Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Animal Science
Department of Biomedical Sciences and Veterinary Public Health

Porcine viruses in Uganda

– a study of TTSuV and PPV4 in wild and domestic pigs

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Sammanfattning

Uganda har östra Afrikas största grispopulation, och de flesta djuren ägs av småbrukare. Infektionssjukdomar bland grisar har en förödande effekt på försörjningen för dessa lantbrukare, som är i stort behov av avkastningen från grisnäringen. I en metagenomikstudie av den vilda grisarten bushpig (*Potamochoerus larvatus*), påvisades grisvirusen Torque teno sus virus (TTSuV) 1 och 2 och Porcint parvovirus 4 (PPV4). TTSuV1 och 2 är allmänt utbredda i världens grispopulation, och PPV4 har hittats i USA och Kina, men inget av dem har studerats i Afrika tidigare. Om virusen har förmåga att orsaka sjukdom är okänt. Syftet med detta projekt var att göra en första uppskattning av prevalensen av dessa virus hos grisar i Uganda, och att genetiskt karaktärisera virus påvisade i blod från bushpigs. Prevalensstudien gjordes på serumprov från tamsvin med PCR, med genotypspecifika primers för TTSuV och PPV4-primers som utformats från en PPV4-sekvens från bushpig. Positiva prover sekvenserades och fylogenetiska analyser utfördes i syftet att jämföra de ugandiska sekvenserna med andra redan publicerade sekvenser. Prevalensen av TTSuV1 och 2 i tamsvinen i denna studie var 16,7% respektive 47,9% med en co-infektion i 14,6% av grisarna. PPV4 hittades inte hos tamsvin genom användandet av bushpig-primrar. Genomet för TTSuV2 från bushpig karaktäriserades till 55% och befanns vara 66-74% identiskt med andra TTSuV2-sekvenser. Genomet för PPV4 från bushpig karaktäriserades till 70% och var 58% identiskt med tillgängliga PPV4-sekvenser. Ytterligare studier måste utföras för att karaktärisera hela virusgenomen från bushpig, och för att utvärdera omfattningen och betydelsen av virusutbyte mellan bushpigs och tamsvin i Uganda.

Nyckelord: Grisvirus, Torque teno sus virus (TTSuV), Porcint parvovirus 4 (PPV4), prevalens, bushpig (*Potamochoerus larvatus*), Uganda

Abstract

Uganda has the largest pig population in east Africa, and most of the animals are owned by smallholders. Infectious diseases among pigs have a devastating impact on the livelihood of these farmers, which are dependent on the proceeds from pig rearing. In a metagenomics study of the wild pig species bushpig (*Potamochoerus larvatus*), the porcine viruses Torque teno sus virus (TTSuV) 1and 2 and Porcine parvovirus 4 (PPV4) were detected. TTSuV1 and 2 are ubiquitous in the world's swine population, and PPV4 has been found in USA and China, but neither of them has been studied in Africa previously. The pathogenic properties of these viruses are unknown. The aim of this project was to do a first estimate of the prevalence of these viruses in pigs in Uganda, and to genetically characterize viruses detected in samples from bushpigs. The prevalence studies were made on serum samples from domestic pigs using PCR with genotype specific primers for TTSuV and PPV4 primers designed from the bushpig PPV4 sequence. Positive samples were sequenced, and phylogenetic analyses were performed in order to compare the Ugandan sequences with others already published. The prevalence of TTSuV1 and 2 in the studied domestic pigs was estimated at 16.7% and 47.9% respectively, with a co-infection of 14.6%. PPV4 was not found in domestic pigs with the bushpig primers. The bushpig TTSuV2 genome was characterized to 55% and was found to be 66-74% identical with other TTSuV2 sequences. The bushpig PPV4 genome was characterized to 70% and was 58% identical with available PPV4 sequences. Further studies must be performed to characterize the entire virus genomes from bushpig, and to evaluate the magnitude and significance of viral exchange between bushpigs and domestic pigs in Uganda.

Keywords: Porcine viruses, Torque teno sus virus (TTSuV), Porcine parvovirus 4 (PPV4), prevalence, bushpig (*Potamochoerus larvatus*), Uganda

Table of contents

1 Introduction

1.1 Emerging infectious diseases

Out of the emerging infectious diseases that have affected humans during the last decades, as much as 75% have originated from animals. These animal diseases, caused by viruses, bacteria or parasites which can be transmitted to humans, are known as zoonoses. Well known examples of zoonotic diseases include SARS and avian influenza, caused by animal viruses that evolved to cause severe disease in humans as well. According to the World Health Organization (WHO), an *emerging infectious disease* is "[a disease] that has appeared in a population for the first time, or that may have existed previously but is rapidly increasing in incidence or geographic range" (WHO, FAO & OIE, 2004). Factors leading to the onset of emerging infectious diseases include environmental changes, demography of humans and domestic and wild animals and ecological changes such as the expansion and globalization of agriculture. As humans occupy new territories, microbes that have since long resided in their natural hosts are given the opportunity to invade a new host species, leading to human disease (Morse, 1995). Human demography and agricultural expansion can also cause emerging diseases among both wild and domestic animals (Daszak et al., 2000). One example is African swine fever (ASF), a viral hemorrhagic fever that was first recognized by European settlers bringing domestic pigs (*Sus scrofa*) to Africa (Jones et al., 1997). ASF is fatal in almost 100% of the cases in domestic pigs, but the natural host of the ASF virus on the other hand, the warthog (*Phacochoerus africanus*), shows no signs of disease. (Jori & Bastos, 2009). As livestock production is increasingly intruding upon the habitats of wild species, the possibilities for new emerging diseases to occur among animals will increase as well (Daszak et al, 2000).

1.2 Pig farming in Uganda

The well-being of domestic animals is an important economic issue, and a question of survival for millions of livestock owners. In Uganda, an east-African country with approximately 32 million inhabitants, three-fourth of the households are subsistence farmers, farming to cover their own needs with little surplus. Most of the country's livestock is owned by these smallholders, out of which 45% are considered to be poor (FAO & AGAL, 2005). Pig production has been the largest growing livestock category in Uganda since the 1980's, and the country is now reported to have the largest pig population in whole east Africa (ILRI, 2009). Disease among domestic pigs will therefore have a devastating impact on the livelihoods of the poor farmers that make up the vast majority of the country's pig owners.

Figure 1. Pigsty in village in Busia district, Uganda, housing a pig of local breed. Private photo.

1.3 Viruses in wild species

Preparedness is an important factor in managing emerging infectious diseases, including identifying risk factors beforehand (WHO, FAO & OIE, 2004). Studying which viruses are circulating in wild porcine species is one approach in monitoring potential risks. A metagenomics study of pooled serum samples from three bushpigs caught in Uganda during 2010 (Blomström, unpublished data) revealed the

presence of several different porcine viruses, among them Torque teno sus virus 1 (TTSuV1) and 2 (TTSuV2) and Porcine parvovirus 4 (PPV4), which have previously been studied in domestic pigs (Leary et al., 1999; Niel et al., 2005; Cheung et al., 2010). The bushpig (*Potamochoerus larvatus*) (Figure 2) is a wild pig species belonging to the family Suidae, just like the domestic pig and the common warthog. Bushpigs are found in southeast Africa, where they live in forests and shrublands with dense vegetation (Seydack, 2008).

Figure 2. Bushpig with piglet. Photo:Michael Jefferies (http://www.flickr.com/photos/ogcodes/384294209). Used with permission according to Creative Commons license (http://creativecommons.org/licenses/by-nc/2.0/deed.en).

Most studies that have been conducted on bushpigs concern their role as reservoirs for ASF. However, the nocturnal lifestyle of bushpigs and their habit of not living in burrows makes them difficult to study, and they are considered less important in the epidemiology of ASF as they exists in lower numbers than warthogs do. It has also been claimed that bushpigs are less likely to come in contact with domestic pigs than warthogs are, but nevertheless, in rural areas bushpigs and domestic animals do encounter, as bushpigs are attracted to crop land and are hunted and brought to villages as game (Jori & Bastos, 2009). The often relatively simple housing of domestic pigs in Uganda (Figure 1), and common practice of letting pigs range free on the farm do also increase the possibility of contact between bushpigs and domestic pigs.

1.4 Torque teno sus virus

Swine TTSuV has a 2.9 kb circular, single-stranded DNA genome (Okamoto et al., 2002). TTSuV was first discovered in pigs in 1999 (Leary et al., 1999), but there is evidence that it has been circulating in Spanish pigs since at least 1985 (Segalés et al., 2008). TTSuV, belonging to the genus *Anellovirus*, has been classified into two genogroups: TTSuV1 and TTSuV2 (Niel et al., 2005), though more recent research has suggested that TTSuV1 and TTSuV2 should actually be classified as different species, due to the low sequence identity between them (Huang, Y.W. et al., 2010).

TTSuV have 3 open reading frames (ORFs): ORF1 coding for the capsid, ORF2 for a replication protein and ORF3, which is produced from a splicing event, coding for a protein of so far unknown function (Kekarainen & Segalés, 2009).

Both TTSuV species are believed to be common in swine populations all over the world (Niel et al., 2005). A study of the prevalence of TTSuV1 in six different countries in North America, Europe and Asia showed that between 33% and 100% of the analyzed pigs tested positive for TTSuV1 DNA (McKeown et al., 2004). Serum samples from Spanish pigs collected between 1985 and 2005 showed an overall prevalence of 33.3% for TTSuV1 and 55.6% for TTSuV2. A co-infection of both species occurred in 23.5% of the cases (Segalés et al., 2008). In another study in Spain, where samples consisted of serum from pigs with symptoms like wasting and diarrhea, a co-infection was seen in 55% of the cases (Kekarainen et al., 2006). DNA from TTSuV1 and 2 have also been shown to be common in European wild boar (Martínez et al., 2006).

TTSuV has so far not been shown to cause disease on its own, and its pathogenic potential is debated. Torque teno virus of a strain which is different from those infecting swine is known to frequently infect humans. Epidemiologically, this virus has been linked to both liver and respiratory conditions as well as cancer, but no direct evidence has been presented (Okamoto, 2009). The porcine virus has been suspected to be involved as a trigger of the swine disease Post-weaning multisystemic wasting syndrome (PMWS), along with the causative agent Porcine circovirus type 2 (PCV-2). Pigs with PMWS were shown to have a 1.25 times higher risk than healthy pigs of being infected with TTSuV2, with a TTSuV2 prevalence of 91% in sick pigs and only 72% in healthy ones. This difference was however not significant for TTSuV1 (Kekarainen et al., 2006). Another study of PMWS-affected pigs did on the other hand not find a higher prevalence of TTSuV2 in the sick pigs (Blomström et al., 2010).

1.5 Porcine parvovirus 4

PPV4 was first discovered in association with an outbreak of an acute-onset disease of domestic pigs in North Carolina, USA, that took place in 2005 (Cheung et al., 2010) and was later also found in Chinese swine herds (Huang, L. et al., 2010). The virus has as a linear, single-stranded DNA genome of approximately 5.4-5.9 kb. PPV4 is a member of the family *Parvoviridae*, subfamily *Parvovirinae*, but the genus classification is uncertain. The virus with closest sequence similarity to PPV4 is Bovine parvovirus 2 (BPV2), but the genome organization of PPV4 resembles that of Bocaviruses. In addition to the ORFs generally found in viruses from the subfamily *Parvovirinae*, ORF1 coding for a replicase and ORF2 for the capsid, PPV4 has an additional ORF, just like the genus *Bocavirus* do, which encodes a 204 amino acid long protein with unknown function. ORF3, situated between ORF1 and 2 in the PPV4 genome, is however only 4.9-11.2% similar to Bocaviruses ORF3 on amino acid level (Cheung et al., 2010).

The prevalence of PPV4 in the Chinese study was 2.09% in 573 tested sick pigs suffering from symptoms like trembling, fever and death, and 0.76% in 132 healthy pigs. The sequence similarity between the American and Chinese isolates was high and it was suggested that PPV4 was introduced in China through the importation of pigs from North Carolina (Huang et al., 2010). The pathogenicity of PPV4, and whether it is capable of causing disease on its own, remains to be resolved (Cheung et al., 2010).

1.6 Purpose of the project

This project had two main aims: to do a first estimate of the prevalence of TTSuV1, TTSuV2 and PPV4 among pigs in Uganda and to genetically characterize viruses from bushpigs and compare them with previously characterized isolates from other parts of the world. Both TTSuV1 and TTSuV2 are ubiquitous in domestic pig populations in America, Europe and Asia, but the situation in Africa for these viruses has not been studied previously.

2 Materials and methods

2.1 Samples

Serum samples from 96 domestic pigs, three bushpigs and one warthog were analyzed. Bushpigs are, as described previously, difficult to study and in order to obtain serum samples from them both permission from Uganda Wildlife Authority and advanced trapping equipment and skills are needed. In lack of extensive bushpig material to analyze, prevalence studies were made on domestic pigs from farms in different parts of Uganda. The domestic pig samples were collected during year 2010 and 2011, as a part of an African swine fever research project (Figure 3). Half of the samples came from farms that were suspected to have ongoing outbreaks of ASF.

Figure 3. Collection of blood samples from pig in village in Busia district, Uganda. Private photo.

The bushpigs and the warthog were sampled in Lake Mburo National Park, Uganda, during 2010. The bushpigs were the same as those sampled in the metagenomics study by Blomström et al. (personal communication) mentioned in section 1.3.

2.2 RNA and DNA extraction

RNA was extracted from part of the samples using TRIzol (Invitrogen) according to the manufacturer's instructions. The RNA was stored in -80 ºC awaiting further use. From another set of samples, RNA was extracted using a TRIzol protocol combined with a Viral RNA mini kit (Qiagen). DNA was then further extracted from the TRIzol with a combination of a TRIzol protocol and QIAamp DNA mini kit. Seven hundred fifty microlitre back extraction buffer (4 M Guanidine Thiocyanate, 50 mM Sodium Citrate, 1 M Tris) was added to the part of the sample that was left after having removed the aqueous RNA phase, and samples were mixed by inversion for 3 minutes and centrifuged for 30 minutes at 12 000 x g. The aqueous upper phase now containing DNA was transferred to a new tube and mixed with the same volume 70% ethanol before it was loaded onto a DNeasy Mini Spin Column and centrifuged at 12 000 x g for 30 seconds. The column was washed with 500 µl AW1 and AW2 buffer respectively before the DNA was finally eluted in 50 µl AE buffer. From a majority of the samples however, extraction was made using DNeasy Blood & Tissue kit (Qiagen) according to the protocol "Purification of Total DNA from Animal Blood or Cells" provided by the manufacturer. Two hundred microlitre serum was used from each sample and the DNA was eluted in 100 µl AE buffer. The extracted DNA was stored in -20 ºC until further use.

2.3 Virus detection

The presence of TTSuV1, TTSuV2 and PPV4 was detected using conventional polymerase chain reaction (PCR). For TTSuV1 and TTSuV2, genotype specific primers described by Segalés et al. (2009) were used. PPV4 primers had been designed from the PPV4 sequence obtained from bushpigs in the metagenomics study by Blomström et al. (personal communication). Each reaction consisted of 1x PCR buffer, 2.5 mM $MgCl_2$, 1.0 mM dNTP, 0.4 µM F primer, 0.4 µM R primer and 1.25 u AmpliTaq Gold DNA polymerase. Two or 4 µl of extracted DNA was added, depending on the DNA concentration achieved from the two different ex-

traction methods. Amplification was done with the following reaction conditions: a 12 minute denaturating step at 95 ºC followed by 39 cycles of 95 ºC for 30 seconds, 58 ºC for 30 seconds and 72 ºC for 90 seconds, finishing with 10 minutes of 72 ºC. The PCR products were visualized on a 1.3% agarose gel with ethidium bromide. The PCR positive products were then purified using a PCR purification kit (Qiagen) and eluted in 30 µl EB. The purified products were sent to Macrogen, Korea, for sequencing.

2.4 Genetic characterization – TTSuV2 and PPV4 from bushpig

In order to characterize TTSuV2 and PPV4 from bushpig, the bushpig samples were subjected to PCR with primers meant to cover more of the genomes. PCR conditions were the same as for the virus detection, as was the purification of the product and sequencing. Half of the sequences used for characterization were produced in another study (Blomström et al., unpublished data) and these were analyzed like the rest.

2.5 Phylogenetic studies

The sequenced PCR products were edited in SeqMan (Lasergene 9, DNASTAR). The edited sequences were subsequently aligned by ClustalW in BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and the sequence identity was compared. The phylogenetic relationship among TTSuV1 and TTSuV2 sequences from this study was compared to previously published sequences available from GenBank (http://www.ncbi.nlm.nih.gov/genbank) using Mega 5 (Tamura et al. 2007). Also PPV4 was compared to PPV4 sequences from GenBank, both on DNA and protein level. The PPV4 capsid gene was translated using Sixpack (EMBOSS Explorer).

3 Results

3.1 Prevalence of TTSuV1 and TTSuV2

TTSuV1 and TTSuV2 were not detected by the PCR assay in neither the bushpig samples nor in the warthog sample using the genogroup specific primers described by Segalés et al. (2009). In the serum samples from domestic pigs however, TTSuV1 was found in 16 out of 96 samples (16.7%) and TTSuV2 in 46 out of 96 samples (47.9%). A co-infection of both the species was seen in 14 out of 96 samples (14.6%). Fifteen out of 16 TTSuV1-positive samples and 43 out of 46 TTSuV2-positive samples had high enough DNA concentration to be sent for sequencing. The TTSuV prevalence for each district where positive samples were found is presented in Table 1. A geographical location of districts with positive samples can be seen in Figure 4.

Table 1. TTSuV1 and 2 prevalence in districts of Uganda where positive samples were found

	District					
	Gulu	Lira	Luwero	Masaka	Mitanya	Rakai
No. of samples	21	8	7	24	11	24
TTSuV1 pos.	6 (29%)	0	2 (29%)	2 (8%)	3 (27%)	3 (13%)
TTSuV2 pos.	9 (43%)	7 (88%)	6 (86%)	8 (33%)	6 (55%)	10 (42%)
Co-infected	4 (19%)	0	2 (29%)	2 (8%)	3 (55%)	2 (8%)

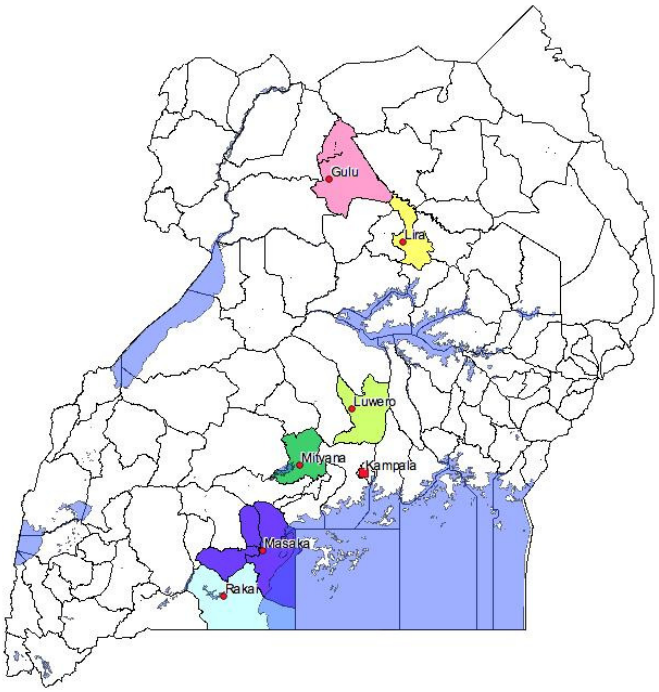

Figure 4. Map of Uganda showing the districts were TTSuV1 and TTSuV2 were found in the present study.

3.2 Phylogenetic studies of TTSuV1 and TTSuV2 in domestic pigs

Fourteen TTSuV1 sequences and 36 TTSuV2 sequences of the amplified 5' UTR region were used for sequence analysis. The rest were omitted due to low sequence quality. The TTSuV1 sequences in this study, from pigs from different regions of Uganda, show a sequence similarity of 89-100% to each other. When compared to 17 TTSuV1 sequences from GenBank (accession numbers are found in Figure 5 and 6), originating from other parts of the world, the identity ranges from 90 to 97%. A phylogenetic analysis of the TTSuV1 sequences (Figure 5) confirms the fact that the sequences from Uganda are as divergent from each other as they are from sequences from other parts of the world. No geographical clustering is visible, except for one quite well-supported clade containing 5 Ugandan sequences. Village names and districts corresponding to sample abbreviations can be found in Table 2.

The TTSuV2 sequences from this study have a sequence similarity of 60-100%. When compared to 11 TTSuV2 sequences from America, Asia and Europe the similarity is 61-99%. A phylogenetic analysis (Figure 6) does not give any distinct geographical grouping.

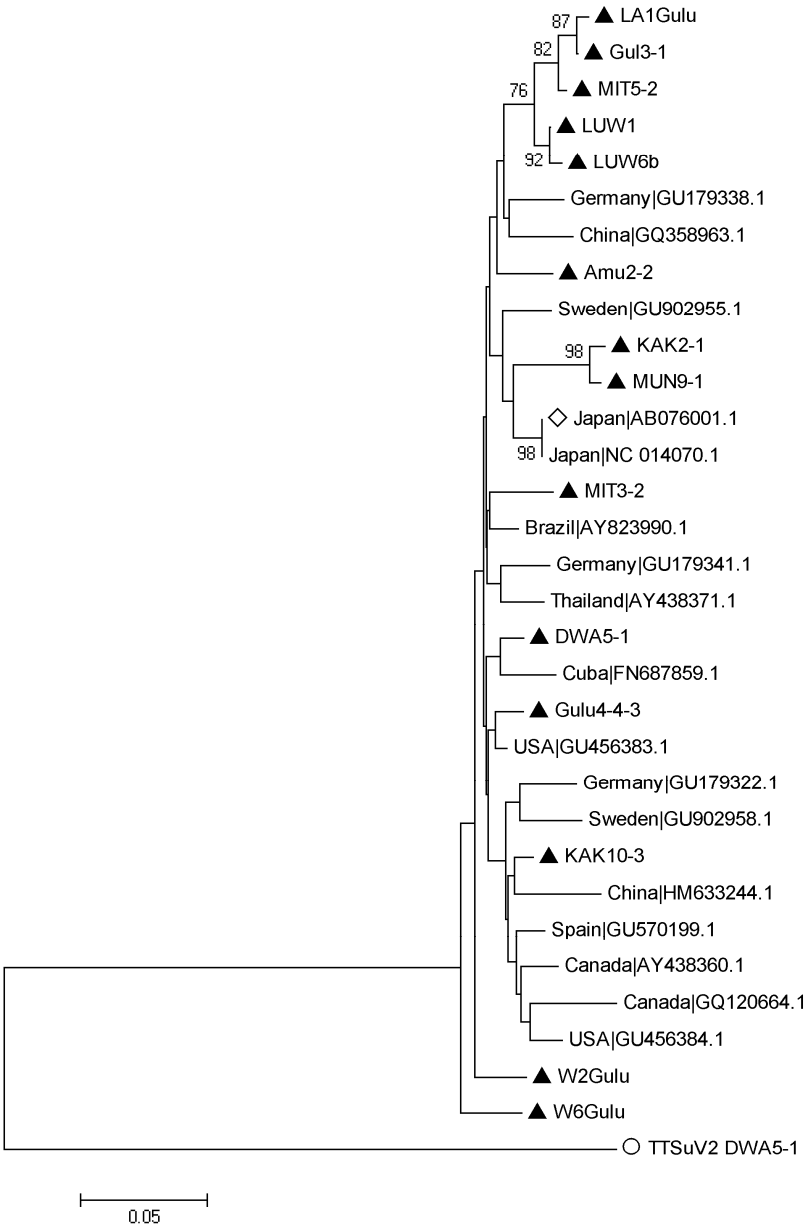

Figure 5. Phylogenetic analysis of TTSuV1 based on a 206 nucleotide long sequence from the 5' UTR region, using Neighbour-joining and a bootstrap value of 1000. Only bootstrap values above 70 are shown. The sequences from this study are marked with ▲. The sequence marked with ◇ is a TTSuV1 reference sequence, and the one marked ○ is a TTSuV2 sequence used as an outgroup.

Figure 6. Phylogenetic analysis of TTSuV2 based on a 185 nucleotide long sequence from the 5' UTR region, using Neighbour-joining and a bootstrap value of 1000. Only bootstrap values above 70 are shown. The sequences from this study are marked with ▲. The sequence marked with ◇ is a TTSuV2 reference sequence, and the one marked ○ is a TTSuV1 sequence used as an outgroup.

Table 2. Key to sample abbreviations and their corresponding villages and districts

Abbreviation	Village	District
Buk	Bukakata	Masaka
BUW	Buwunga	Masaka
DWA	Dwaniro	Rakai
Gul	Gulu	Gulu
KAK	Kakuuto	Rakai
Kal	Kalungo	Masaka
KAS	Kasasa	Rakai
KBR	Kabira	Rakai
KIR	Kirumba	Rakai
KIS	Kisekka	Masaka
KIT	Kitanda	Masaka
KLZ	Kaliziso	Rakai
KYN	Kyanamukaka	Masaka
LIR	Lira	Lira
LUW	Luwero	Luwero
LWN	Lwanda	Rakai
MIT	Mityana	Mitanya
MUD	Mityana	Mitanya
Muk	Mukungwe	Masaka
MUN	Masaka	Masaka
NAB	Nabigasa	Rakai

3.3 Prevalence of PPV4

PPV4 was detected by PCR in one out of the three serum samples from bushpig. No PPV4, using the PPV4 bushpig primers, was detected from domestic pigs or the warthog.

3.4 Genetic characterization of PPV4 and TTSuV2 from bushpig

3.4.1 PPV4

The bushpig PPV4 sequence was assembled in SeqMan with two partial PPV4 genome sequences obtained from Blomström et al. (personal communication) resulting in a 3758 nucleotide long contig, covering almost 70% of the total genome.

Both PPV4 ORF1 and ORF2, coding for a replicase and the capsid respectively, were partly covered. Between them lies ORF3, which encodes a 204 amino acid long protein with unknown function (Cheung et al., 2010). A partial replicase gene comparison (818 nucleotides out of 1796 nucleotides of the complete replicase) showed that the bushpig sequence is 62% identical with 8 other PPV4 sequences available on GenBank (accession numbers are found in Figure 7). These other sequences are in turn 99-100% identical. A partial capsid gene comparison (2081 nucleotides out of 2186) showed that the bushpig sequence is only 58% identical with the other PPV4 sequences on this region. On an amino acid level, the similarity is 52%. A phylogenetic analysis of the PPV4 sequences was performed together with PPV1, 2, 3, and a Porcine boca-like virus (Pbo-likeV) and Bovine parvovirus 2 (BPV2) which both have been shown have some sequence identity with known PPV4 sequences (Huang, L. et al., 2010). Bushpig PPV4 clusters together with the other PPV4 sequences in the Neighbour-joining tree in the partial capsid comparison, but is still found in its own clade (Figure 7). The sequence identity between the PPV4 sequences and BPV2 is 42% for the bushpig virus and 45% for domestic pig virus regarding the partial capsid gene.

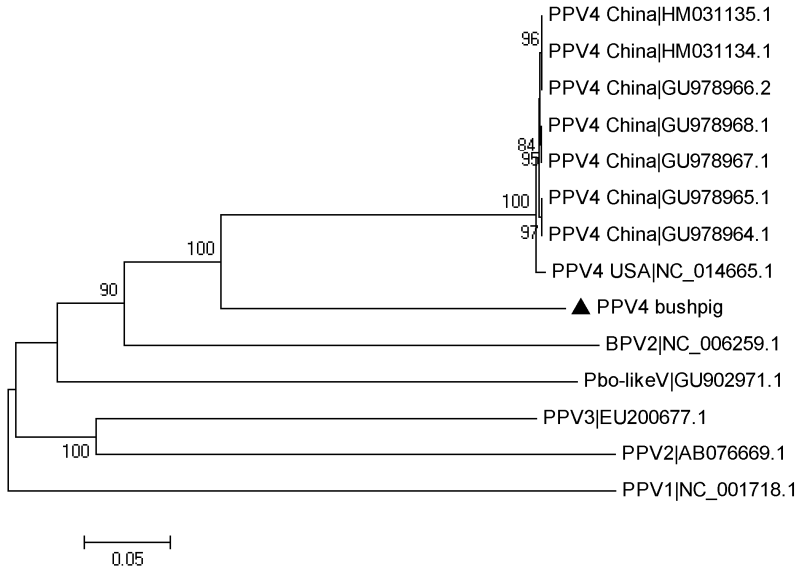

Figure 7. Phylogenetic analysis of PPV4 from bushpig and PPV1, 2, 3 and 4 from domestic pigs. Included is also Bovine parvovirus 2 (BPV2) and Porcine boca-like virus (Pbo-likeV). The analysis is based on a partial capsid DNA comparison (1797 nucleotides), using Neighbour-joining and a bootstrap value of 1000. Only bootstrap values above 70 are shown. The sequence from this study is marked with ▲.

Bushpig PPV4 ORF3 was compared to ORF3 from PPV4 of domestic pigs, 4 different porcine bocaviruses, a human bocavirus, gorilla bocavirus, bocavirus from dog and BPV2 (accession numbers are found in Figure 8). The nucleotide sequence identity of bushpig PPV4 was 44% compared to the other PPV4 sequences, and 15-33% with the bocavirus sequences. A phylogenetic analysis (Figure 8) shows a similar grouping of sequences as in Figure 7 where the capsid gene was compared, with bushpig PPV4 forming its own clade, and BPV2 being the closest related sequence to all PPV4 sequences. All other boca or boca-like sequences included in the analysis groups together.

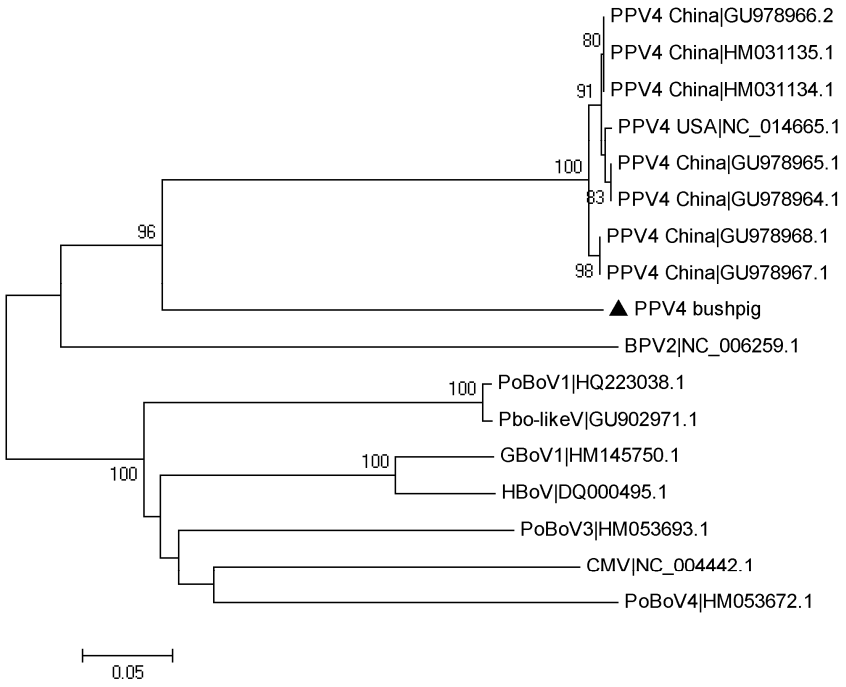

Figure 8. Phylogenetic analysis of ORF3 from PPV4 and members of the genus *Bocavirus*, based on a 441 nucleotide long stretch from the ORF, using Neighbour-joining and a bootstrap value of 1000. Only bootstrap values above 70 are shown. The sequence from this study is marked with ▲. Bovine parvovirus (BPV), porcine bocavirus (PoBoV), porcine boca-like virus (Pbo-likeV), gorilla bocavirus (GBoV), human bocavirus (HBoV), canine minute virus (CMV).

3.4.2 TTSuV2

Two TTSuV2 sequences obtained from Blomström et al. (personal communication) were assembled in SeqMan to cover 1550 nucleotides, or 55%, of the genome. The segment is located within TTSuV2 ORF1, covering approximately 80% of the capsid-coding ORF. This sequence is 66-74% identical with 18

TTSuV2 sequences from GenBank (accession numbers are found in Figure 9). A phylogenetic analysis (Figure 9) shows that the bushpig sequence does not group with any of the others, but forms its own clade.

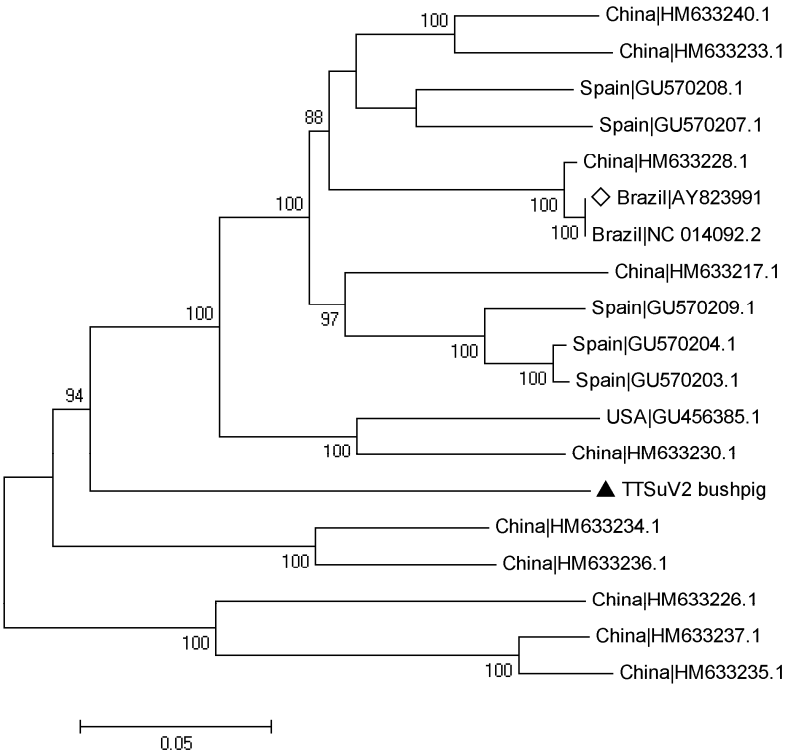

Figure 9. Phylogenetic analysis of TTSuV2 from bushpig and previously studied domestic pigs based on a 1568 nucleotide long sequence, using Neighbour-joining and a bootstrap value of 1000. Only bootstrap values above 70 are shown. The sequence from this study is marked with ▲. The sequence marked with ◇ is a TTSuV2 reference sequence.

4 Discussion and Conclusions

The objective of this project was to estimate the prevalence of TTSuV and PPV4 among pigs in Uganda, and to genetically characterize these viruses from bushpig, a wild Suidae species.

The prevalence study of TTSuV1 and TTSuV2 among domestic pigs indicates that TTSuV infection is common in Uganda, though the prevalence of TTSuV1 (16.7%) is lower than in studies from Spain (33.3-90% in different studies), Canada (46%), Thailand (40%) and the Czech Republic (43%) etc., studies which are based on sera from between 20 and 212 pigs (cf. McKeown et al., 2004; Segalés et al., 2009; Jarosova et al., 2011). The prevalence of TTSuV2 in this study (47.9%) was not as high as in Spain (56%), but corresponds well to the prevalence in the Czech Republic (47%) (McKeown et al., 2004; Jarosova et al., 2011). The co-infection rate of TTSuV1 and 2 was high; only 2 out of 16 TTSuV1-positive pigs were not at the same time infected with TTSuV2.

Previous studies have shown that TTSuV lacks an apparent geographical clustering (McKeown et al., 2004), and this is further confirmed by the present study. The phylogenetic analyses of TTSuV1 (Figure 5) and TTSuV2 (Figure 6) displays the Ugandan sequences evenly distributed among the isolates from other parts of the world. TTSuV seems to have a stable genome with slow evolution rate. Bootstrap values were however generally low for both strains, meaning that the branching patterns of the trees are not highly confident. Using the ORF1 capsid gene as a marker, which has been suggested previously (Segalés et al., 2009), could possibly give a better idea of the evolutionary origin of the sequences, since the capsid gene presumably is under purifying selection pressure.

As TTSuV is widely prevalent in the world's population of domestic pigs as well as in European wild boar, it would not be unexpected to find TTSuV also in the African bushpig. Indeed, TTSuV2 was detected from a pooled sample of the 3 bushpigs in a viral metagenomics study (Blomström et al., unpublished data).

However, the genotype specific primers for TTSuV1 and 2 based on the 5' UTR region that was used for the domestic pigs in this study did not manage to pick up on TTSuV from the same bushpigs. Possibly, this sequence is to diverge in the bushpig virus, and the UTR region was not covered in the genetic characterization of TTSuV2 using sequences from the metagenomics study. Further studies should be made to cover the missing pieces of this circular genome from the bushpig virus, using the known flanking regions in order to design suitable primers. Being able to compare the viral sequences from domestic pigs in Uganda with the bushpig sequence would be very interesting from a phylogenetic point of view. When the 1550 nt TTSuV2 sequence originating from Blomström et al. (unpublished data) were compared to complete genome sequences of TTSuV2 from previous studies of domestic pigs, the bushpig virus showed a moderate sequence identity (Figure 9).

The pathogenicity of TTSuV is unclear, though several studies concern the effect of co-infection of TTSuV1 and 2 with known pathogenic viruses like PCV-2, the causative agent of PMWS (Kekarainen et al., 2006; Ellis et al., 2008). These studies showed among other things a higher prevalence of TTSuV2 in pigs with PMWS, though this has later been questioned (Blomström et al. 2010). When the samples from the current study were screened for ASFV, 17 out of 96 samples were found to be positive for ASFV (Nyberg; Ståhl; personal communication, 18 May 2011). The prevalence of TTSuV among these 17 samples was 23.5% for TTSuV1 and 47.1% for TTSuV2, not significantly different from the 16.7% and 47.9% respectively found it the entire sample set. Twelve percent of the ASFV-positive samples were positive for both TTSuV strains. Consequently, this study does not indicate any connection between TTSuV and ASFV infection. To actually determine whether such a connection does exist or not, and the effect of the co-infection, would require more extensive research.

PPV4 was not found in the domestic pig population in Uganda in this study. However, the primers used were designed for bushpig PPV4 and a BLAST search (Altschul et al., 1997) of the primers gave no significant hits for PPV4. If PPV4 exists in the domestic pig population and it resembles the isolates from China and USA, it might be too divergent from the bushpig virus to be detected in the assay with bushpig primers. The current sample set might also be too small, as the prevalence was only 0.76% among healthy pigs in the Chinese study (Huang, L. et al., 2010).
While the Chinese and American isolates show a very high sequences identity, the bushpig PPV4 isolate is only 58-62% identical in the coding regions on nucleotide

level, and only 52% at amino acid level. Whereas the Chinese and American isolates are clearly closely related, the bushpig PPV4 might belong to a different genogroup. The complete genetic characterization of PPV4 from bushpig awaits to be performed.

When compared to other viruses, PPV4 shows limited sequence identity with both other parvoviruses and bocaviruses, which also has been confirmed in earlier studies (Cheung et al., 2010; Zhang et al., 2011). Despite the quite low sequence identity between PPV4 from bushpig and domestic pig, bushpig PPV4 seems neither more nor less divergent from the parvovirus and bocavirus sequences than the domestic pig viruses do.

In conclusion, the estimated prevalence of TTSuV1 and 2 in this study of 96 domestic pigs in Uganda seems to be similar to the prevalence found in domestic pig populations in other parts of the world. This study has also partly characterized TTSuV2 and PPV4 from bushpig and shown that the TTSuV2 is related to isolates from domestic pigs. For PPV4, the bushpig sequence is only distantly related to the known PPV4 sequences of domestic pigs, but there are limited amounts of sequences to compare with as PPV4 has not been intensively studied so far. The possible pathogenic properties of these viruses remain uncertain, and though they may not be able to cause disease on their own, their interaction with other viruses in the host could lead to the development of disease. It is possible that there is an exchange of viruses between domestic pigs and bushpigs in Uganda, but further studies must be performed to determine the extent of this exchange and its risks to animal health. Bushpigs might act as a reservoir for these viruses, keeping them in the environment even if we were to eradicate them from domestic pigs. One can speculate in that the lack of structured pig breeding and trade infrastructure in Uganda makes the country's pig industry extra vulnerable to disease, and as the pig owners to a large extent are subsistence farmers one can see that this field of research is important from both an economic and poverty-reducing point of view. Whether bushpig variants of TTSuV1, TTSuV2 and PPV4 or other bushpig viruses could cause an emerging infectious disease among domestic pigs is a question still to be answered.

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