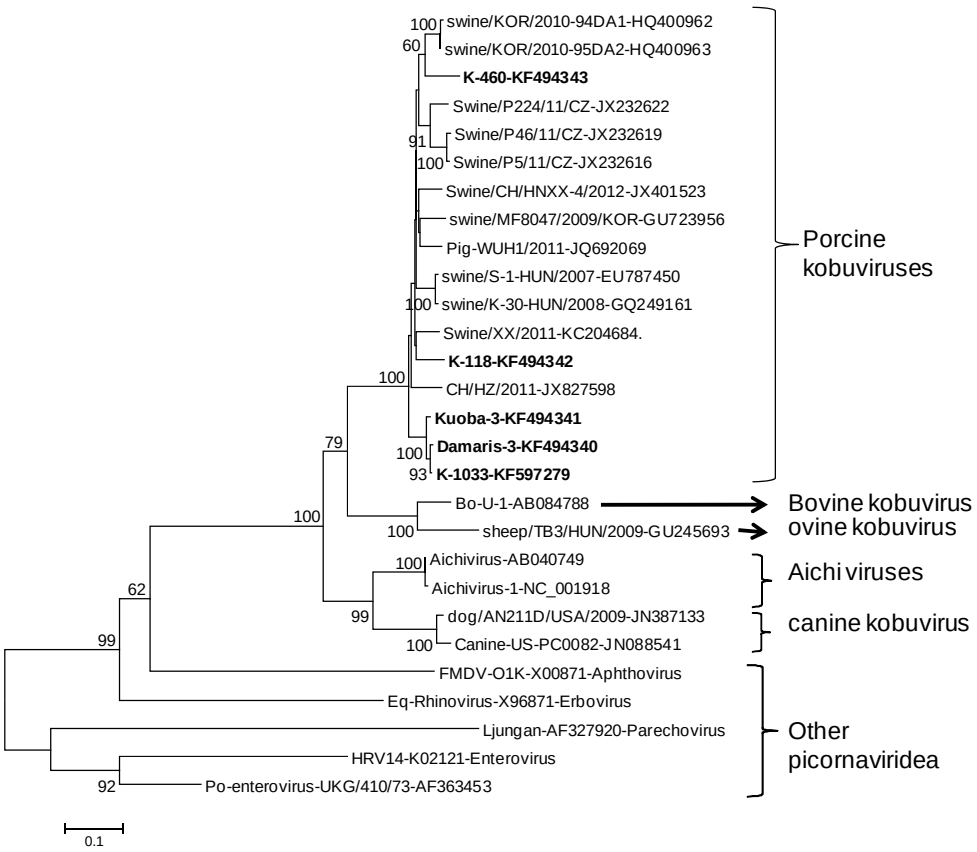

Figure 7.1 Phylogenetic dendrogram of the partial (690bp) nucleotide sequence of the 3D RdRp gene of field kobuvirus strains (bold) compared with similar gene sequences for known kobuviruses and other Picornaviruses in the GenBank. The evolutionary distances were computed using the Tamura-Nei method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site.

Phylogenetic analysis also confirmed that the 5 strains detected were more closely related to the kobuviruses, notably porcine *kobuviruses*, than to any other *picornaviruses*, and this was supported by the 100% bootstrap value (Figure 7.1). Evidence of a close genetic relationship or interspecies transmission among different viruses in the genus kobuvirus has been documented. A sheep kobuvirus strain has high nucleotide sequence identity to bovine kobuvirus (Reuter *et al.*, 2009a), and one porcine kobuvirus is more closely related to bovine kobuvirus than to porcine strains (Khamrin *et al.*, 2010). However, this study did not find kobuvirus strains that were similar to members of kobuviruses from species other than the porcine strains.

7.4.3 Genetic and phylogenetic analysis of porcine astroviruses

In other fecal samples, an approximately 720-nt-long weak, non-specific RT-PCR product was also observed. The nucleotide (nt) sequence of the RT-PCR product of 3 selected samples was determined by direct sequencing, and the result showed that one of the strains (K-268-KF597282) had 86.1% nt identity to porcine astrovirus type 2 RdRp gene in the GenBank database. The other two strains (K-006- KF597280 and K-332- KF597281) had 96.7% identity with porcine astrovirus type 3. This was confirmed by phylogenetic analysis whereby K-006-KF597280 and K-332- KF597281 strains clustered with astrovirus type 3 (PAstV-3-US-MO123-JX556691) detected in the USA while the K-268-KF597282 strain clustered with astrovirus type 2 (PAstV-2/2007/HUN-GU562296) detected in Hungary (Figure 7.2).

Recent studies in the USA revealed high genetic heterogeneity of PAstV strains (Xiao *et al.*, 2013). The importance of co-circulation of PAstVs and the role pigs may play in the transmission and recombination of this virus need further investigation.

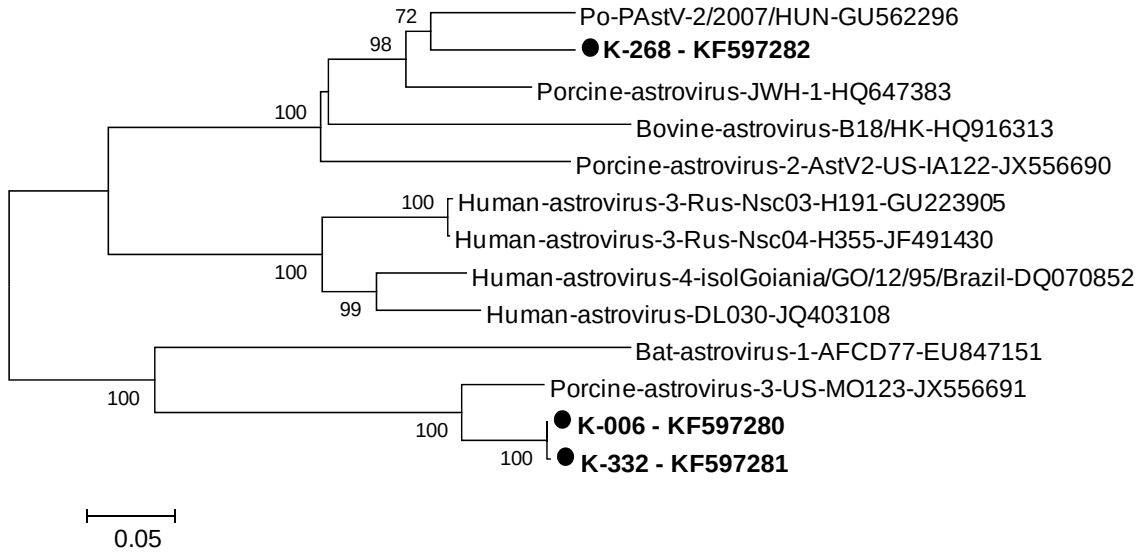

Figure 7.2 Phylogenetic dendrogram of the partial nucleotide sequence of 3D RdRp gene (541nt) of field astrovirus strains (bold) compared with similar gene sequences for known astroviruses in the GenBank. The evolutionary distances were computed using the Tamura 3-parameter method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site.

In conclusion, the findings of this study demonstrate that porcine kobuviruses and astroviruses are present in the swine population in East Africa and to my knowledge this study reports the first detection of porcine kobuvirus in this African region and the first molecular analysis of the detected strains. The presence of these gastroenteritis-producing viruses in clinically healthy pigs represent a source of infection of pigs, and possibly to humans and hence further studies are required to determine their role in gastrointestinal diseases of pigs in this region and to determine their genetic diversity in-order to develop accurate diagnostic tools and implement appropriate control strategies.

CHAPTER 8

8 GENERAL CONCLUSITIONS AND RECOMENDATIONS

The aim of this study was to determine the distribution of RVs (RVA and RVC), the distribution of G and P genotypes of RVAs and zoonotic potential of RV circulating swine population raised under smallholder production systems in Kenya and Uganda and in highly specialized commercial farms in USA. Chapters 1, 2 and 3 include the introduction, literature review and how the study was conducted respectively.

In Chapter 4 the relative distribution and zoonotic potential of RVC circulating in USA farms is described. High incidence of RVC was reported and the study revealed that porcine RVC are widespread and genetically diverse in swine herds in the state of Ohio, USA, which poses challenges for development of diagnostic and prophylactic tools for prevention of RV infections. Additionally in this chapter, one of the RV strain detected (RVC/Pig-wt/USA/RV0143/2012/G6Px) represented a new RVC VP4 genotype. Therefore, the study concluded that a detailed molecular epidemiological surveillance is needed for a better understanding of the diversity of RVCs and their role in diarrhea in swine of various ages. Information acquired from full length sequences of NSP4, VP6 and VP7 and VP4 genes in this study could be useful in the development of accurate diagnostic tools and also in basic research for understanding the functions of these genes.

Chapter 5 describes the prevalence and genetic diversity of RVA in commercial farms in USA. The prevalence of RVA in US farms studied was low, however, the genetic diversity of RVA detected in US farms was high. To date, the only RVA vaccine (ProSystem Rota, Intervet Inc.,

Merck Animal Health) licensed for swine in the US contains only G4 and G5 genotypes of RVA, according to the manufacturer, with no information about P genotype. The study in this chapter reported G4, G9 and G11 genotypes with P[7] and P[13] and since none of the farms surveyed used the RVA vaccine; it is unclear whether the diversity reported in this study may pose a challenge for future prophylactic strategies for prevention and control of RV associated gastroenteritis in nursing and weaned piglets. Therefore, this study concluded that, given the low frequency of RVA detected and the low number of farms surveyed within only OH, large-scale epidemiological investigations are needed to better evaluate both the ecology and genetic diversity of porcine RVAs circulating in US swine farms.

Chapter 6 and 7 of this thesis describes the results of the study carried out in smallholder pig production systems in Kenya and Uganda. Chapter 6 report the results of rotavirus study in the asymptomatic pigs. High prevalence of RVA was reported with different P genotypes reported in East African swine population. This study revealed that age, management system and pig density significantly ($p<0.01$) influenced the incidence of RVA infections, with housed_free-range management system and larger herd size showing higher risks for RVA infection. Further studies on risk factors for RVA infections and the association of this virus with gastroenteritis are necessary, especially in developing countries where a majority of pigs are raised in smallholder production system with varied management. A striking finding was the close genetic similarities between the pig P[6] and P[8] strains detected and the corresponding human strains. Thus, it is important to screen for the presence of rotavirus in asymptomatic animals and to characterize these rotaviruses. Asymptomatic animals could serve as natural reservoirs/sources of new emerging genotypes in both animals and humans, especially in view of the close proximity between them in some geographic regions.

In chapter 7 the first detection and genetic characterization of two porcine viruses that has not been reported in East Africa region i.e. kobuviruses and astroviruses are reported. The study concluded that the presence of these gastroenteritis-producing viruses in clinically healthy pigs represents a source of infection of pigs, and possibly to humans and hence further studies are required to determine their role in gastrointestinal infections of pigs in this region and to determine their genetic diversity in-order to develop accurate diagnostic tools and implement appropriate control strategies for diarrhea in pigs.

In developing countries, such as Kenya or Uganda, with warm climates, high population densities and low hygiene levels the RV genotypes/genogroups distribution is very different from that in developed countries, such as USA. For example this study revealed high incidence RVC genogroup and P[13] genotype of RVA in USA while in East Africa RVA genogroup and P[8] genotype of RVA were the the most dominant.

This study also demostrated the zoonotic potential of RV which is of public health concern. The RVA G4 and P[7] genotypes detected in USA were closely related to human RV D151 (Mexico) and KJ25-1 (S. Korea) strains respectively (Chapter 5), while, the P[6] and P[8] genotypes of RVA detected in East Africa were closely related to human RVA DRC86 (Democratic Republic of Congo Africa) and Wa prototype resepectively (Chaper 6).

In conclusion, since RV infections cannot be diagnosed solely based on clinical signs, laboratory tests are necessary for reliable RV surveillance, and can be useful in clinical settings to avoid inappropriate use of antibiotics which cannot prevent viral diarrheas. Since it was not possible to characterize G and P genotypes for some RVA positive samples, especially those circulating in smallholder farms in Kenya and Uganda, which may be attributed to either low viral load in the fecal sample of asymptomatic pigs or variability in the primer binding sites due to genetic

diversity at the nucleotide level (Kerin *et al.*, 2007), it is necessary to constantly review and update genotyping primers as more sequencing data becomes available as was done in this study with USA samples. However, it was not possible to update primers for genotyping porcine RV circulating in East Africa since no data was available. Thus, this study recommends that the future work should include full genome sequencing of the RV circulating in East Africa region using next generation sequencing (Illumina miseq/454 pyrsequencing) to obtain more sequence data that could be used to design/update genotyping primers for the RV strains circulating in this region

Acquiring information on the epidemiology of RVAs, non-group A RVs and other viral enteric pathogens will be important to design effective prophylactic measures, to decrease the impact of enteric infections in swine and to minimize the financial loss for swine producers. Ultimately, the dominant rotavirus genogroups and/or genotypes should remain the prime targets for vaccine development.

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10 APPENDICES

Appendix 1 Questionnaire used to collect epidemiological data

Rotavirus Epidemiological Data Collection Sheet

Qno. ____

HH name ____ Date ____

Enumerator ____ County ____

Village ____

Location: Rural ____ Urban ____

Watering: Pond/river ____ Borehole ____ Tap ____

Production system: Free range ____ Semi-intensive/Backyard ____ Intensive/Commecialize ____ others specify ____

Feeding: Freerange ____ Swirl (kitchen/hotel) ____ Commercial feed ____

No. of Pigs: Boars ____ Sows ____ Weaners ____ Piglets ____

Labour: Family ____ Hired ____

Housing: Concrete floor ____ wooden floor ____ Raised slatted wooden floor ____ Earthen ____

Other animals kept: Cattle ____ Sheep/goats ____ dog/cats ____ Others ____

Fecal Samples from piglets

Sample ID	Animal ID	Age	Sex	Litter size	Breed	Health status		Vaccination status	
						Not Diarrheic	Diarrheic	Vaccinated	Not vaccinated

Appendix 2 GenBank accession numbers for each gene segment of RVC strains used in this study

Gene	Strain	Accession	Gene	Strain	Accession
VP6	RVC/Pig-wt/USA/Cowden/1980/G1Px	M94157	VP7	RVC/Pig-wt/ITA/118/05-1/2005/G1Px	EF464651
nucleotide	RVC/Pig-wt/KOR/CUK-5/2011/G5Px	HQ833829	nucleotide	RVC/Pig-wt/IRL/45/06/Cork/2006/G1Px	EU624405
	RVC/Pig-wt/KOR/CUK-6/2011/G1Px	HQ323753		RVC/Pig-wt/USA/Cowden/1980/G1Px	M61101
	RVC/Pig-wt/KOR/CA-2/2009/GxPx	GQ925781		RVC/Pig-wt/KOR/CUK-6/2011/G1Px	HQ323754
	RVC/Pig-wt/BRA/BRA61/10/2010/GxPx	JF810453		RVC/Pig-wt/USA/WH/xxxx/G1Px	U31749
	RVC/Pig-wt/BRA/BRA905/07/2007/GxPx	JF810449		RVC/cow-wt/USA/Shintoku/xxxx/G2Px	U31750
	RVC/Human-wt/NGA/Jajeri/1999-00/G4Px	AF325802		RVC/Cow-wt/JPN/Yamagata/2002/G2Px	AB108681
	RVC/Human-wt/NGA/Moduganari/1999-00/G4Px	AF325806		RVC/Pig-wt/USA/HF/xxxx/G3Px	U31748
	RVC/Human-wt/RUS/Nsk09-B43/2009/G4Px	GU592519		RVC/Human-wt/BRA/Belem/xxxx/G4Px	X72256
	RVC/Human-wt/KOR/CAU-10-312/2010/G4Px	HQ896714		RVC/Human-wt/KOR/CAU-10-312/2010/G4Px	HQ896715
	RVC/Human-wt/JPN/OH567/2003/GxPx	HQ185667		RVC/Human-wt/NGA/Jajeri/1999-00/G4Px	AF325805
	RVC/Human-wt/GBR/Preston/xxxx/G4Px	M94156		RVC/Human-wt/NGA/Moduganari/1999-00/G4Px	AF325806
	RVC/Human-wt/BRA/Belem/xxxx/G4Px	M94155		RVC/Human-wt/RUS/Nsk09-B43/2009/G4Px	JN934901
	RVC/Human-wt/GBR/Bristol/xxxx/G4Px	X59843		RVC/Human-wt/JPN/OK450/1989/G4Px	D87544
	RVC/Cow-wt/JPN/Yamagata/2002/G2Px	AB108680		RVC/Human-wt/GBR/Preston/xxxx/G4Px	X77258
NSP4	RVC/Human-wt/JPN/Ehime9301/1993/GxPx	D88353		RVC/Pig-wt/ITA/134/04-18/2004/G5Px	EF464653
nucleotide	RVC/Human-wt/JPN/BK0830/2008/GxPx	HQ185682		RVC/Pig-wt/KOR/CUK-5/2011/G5Px	HQ833830
	RVC/Human-wt/CHN/208/xxxx/G4Px	AB008673		RVC/Pig-wt/ITA/134/04-2/2004/G6Px	EF464655
	RVC/Human-wt/GBR/Bristol/xxxx/G4Px	X83967		RVC/Pig-wt/IRL/1GA/05/Cork/2005/G6Px	EU624403
	RVC/Human-wt/KOR/CAU-10-312/2010/G4Px	HQ896719		RVC/Pig-wt/IRL/281/07/Dublin/2007/G6Px	EU624404
	RVC/Human-wt/JPN/OH567/2003/GxPx	HQ185671		RVC/Pig-wt/ITA/344-04-7/2004/G6Px	EF464654
	RVC/Human-wt/IND/V508/2001/GxPx	AY770976		RVC/Pig-wt/ITA/43/06-16/2006/G6Px	EF464656
	RVC/Human-wt/RUS/Nsk09-B43/2009/G4Px	JN969080		RVC/Pig-wt/ITA/43/06-22/2006/G6Px	EF464657
	RVC/Pig-wt/USA/Cowden/1980/G1Px	AF093202/ AF093203			
VP4	RVC/Human-wt/KOR/CAU-10-312/2010/G4Px	HQ896713	VP4	RVC/Human-wt/KOR/CAU-10-312/2010/G4Px	AEJ21069
nucleotide	RVC/Human-wt/BGD/BS347/2005/G4Px	HQ185635	amino acid	RVC/Human-wt/BGD/BS347/2005/G4Px	ADP76599
	RVC/Human-wt/CHN/208/xxxx/G4Px	AB008670		RVC/Human-wt/CHN/208/xxxx/G4Px	BAB83827
	RVC/Human-wt/JPN/Y11-1/2011/G4Px	AB648917		RVC/Human-wt/JPN/Y11-1/2011/G4Px	BAK53408
	RVC/Human-wt/NGA/Jajeri/1999-00/G4Px	AF323980		RVC/Human-wt/NGA/Moduganari/1999-00/G4Px	AAK26531
	RVC/Human-wt/NGA/Moduganari/1999-00/G4Px	AF323981		RVC/Human-wt/NGA/Jajeri/1999-00/G4Px	AAK26532
	RVC/Pig-wt/USA/Cowden/1980/G1Px	M74218		RVC/Pig-wt/USA/Cowden/1980/G1Px	AAB00802
	RVC/cow-wt/USA/Shintoku/xxxx/G2Px	U26551		RVC/cow-wt/USA/Shintoku/xxxx/G2Px	AAB01672
	RVC/Pig-wt/KOR/CUK-5/2011/G5Px	HQ833828		RVC/Pig-wt/KOR/CUK-5/2011/G5Px	AEK20775
	RVC/Pig-wt/KOR/CUK-6/2011/G1Px	HQ833827		RVC/Pig-wt/KOR/CUK-6/2011/G1Px	AEK20774

Appendix 3 GenBank accession numbers for each gene segment (VP7 and VP4) of RVA field (bold) and available known strains used in this study

Gene	Reference Strains	Host	Accession No		Reference Strains	Host	Accession No
VP7	B3458	Human	EF990708	VP7	CMP178	Porcine	DQ515961
	CAU-1	Human	HQ198807		CMP213	Porcine	DQ786576
	D151	human	AJ488586		CMP77	Porcine	DQ683521
	CIT-176RV	Human	AF254139		FGP28	Porcine	AB573873
	Cr117	Human	AF450294		FGP35	Porcine	AB573875
	CUK1	Human	EF121951		FGP36	Porcine	AB573877
	IAL-28	Human	L79916		GUB72	Porcine	AB573649
	JP8485	Human	HQ230034		Hokkaido-14	Porcine	AB176677
	Melb-G9-19	Human	AY307089		HP113	Porcine	DQ003292
	MI09	Human	HQ018934		HP140	Porcine	DQ003293
	MS037-03	Human	AB436831		Ire/60/2007	Porcine	FJ617260
	R479	Human	DQ873680		JP13-3	Porcine	AB176679
	VN-9	Human	FJ598040		JP32-4	Porcine	AB176682
	1/07/Ire	Porcine	FJ617255		JP35-7	Porcine	AB176683
	A46	Porcine	L35054		Arg/P3	Porcine	AY115862
	CMP003	Porcine	AY707787		Arg/P14	Porcine	AY115861
	CMP121	Porcine	DQ683522		Arg4671	Human	FJ712692
	RVA/Pig-wt/USA/QW359/2004	**Porcine**	**KC244310**		**RVA/pig-wt/USA/RV0084/2011**	**Porcine**	**KC244286**
	RVA/Pig-wt/USA/QW362/2004	**Porcine**	**KC244311**		**RVA/pig-wt/USA/RV0120/2011**	**Porcine**	**KC244312**
	RVA/pig-wt/USA/RV0036/2011	**Porcine**	**KC244285**		**RVA/pig-wt/USA/RV0132/2011**	**Porcine**	**KC244313**
					RVA/pig-wt/USA/RV0146/2012	**Porcine**	**KC244287**
VP4	VMRI	Bovine	U53923	VP4	I321	Human	L07657
	GRV	Caprine	AB055967		K8	Human	D90260
	CH3	Equine	D25228		KTM368	Human	GU199495
	FI23	Equine	D16342		Mc323	Human	D38052
	L338	Equine	JF712558		Mc35	Human	D14032
	Cat97	Feline	EU708948		TB-Chen	Human	AY787644
	FRV64	Feline	D14723		US1205	Human	AF079356
	A_B10	Human	HM627556		Wa	Human	JX406750
	RMC321	Human	AF523677		Murine_EB	Murine	U08419
	116E	Human	L07934		Murine_EC	Murine	U08421
	69M	Human	M60600		Murine_EHP	Murine	U08424
	AU-1	Human	D10970		Lamb_L11599	Ovine	L11599
	B4106	Human	AY740738		Lamb_Lamb-NT	Ovine	FJ031027
	Dhaka6	Human	GU199520		134/04-15	Porcine	DQ061053
	DS-1	Human	HQ650119		344-04-1	Porcine	DQ242615

Gene	Reference Strains	Host	Accession No	Reference Strains	Host	Accession No
	Ecu534	Human	EU805773	61/07/Ire	Porcine	FJ492835
	G9_F45	Human	U30716	A46	Porcine	AY050274
	Ch-04V0027	Avian	EU486961	BMI-1	Porcine	L07887
	AvRV-2	Avian	JQ085405	CMP034	Porcine	DQ534016
	Ch-06V0661	Avian	EU486962	CMP178	Porcine	QD536362
	PO-13	Avian	AB009632	CMP213	Porcine	DQ786578
	Tu-03V0002E10	Avian	EU486958	FGP28	Porcine	AB573874
	993-83	Bovine	D16352	FGP35	Porcine	AB573876
	A5	Bovine	D13395	FGP36	Porcine	AB573878
	AzuK-1	Bovine	AB454420	GUB72	Porcine	AB573650
	C486	Bovine	Y00127	HP113	Porcine	DQ003290
	CACC	Bovine	AB486010	HP140	Porcine	QD003291
	Dai-10	Bovine	AB513836	JL94	Porcine	AY523636
	Hg18	Bovine	AF237665	PRG921	Porcine	JF796715
	KJ25-1	Bovine	HM988968	OSU	Porcine	X13190
	KV0407	Bovine	EU873009	PRG9121	Porcine	JF796737
	M63267	Bovine	M63267	Rhesus_TUCH	Simian	FJ816611
	M33516	Porcine	M33516	SA11	Simian	D16346
	MDR-13	Porcine	L07886	**RVA/pig-wt/USA/RV0146/2012**	**Porcine**	**KC244298**
	RVA/pig-wt/USA/QW359/2004	**Porcine**	**KC244301**	**RVA/pig-wt/USA/RV0147/2011**	**Porcine**	**KC244303**
	RVA/pig-wt/USA/QW362/2004	**Porcine**	**KC244290**	**RVA/pig-wt/USA/RV0148/2011**	**Porcine**	**KC244304**
	RVA/pig-wt/USA/RV0036/2011	**Porcine**	**KC244291**	**RVA/pig-wt/USA/RV0154/2011**	**Porcine**	**KC244305**
	RVA/pig-wt/USA/RV0042/2011	**Porcine**	**KC244292**	**RVA/pig-wt/USA/RV0160/2012**	**Porcine**	**KC244299**
	RVA/pig-wt/USA/RV0044/2011	**Porcine**	**KC244302**	**RVA/pig-wt/USA/RV0161/2012**	**Porcine**	**KC244288**
	RVA/pig-wt/USA/RV0047/2011	**Porcine**	**KC244293**	**RVA/pig-wt/USA/RV0254/2011**	**Porcine**	**KC244306**
	RVA/pig-wt/USA/RV0084/2011	**Porcine**	**KC244294**	**RVA/pig-wt/USA/RV0258/2011**	**Porcine**	**KC244307**
	RVA/pig-wt/USA/RV0120/2011	**Porcine**	**KC244295**	**RVA/pig-wt/USA/RV0262/2011**	**Porcine**	**KC244300**
	RVA/pig-wt/USA/RV0132/2011	**Porcine**	**KC244296**	**RVA/pig-wt/USA/RV0263/2012**	**Porcine**	**KC244289**
	RVA/pig-wt/USA/RV0145/2012	**Porcine**	**KC244297**	**RVA/pig-wt/USA/RV0264/2012**	**Porcine**	**KC244308**

Appendix 4 GenBank accession numbers for VP4 gene segment of RVA field (bold) and available known strains used in this study.

Strains	P-types	Host	Accession no.
Hu/BEL/BE2001/2009/G9P6	P[6]	Human	JQ993319
Human_R479	P[6]	Human	GU189554
Human_Ryukyu-1120	P[6]	Human	AB741652
Porcine_MDR-13	P[13]	Porcine	L07886
Porcine_FGP28	P[13]	Porcine	AB573874
Porcine_FGP35	P[13]	Porcine	AB573876
Porcine_A46	P[13]	Porcine	AY050274
Porcine_GUB72	P[13]	Porcine	AB573650
Porcine_HP140	P[13]	Porcine	QD003291
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	P[8]	Human	JN849113
RVA/Human-wt/AUS/CK00063/2007/G1P[8]	P[8]	Human	JX027722
RVA/Human-wt/Bel/BE00001/2004/G1P[8]	P[8]	Human	KC193615
Human-Wag7/8re-G1,P1A[8]	P[8]	Human	FJ423138
RVA/Human-wt/USA/DC412/1974/G1P[8]	P[8]	Human	KC579545
Porcine_PRG921	P[23]	Porcine	JF796715
Porcine_PRG9121	P[7]	Porcine	JF796737
Porcine_OSU	P[7]	Porcine	X13190
Porcine_Gottfried	P6	Porcine	M33516
Ke-003-5	P[6]	Porcine	**KJ469876**
Ug-49	P[13]	Porcine	**KJ469877**
Ug-453	P[13]	Porcine	**KJ469878**
Ke-063	P[8]	Porcine	**KJ469879**
Ke-206	P[8]	Porcine	**KJ469880**
Ke-451	P[8]	Porcine	**KJ469881**
Ke-460	P[8]	Porcine	**KJ469882**
Ke-456	P[8]	Porcine	**KJ469883**
Ke-450	P[8]	Porcine	**KJ469884**
Ke-307	P[8]	Porcine	**KJ469885**
Ke-293	P[8]	Porcine	**KJ469886**
Ke-118	P[8]	Porcine	**KJ469887**
Ke-Damaris-3	P[8]	Porcine	**KJ469888**
Ke-Kuoba-3	P[8]	Porcine	**KJ469889**
Ke-214-2	P[8]	Porcine	**KJ469892**
Ug-218	P[8]	Porcine	**KJ469890**
Ug-231	P[8]	Porcine	**KJ469891**
Ug-339	P[8]	Porcine	**KJ469893**