TRANSMISSION AND PATHOGENESIS OF SWINE TORQUE-TENO VIRUS 1 (TTSUV1)

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Torque-teno viruses (TTVs) are small ubiquitous non-enveloped single-stranded circular DNA viruses. Since their discovery in a post-transfusion hepatitis patient, they have been isolated in several vertebrate hosts with over 90% prevalence, including swine. They have been detected in the environment, water sources, human drugs, vaccine and blood product as contaminants. Intriguingly, the role of TTVs in human disease causation is still not fully understood. Several epidemiological studies have associated TTVs to human diseases, like cancers, hepatitis, and autoimmune diseases, but no clear link between infection and clinical disease has been demonstrated yet. In contrast, experimental studies done in pigs demonstrated that swine TTVs (TTSuVs) could an act as sole pathogens. Other studies also demonstrated that TTSuVs could exacerbate symptoms of other viral pathogens in coinfections. Here, we showed that TTSuV1 could be zoonotic, as we detected TTSuV1 DNA in human serum samples. We also showed that TTSuV1 could replicate in human immune cells, and consequently suppress their ability to respond to immune stimuli. Further *in-vivo* studies, to elucidate host immune regulation by TTSuVs, showed a delayed antibody response and minimal viremia. Also, we found that viral sensing could be limited to interferon-inducing sensors (DHX36), while upregulation of PD-1 could demonstrate how these viruses may establish chronic infections. In another study, we showed the use of our novel recombinant TTSuV1 culture system to study the synergistic interactions between TTSuV1 and porcine circovirus 1 (PCV1). When both viruses were cultured together *in-vitro*, their respective viral titers were increased, compared to the single virus infections. We also demonstrated that increased *in-vitro* replication of TTSuV1 could be relying on expression of PCV1 replicase. In addition, molecular mechanisms were used to explain this synergistic relationship; a strong promoter activity by the putative major promoter

of TTSuV1 was shown to be blocked PCV1 and TTSuV1 replicase proteins, but protein-DNA interaction assays need further optimizations to demonstrate physical interaction between these viruses. In conclusion, our result showed new information about TTSuV1 transmission, pathogenesis, host innate immune regulation, and their role in coinfections.

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DEDICATION

To my dad, Samuel K. Miiro, Sisters (Grace, Viola and Esther), Pink-house crew (many) and above all, God.

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CHAPTER ONE: GENERAL INTRODUCTION

Organization of the dissertation

This dissertation consists of five (5) chapters, namely; the general introduction and literature review section, the chapters 2-4 focus on the specific studies (manuscript format), and then general conclusions and future directions. The *first* chapter presents a review of the recent literature and how this study relates to the field, identifies the gaps in knowledge, and also highlight the significance and objectives of my study. The *second* chapter discusses detection of heterologous torque-teno viruses (TTVs) in humans and swine, showing a potential zoonotic transmission mechanism of these viruses (published work). The *third* chapter details using a swine model to study the host immune regulation of TTVs. The *fourth* chapter includes the potential role of helper viruses could have in the replication of TTVs, while using a novel technique to propagate TTVs to higher titers in an *in-vitro* system. Finally, the *last* chapter discusses general conclusions from all three studies and also suggests future directions. For each individual chapter, apart from chapter 5, there is a list of references cited at the end.

Torque-teno viruses (Family: *Anellorividae*)

Torque-teno viruses (TTVs) are one the most recently isolated viruses. They were first discovered in 1997 from a Japanese patient serum with hepatitis of unknown cause (1). The virus was first named with the initials of the patient (T.T), but due to continued detection in post-transfusion hepatitis patients, the name was changed to transfusion-transmitted virus (2); and later, due to the diversity and shape of its genome, it was renamed "torque-teno" virus, an Italian phrase to mean "thin necklace" (2). TTVs are small non-enveloped and icosahedral viruses, with a circular single-stranded antisense-DNA genome, ranging between 20-30nm diameter. Since its

genome organization resembled that of circoviruses, small DNA-viruses not known to infect humans (Figure 1), TTVs were initially grouped in the *Circoviridae* family (2, 3).

Consequently, due to their diversity in genome size and host-range, a new family: *Anelloviridae* was formed. TTVs have been isolated in several vertebrate hosts, including non-human primates, domesticated animals, wildlife, and sea-life (4-9). However, although TTVs have very diverse genome sizes (2.1-3.8kb), their genomic structure and organization remain very similar among all host-specific strains (10) (Figure 1). The genome usually consists of two major regions; i) a conserved non-coding GC region (untranslated region or UTR) with regulatory binding sites for transcription and promoter activity, and ii) a variable coding region which could contain 3-4 open reading frames (ORFs).

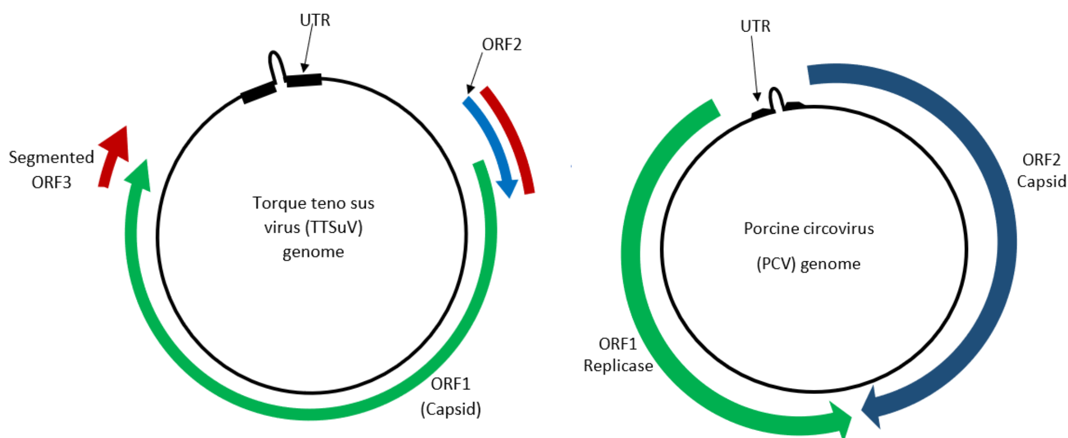

Figure 1: Genome organization of torque teno virus (Left). UTR represents the conserved GC-rich non-coding region, open reading frames (ORFs) represent variable coding regions. A similar genome organization is seen in Porcine circovirus (Right), with the origin of replication (in the non-coding UTR region) and coding region with the capsid protein ORF2 (Capsid) and the replicase protein ORF1 (Replicase).

According to the International Committee on Taxonomy of Viruses (ICTV), the *Anelloviridae* family currently includes 12 genera, depending on the host species (11). Human TTVs belong to three genera, namely; *Alphatorquevirus* (torque teno virus), *Betatorquevirus* (torque teno mini virus), and *Gammatorquevirus* (torque teno midi virus). Initially, swine TTVs

were first named SdTTV31 when they were isolated in Japanese domestic pigs (4). With time, Niel *et al*. (31) isolated two very diverse clones from Brazilian pigs, designated clone 1p and 2p. Later, these two clones were renamed swine TTV1 and TTV2 respectively (12, 28). But, due to the continued isolation of new diverse genotypes of these viruses, the ICTV grouped them into two major groups; *Iotatorquevirus* (torque teno sus virus 1) and *Kappatorquevirus* (torque teno sus virus k2). Both human and swine TTVs are very diverse and many phylogenetic studies have showed 40-60% similarities between genera (12, 13). In addition, the respective genera of both swine and human TTVs include several subtypes (12,13). The other genera include; Sea-lion TTVs (*Lambdatorquetenovirus* and *Nutorquevirus*); Cat TTVs (*Etatorquetenovirus*); Dog TTVs (*Thetatorquetenovirus*); Horse TTVs (*Mutorquevirus*); and Tamarin TTVs (*Epsilontorquetenovirus*). The 12th genus contains *Gyrovirus* (Chicken anemia virus). However, more TTVs are still being isolated from new host species and newer genera have recently been suggested, like *Sigmatorquevirus* and *Omegatorquevirus* in rodents (9, 11, 14, 15).

Infection with several TTV genotypes or subtypes within the same host has previously been reported, but not extensively studied. One study has previously demonstrated presence of human TTVs in non-human primates (7). Another study detected human TTVs in buffalo milk, although this was later suspected to be a food-chain contamination (16). In conclusion, very few studies have explored the role of TTVs in cross-species transmission between host species. Furthermore, because these viruses are very ubiquitous, they have been detected in the environment, water sources, human drugs, vaccine and blood product as contaminants (17-19), and this poses risk of transmission between host species.

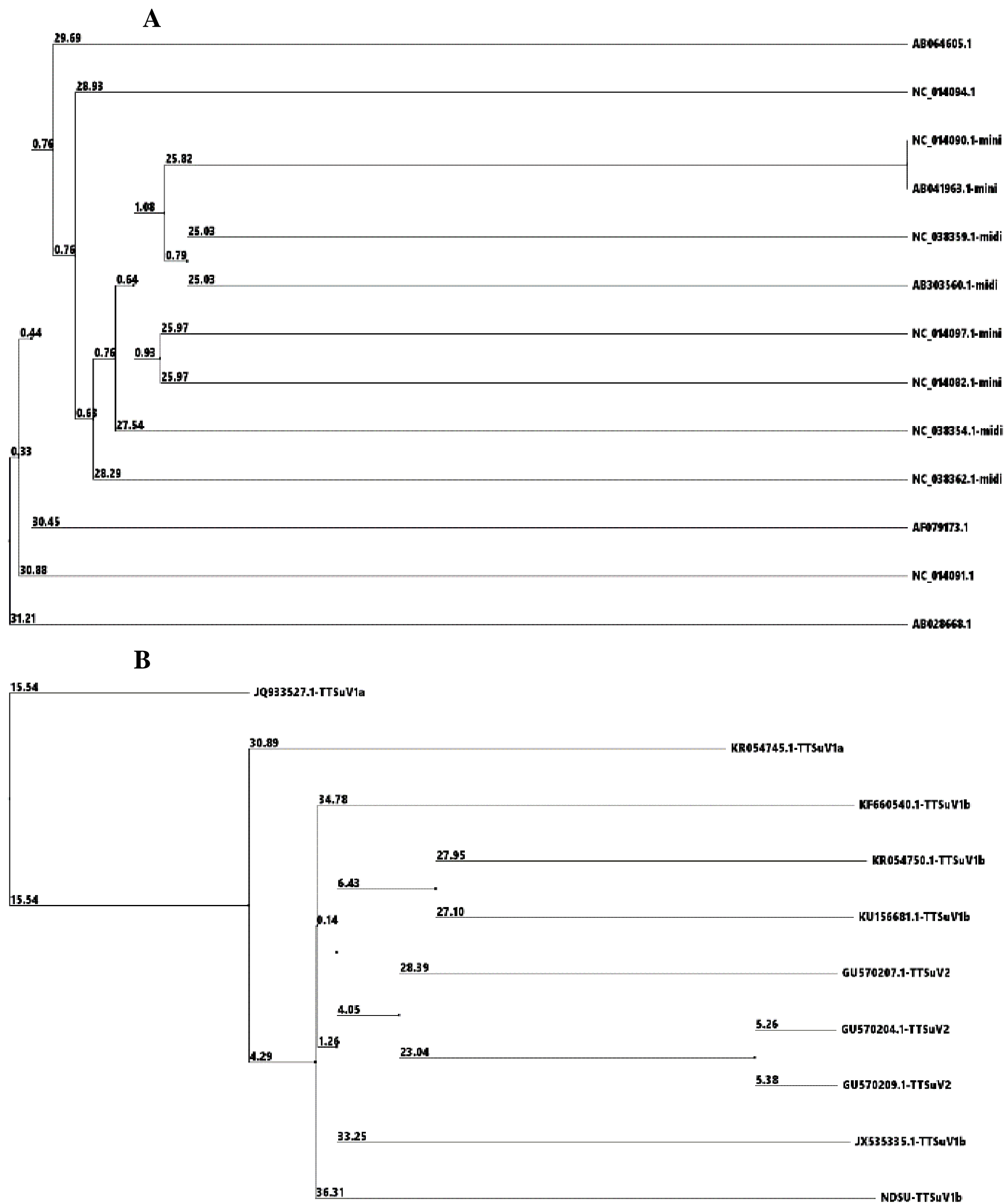

Figure 2: Illustrations of diversity of TTVs by phylogenetic trees based on percentage identities of complete genomes, using the neighbor-joining phylogenetic method. The left tree (A) shows diversity of human TTV genomes, while the right tree (B) shows diversity of swine TTV (TTSuV) genomes.

Molecular biology of human and swine TTV

According to the Ninth Report of the International Committee on Taxonomy of viruses, TTVs consist of at least two major open reading frames (ORFs), together with a GC-rich untranslated region (UTR) (20) , as shown in figure 1. Although swine TTVs (TTSuVs) have similar genomic organization as human TTVs, they only share about 45% nucleotide similarity (21). Also, the TTSuV genome is approximately 2.9kb in length and contains three ORFs, in comparison to the 3.8kb human TTV genomes. Generally, TTVs have high variability (47-70%) in the coding regions, with hypervariable sequences, while the UTR is usually conserved among isolates, and has regulatory and replication initiation sites (22-24). In addition, transcription initiation sites were previously identified within the UTR of the human TTVs (24, 25); however, no similar work has been done in TTSuVs. In both human TTVs and TTSuVs, the ORF1 gene is the largest and encodes the capsid protein, which also contains conserved rolling-circle replication motifs which could help with initiation of viral replication (26); ORF2 gene encodes non-structural proteins for viral replication and immune evasion (27). In addition, TTSuVs have an additional gene, ORF3, which encodes a non-structural protein of unknown function (28). Alternative splicing of ORF1 and/or ORF3 mRNAs to encode different protein isoforms has also been demonstrated in both human TTVs and TTSuVs (28, 29).

TTVs have very similar genome organization as other single-stranded circular viruses, especially porcine circoviruses (PCVs). In fact, TTVs were initially classified as the first human circoviruses (2), viruses not known to infect humans. PCVs have been well studied and their genes characterized. Both these viruses encode similar proteins, and have a conserved untranslated region (UTR) which contains regulatory sites (Figure 1 and 3) (24, 30). Based on this, both viruses are reported to use rolling-circle replication mechanism, which usually starts

with a nick on the stem-loop (origin of replication), by the replicase proteins (30, 31). The stem-loop sequence is highly conserved among virus species (Figure 3) (32). The replicase protein of PCVs has previously been shown to physically bind to the stem-loop and initiate replication (30). We therefore hypothesize that TTSuV ORF1 protein would act the same way since it contains replication motifs in its sequence (26). Similarly, we suspect PCV replicases could bind to the same site to assist with TTSuV replication. This could as well explain the reported synergistic relationship between these viruses in coinfection systems, and this was the hypothesis test in chapter 4 of this dissertation.

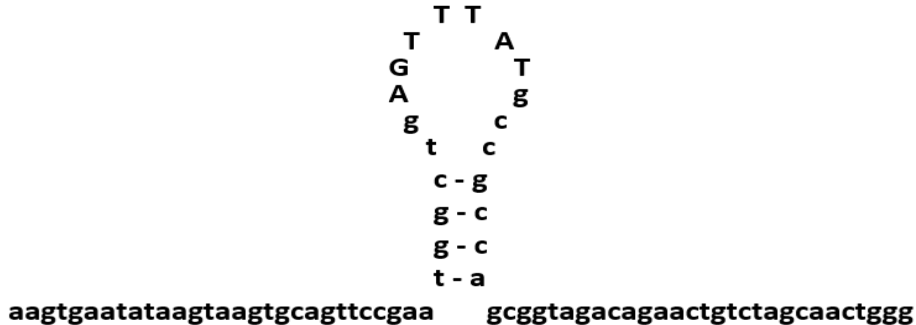

Figure 3: A. Illustration of putative conserved stem-loop of TTSuV1 (KT037083), showing the origin of replication. This genome organization is similar in other circular DNA viruses (32).

Role of TTVs in diseases and coinfections

Since their discovery in 1997, TTVs have been implicated in many disease complications, like acute hepatitis, cancer, respiratory complications (1, 20, 33). Several epidemiological studies have continued to associate TTVs with other infectious and non-infectious diseases, but very few have implicated them as the sole causative agents of diseases. As a result, different pools of thought about TTVs exist; with some suggesting TTVs as commensals while some suggest they could be pathogens, whether singly or in co-infection cases (34). Since TTVs are very closely related to other pathogenic animal anelloviruses, especially

chicken anemia virus (Genus: *Gyrovirus*) (35), they could be linked to disease manifestations in their hosts; however, such evidence is yet to be reported. Actually, some studies have suggested that long-term co-evolution of TTVs with their hosts could have resulted in the low host immune responses, hence no clinical signs of disease (22, 36). To date, few studies have explored the pathogenesis, transmission and general dynamics of the viruses. This setback has been highly attributed to the lack of cell-culture system or animal model to propagate and study these viruses (20, 34). In addition, the lack of good controls has also contributed to this enigma, since TTVs have been reported in over 90% of healthy populations with no clinical symptoms.

In humans, several epidemiological studies have showed and linked TTVs to disease causation or suggest they somehow contribute to disease causation. TTVs were first isolated in a Japanese post-transfusion patient of non-A-G hepatitis (1). In this same study, they detected elevated alanine transaminase in TTV infected patients compared to those uninfected (1). Similar studies also showed increased TTV viral loads in hepatitis patients compared to controls (26). Again, other studies have reported that TTV-related hepatitis could depend on specific virus genotypes which cause persistent liver failure (37). Other TTV hepatitis cases were reported in the Middle East (38, 39). In addition, co-infection with known hepatitis viruses (HCV or HBV) with TTVs has greatly been shown to cause increased mortalities or onset of liver cancers (40-42); although, in these studies, their role in disease progression was not demonstrated. Similarly, high TTV viral titers have been detected in several other cancers, like Kaposi's sarcoma, leukemia, carcinomas and lymphomas (43-45). Also, co-infection with human papillomavirus showed exacerbated cancer manifestation. TTVs have also been implicated in several respiratory disorders. A study in Turkey showed that TTVs were highly associated with acute respiratory disease in children, showing high viral loads compared to those with mild conditions (33).

Another report showed similar conclusions, and suggested that TTVs could play a role in the onset of asthma (46). Further still, other studies have implicated TTVs in autoimmune diseases and hematological disorders (22, 47), but their contribution to disease is still unclear. In all these epidemiological studies, no experimental evidence for human disease causation has been demonstrated.

Similarly, several epidemiological studies have linked TTSuVs to several diseases, especially porcine respiratory disease complex (PRDC) and porcine wasting syndrome (PMWS). A study by Rammohan *et al.* (2012) showed a strong epidemiological presence of TTSuVs in coinfection with other swine pathogens; for example, over 70% of PCV2, porcine respiratory and reproductive syndrome virus (PRRSV) and swine influenza (SIV) cases were also positive for TTSuV respectively (52). Another study in Spain showed detection of high viral loads of TTSuV2 in PMWS pigs compared to non-PMWS pigs (88-90), further suggesting their role in onset of PMWS. Association of TTSuVs with other infectious diseases, like PCV3, PEDV, among others, have been reported (Table 1). However, a few studies have also showed contradictory results. For example; one study demonstrated that in 200 sera and tissue samples from PMWS-positive pigs, only about 30% were positive for TTSuVk2b, and no correlation was observed with PMWS (91). Similar results were reported by Ramos *et al.* (2018), although high prevalence of TTSuVs was seen in Uruguayan herds, no correlation with PCV2 incidence was seen. These results further elicit debate on the role on TTSuVs in disease causation, with some researchers suggesting they are could only be normal flora or co-factors in other infections.

Interestingly, experimental studies done in gnotobiotic pigs recently reported that swine TTVs (TTSuV1) could cause PMWS or PRDC-like symptomatic lesions in several organs, including kidneys, liver, heart, and lungs (48, 49). This could demonstrate a possible primary

etiological role for TTSuVs in swine diseases. For example, pathological changes were demonstrated as more cellular debris was observed in the lung alveolar compared to the low fibrous exudates seen on day 7 post-infection (49). Similarly, Mei *et al*. (2011) showed that infection of gnotobiotic pigs, using liver lysate form a TTSuV1-positive pig, resulted in histopathological lesions in different organs. In addition, TTSuVs were shown to worsen clinical manifestations of other swine viral infections (50, 51). They demonstrated an acute onset of the wasting syndrome in gnotobiotic piglets co-infected with TTSuV and porcine circovirus 2 (PCV2) – the causative agent of wasting syndrome (PMWS), compared to when piglets were singly infected with PCV2 (50). However, in this study, no disease-like symptoms were observed when only TTSuV1 was inoculated. Additionally, more adverse PRDC-like symptoms were seen when both TTSuV1 and PRRSV were inoculated in pigs, compared to when a TTSuV1-only homogenate was used in the pigs, which only showed mild pneumonia (51). Results from these experimental studies demonstrate that TTSuVs could act as a primary pathogen in swine, and/or could worsen other swine diseases.

In conclusion, detection of TTVs in food, drugs and water sources for human use, together with the limited knowledge about their zoonotic capabilities, heightens the need for identification of better *in-vitro* and *in-vivo* systems to study these viruses. This would clearly demonstrate TTVs' role in disease complications and show whether TTVs could act as primary or opportunistic pathogens, or a co-factor in case of coinfections with other pathogens. In addition, although many epidemiological studies have showed a synergistic association of TTSuVs with PCV2 (Table 1) (50, 52), molecular mechanisms responsible for this relationship have not been reported yet. This was the main goal of chapter 4 in this dissertation. This synergistic relationship could be because both PCVs and TTVs interchangeably use their viral

proteins for their respective replication. Also, TTVs could be causing immunosuppression (53, 54), and that is why other pathogens thrive in coinfection systems.

Table 1: Association of TTSuVs with other swine pathogens

TTSuV-coinfection with…	**Reference**	
Porcine circovirus 2 (PCV2)	Prevalence of swine Torque teno virus in post-weaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs in Spain	Kekarainen and Sibila (2006)
	Studies of porcine circovirus type 2, porcine boca-like virus and torque teno virus indicate the presence of multiple viral infections in postweaning multisystemic wasting syndrome pigs.	Blomström *et al.* (2010)
	Torque teno sus virus 1 and 2 viral loads in postweaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS) affected pigs.	Kekarainen (2011)
	Dynamics of Torque teno sus virus 1 (TTSuV1) and 2 (TTSuV2) DNA loads in serum of healthy and postweaning multisystemic wasting syndrome (PMWS) affected pigs.	Nieto *et al*. (2011)
	The pathogenic role of torque teno sus virus 1 and 2 and their correlations with various viral pathogens and host immunocytes in wasting pigs. Vet Microbiol.	Lee *et al.* (2015)
Classical swine fever virus	Increased viral load and prevalence of Torque teno sus virus 2 (TTSuV2) in pigs experimentally infected with classical swine fever virus (CSFV).	Aramouni *et al.* (2013)
Porcine circovirus 3 (PCV3)	Presence of Torque teno sus virus 1 and 2 in porcine circovirus 3-positive pigs.	Zheng *et al.* (2018)
Porcine epidemic diarrhea virus (PEDV)	Viral metagenomics analysis demonstrates the diversity of viral flora in piglet diarrhoeic faeces in China.	Zhang *et al.* (2014)

Transmission and geographical distribution of TTVs

TTV infections have been reported to be worldwide, almost in all continents (22). In many different countries where TTVs have been isolated, the link between TTVs to disease causation is still lacking. This has been mainly attributed to the high incidence of the viruses in both diseased and healthy individuals, hence lack of good controls. However, these differences in TTV prevalence rates could be attributed to differences in detection methods used, both in humans and swine (5, 22). For example, Spandole *et al*. 2015 used a map illustration to demonstrate that TTV prevalence estimates in humans are usually higher when UTR-based primers are used for detection compared to ORF-based primers (22). The reason for this difference is because the TTV-UTR sequences are highly conserved among all TTVs, and so primers based on these sequences will detect many different TTVs at the same time. In contrast, when primers are designed based on more variable regions of the genome (ORF-based), only a few or specific TTVs will be detected. Similarly, different estimates of swine TTV prevalence were reported while using a panel of PCR primers; with higher prevalence observed when using UTR-based primers compared to ORF-based primers (5).

TTV prevalence was estimated at 94% in healthy human population in Russia, and yet they showed no clinical signs of disease (55). Similar prevalence was reported in healthy humans in China (56) and middle east (57) respectively. In swine populations, similar prevalence rates were previously reported. For example, a study in Iowa (a big pork production state) tested 300 randomly selected swine sera from six major swine production zones in Iowa, and reported an overall prevalence of 52%, with 83/300 (47%) samples were positive for TTSuV1 and 15/300 (25%) for TTSuV2; 59/300 were co-infected with both genotypes (52). However, no specific geographical patterns or correlation with swine density was observed. Also, Sibila and group

(2009) reported 75% and 43% prevalence of TTSuV1 and TTSuV2 in sows in Spain respectively. Similarly, although no correlation was observed with PCAD incidence in pigs, high prevalence of TTSUV1 and TTSuV2 was also reported in S. Korea (Lee *et al.* 2010). Other studies found that TTVs have also been detected in african domestic and bush pigs, and these showed 90-98% similarity with those isolated elsewhere in the world (58, 59). Again, these animals were healthy with no disease. Another study in European wild boars found 85% prevalence of a TTV strain similar to one infecting domestic pigs (60).

TTV transmission is not completely understood. Transmission through the contaminated body fluids was first reported, since the virus was first isolated in a transfused patient in Japan (1). Presence of the viruses in blood and blood products has mainly been reported (61, 62). Other studies have shown the parenteral route as another mode of transmission (63). Although earlier studies had suggested minimal chances of vertical transmission (64), TTVs have also been demonstrated to transmit via the mother-to-baby route, both in animals and humans (65-67). However, due to the high prevalence in the population, more recent studies have suggested other routes of transmission to be more important. The fecal-oral route has been demonstrated (68, 69); TTVs have been isolated in feces and in nasopharyngeal fluids, with higher viral titers compared to serum (70), implying that this could be a crucial route for viral transmission. The fecal-oral route is very important since swine TTVs have already been isolated from animal food products, water sources and drugs for human use (18, 71). Additionally, aerosol transmission has also been suggested (72).

TTVs have a wide range of hosts they infect, from humans and non-human primates to farm animals to sea mammals to pets (20, 56, 73), among others. Currently, most TTVs are species-specific, with few or no zoonotic transmission studies reported. Only one study

confirmed detection of TTV DNA from non-human primates (7). In another study by Roperto *et al*. (16), they showed possible contamination of buffalo milk with human TTVs; although poor handling practices could have caused this contamination. Since swine TTVs were previously demonstrated as possible primary pathogens, it is important to know if they can infect humans, and this was the primary goal of Chapter 2 of this dissertation. Since both human and swine TTVs have similar genome organization, and encode similar proteins (20), there could be a possibility that TTSuVs could infect humans. However, due to lack of a proper cell culture system to propagate these viruses, clear insights into such possibilities are yet to be known. Moreover, swine TTVs have been isolated from human drugs and food products (18, 74, 75). Such findings present great risk to human health because anatomically, pigs are known to be very similar to humans; and so cross-species infection could be possible. Not surprising, some researchers have also suggested the use of pigs as models for studying human TTV studies (73).

Host immune regulation by TTVs

TTVs establish life-long infections, starting at very early ages (76, 77). However, very few studies have explored or demonstrated how these viruses are capable of host immune modulation or evasion (78, 79). Since the rate of mutations in DNA viruses is usually lower, some studies have also suspected that presence of hypervariable regions within TTV genomes could be responsible for this tolerance to the host immune responses, as the viruses make several quasispecies, hence establishing life-long infections (80). Little information exists to explain how TTVs establish infections or how they modulate the host immunity. This has been mainly attributed to the lack of an animal model to study their role in pathogenesis. Although an infectious clone for TTSuVs was initially used in *in-vitro* experiments to make viral particles

(81), improvements to this system are still required to use such viral particles in *in-vivo* studies to understand immune regulation by TTSuVs.

The host innate immunity is the first line of defense, with macrophages playing great role. *In-vitro* studies with macrophages showed that major TTSuV1 proteins modulate the host immune responses (78, 82). Although ORF1 was reported to elicit an early anti-viral response, it later upregulated immune-suppressive proteins (78). Further still, ORF2 protein has been reported to diminish NF-kB pathways, important in inflammation (27), and also downregulate IFN-β expression (78). Moreover, a recent study by Kincaid *et al*. (83) also showed that human TTVs encode for miRNA which suppresses the interferon expression, a great tool in clearing viral infections. Another study, in children with respiratory infections, found that human TTVs can regulate the innate immunity by inducing pro-inflammatory cytokines (22, 84). Furthermore, it was suggested that human TTV ORF3 encodes a non-structural protein similar to one in human cytomegalovirus (HCV protein 5A) which is responsible for interferon-induced responses (22), and this could be one of the ways TTVs modulates host immunity for its own replication or other co-infecting agents, or evades it.

Another line of defense is the adaptive immunity, and this kicks in later after the innate response and it is very specific. Both ORF1 and ORF2 proteins have been shown to be immunogenic, in both humans and swine (8, 62, 85). Antibodies to these two proteins (ORF1 and ORF2) have also been detected in the population. A previous study detected TTV DNA in patient samples and also suggested that anti-ORF1 antibodies may not be neutralizing (62). However, a more recent study showed that vaccination with ORF1-ORF2 protein cocktails in swine could help reduce or delay infection in herds (86). Further still, TTVs are well documented to replicate and thrive in lymphocytes (22), cells that play a critical role in adaptive immune

responses. A negative correlation was observed in HIV-positive patients, where high TTV viral loads were detected in low $CD4^+$ T-cell count patients (87). Similarly, high B-cell counts were observed in high TTV titer cases (46). Both these evidences suggest a role of TTVs in regulating host adaptive immune responses. Furthermore, *in-vitro* studies showed that swine TTV ORF3 protein could reduce adaptive immune responses by inhibiting IL4 and IL13 expression (82). Moreover, the same studies also showed that TTVs upregulated expression of PD1 and SOCS-1 genes (78), two proteins known to suppress the cell-mediated immune responses, through the blocking of T-cell responses.

In conclusion, TTVs have been well documented to possibly cause immune suppression, to be associated with other viral pathogens, and also establish chronic infections. However, few or no *in-vivo* studies have characterized how TTVs could accomplish this. Developing an *in-vivo* model for studying TTVs is critical to answer or confirm hypotheses surrounding TTV immune regulation. Here, we considered known host DNA sensors and cytokines involved in viral DNA sensing and their downstream signaling cytokines which lead to an antiviral state, in case of viral DNA infections (Figure 4). Ultimately, based on our results, we would like to propose a TTV infection and persistence model, and this was the goal of chapter 3 of this dissertation.

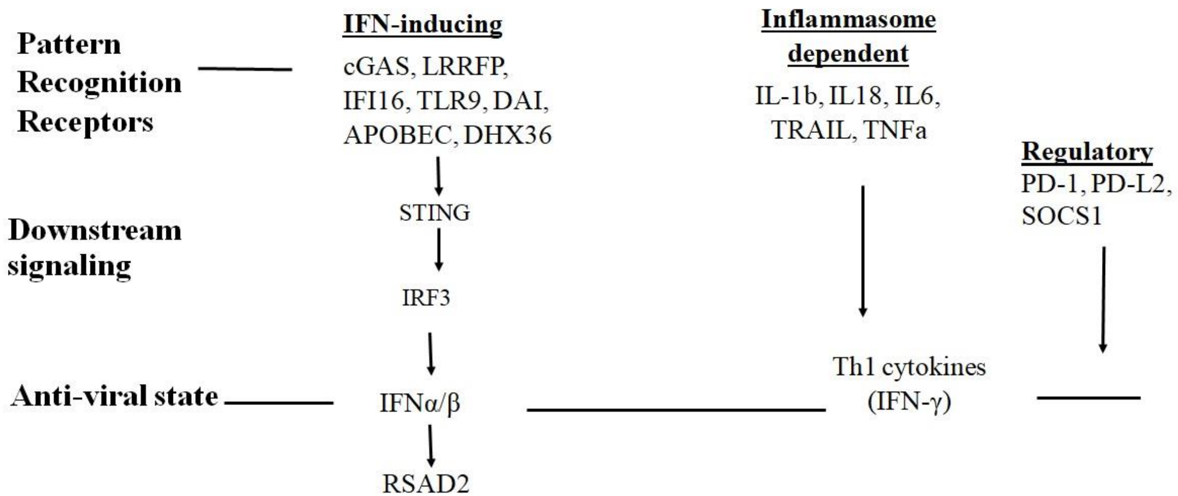

Figure 4: Flow-chart showing some of the viral-DNA sensors responsible for host innate and adaptive immune regulation by other DNA viruses.

Objectives of this dissertation

This dissertation therefore mainly focused on investigating three (3) research objectives, namely;

1. To demonstrate potential zoonotic transmission of torque-teno viruses (TTVs) between humans and swine (Chapter 2).
2. To study the immune regulation of TTVs, using a swine model (Chapter 3).
3. To show potential synergistic relationship between replication of TTVs and porcine circoviruses (PCVs) (Chapter 4).

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CHAPTER TWO: IDENTIFICATION OF HETEROLOGOUS TORQUE TENO VIRUSES IN HUMANS AND SWINE[1]

Abstract

Torque teno viruses (TTVs) are ubiquitous viruses which are highly prevalent in several mammalian species. Human TTVs are epidemiologically associated with several human disease conditions such as respiratory illnesses, auto-immune disorders and hepatitis. Recently it was found that swine TTVs (TTSuVs) can act as primary pathogens. The common occurrence of TTVs as environmental contaminants and the increasing interest in the use of swine organs for xenotransplantation lend importance to the question of whether TTVs can cross-infect across species. In this study, we examined human and swine sera by swine or human TTV-specific PCRs, to determine whether TTSuV DNA can be detected in humans and vice versa. Surprisingly, both human TTV and TTSuV DNA were present in a majority of the samples tested. Transfection of human PBMCs with TTSuV1 genomic DNA resulted in productive viral infection which was sustained for the three serial passages tested. Lymphoproliferative responses in infected human PBMCs were diminished when compared to the controls. Furthermore, mild to moderate antibody responses against the TTSuV1 ORF2 protein was detected in 16 of the 40 human sera by ELISA. Therefore, these study findings provide initial and fundamental evidence for possible cross-species transmission of TTVs.

Introduction

Torque teno viruses are small DNA viruses which were discovered as a possible cause of post-transfusion hepatitis in humans (1). Since then, TTVs have been detected in many

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mammalian hosts; including dogs, cats, chimpanzees and swine (2). While the prevalence of TTVs in other species has not been studied extensively, they are reported to range from 5-90% in humans (3, 4), and about 55% -100% in swine (5, 6). The virus is detected in all major organs, secretions, excretions, blood and blood cells. The tissue distribution and localization of TTVs are similar in humans and swine (3, 7).

Generally, TTVs establish chronic infections without causing overt pathology. Hence their role as primary pathogens is a subject of scientific debate. Several epidemiological studies have associated TTVs with a spectrum human diseases such as hepatitis B or C, multiple sclerosis, hepatocellular carcinomas, respiratory infections, blood disorders and autoimmune diseases (8, 9). In swine, experimental infection of gnotobiotic pigs with swine torque teno virus 1 or 2 (TTSuV1 or 2) causes mild to moderate respiratory, hepatic and nephritic lesions, indicating that TTSuVs can act as a primary pathogen in swine. In experimental coinfections, TTSuVs exacerbated other swine viral diseases (10, 11). Therefore, the question of whether TTVs can establish cross-species infections is of considerable importance.

The lack of a reliable cell culture system has limited the exploration of the molecular biology and pathogenesis of TTVs. However, recent studies show that TTV proteins encode auto-reactive epitopes which are also detected in multiple sclerosis and lupus patients (12), and that a TTV encoded miRNA depresses host interferon signaling (13). Viremia in TTV-infected individuals is inversely correlated with immune status. Indeed, it has been suggested that TTV DNA loads can be used as an indicator of immuno-suppression (14, 15). Therefore, in immuno-compromised individuals, the possibility that TTVs could replicate to high levels and facilitate pathology cannot be ruled out.

Widespread environmental contamination, based on the detection of human TTV (huTTV) DNA, is extremely common in water sources (16-18), sewage (19) and in air or on surfaces, especially in hospitals (20). Contamination of some veterinary vaccines and swine-derived laboratory enzymes, such as trypsin, with TTSuVs is also reported (21). Current screening protocols for blood donors do not include detection of TTVs. However, given their ubiquitous nature, TTVs are also potential contaminants of the blood supply (22). Humans are likely to frequently ingest TTSuVs in food and water. Both pork products and human feces contain TTSuV DNA (23, 24). Moreover, with the availability of improved technology, there is an increased interest and potential for the use of swine-based xenotransplantation products (25). Therefore, from a public health perspective, it is especially critical to determine whether TTSuVs can establish infections in humans.

In this study, to test the hypothesis that TTVs could be transmitted across host-species, we examined sera from humans and swine for the presence of TTSuV and human TTVs (huTTV) DNA by PCR. Interestingly, both TTSuV and huTTV DNA were detected at high levels in both species. We also determined that TTSuV1 can serially infect human PBMCs and reduce their ability to proliferate in response to mitogens. Antibody responses to TTSuV1 were detected in some human samples, indicating that TTSuVs can potentially establish infections in humans. Our data provides key, primary evidence for the possible transmission of TTVs between mammalian species and is significant in understanding the ecology and pathogenesis of this highly prevalent virus.

Materials and methods

Serum samples

A total of 60 sera, comprising human (N=40) and swine (N=20) were tested by swine or human TTV-specific PCRs as indicated in Table 2. Swine samples were obtained from a herd maintained as a source of experimental animals for university research. The human sera purchased were collected with informed consent and the approval of the institutional review boards of two different commercial vendors (Valley Biomedical, Winchester, VA or Bioreclamation IVT, Long Island, NY). All human samples were screened to be negative for HBsAG, HIV 1/2 Ab, HCV Ab, HIV-1 RNA, HCV RNA and STS by the vendor. The end users were blinded to the identity of the donors of the samples. All experimentation was carried out with the approval of the North Dakota State University's institutional biosafety committee and in accordance with the approved guidelines.

PCR detection of human TTV DNA

For the detection of human TTV DNA, a previously described pan-human TTV (huTTV) PCR (26) was adapted to a real-time PCR format. Briefly, DNA was extracted from the sera using the QiaAmp DNA Mini kit (Qiagen, Valencia, CA), Primers (Table 2) were added to the iTaq™ Universal SYBR® Green Supermix (Biorad, Hercules, CA), with 25ng of template DNA, cycled 40times (iCycler CFX96 Touch Real Time PCR Detection system, Biorad, Hercules, CA) at a Tm of 60°C.

PCR detection of TTSuV DNA

To ensure reliable detection, four different PCRs (Pan TTSuV UTR PCR 1, TTSuV1 UTR PCR2, TTSuV1 ORF2, and TTSuV1 ORF3 - Table 2) were used for the detection of TTSuV DNA in the human and swine sera. Two PCR's designated as the pan-TTSuV UTR PCR

1 and TTSuV1 UTR PCR2 targeted two non-overlapping regions of the conserved untranslated region (UTR). The pan-TTSuV PCR1 detected both the TTSuV1 and 2 genotypes while the TTSuV1 UTR2 targeted the UTR of the more commonly prevalent TTSuV1 genotype (5). Two other PCRs (TTSuV1 ORF2 & 3) targeted the more variable ORF2 and 3 regions of the TTSuV1 genotype (Table 2). The pan-TTSuV PCR1 was carried out essentially as described (6) except that iTaq™ Universal SYBR® Green Supermix (Biorad, Hercules, CA), with 25ng of template DNA, primers, a Tm of 57°C and 36 amplification cycles was used. The TTSuV1 PCR 2 was carried out as previously described (5), except that the TTSuV2 primers were not used. The PCRs targeting the TTSuV1 ORF1 and 2 were carried out using the primers listed in Table 2. 25ng of template DNA in a commercial PCR master mix (ReadyMix™Taq PCR Reaction Mix, Sigma), and a Tm of 56°C for 35 cycles. For all PCRs, two no template controls were included, and samples were tested in duplicate or nested. The specificity of all PCR assays was determined by a nucleotide BLAST analysis of two sequenced amplicons from each species.

Table 2: Human or swine TTV- specific PCR assays used for the assessment of the human and swine sera

PCR	PCR Type	Target	Forward Primer	Reverse Primer	Size (bp)	BLAST results
Pan - Human TTV	qPCR	Untranslated region (Conserved)	5'gtaagtgcacttc cgaatggctgag3'	5'gcccgaattgc ccttgac3'	132	[a]AB041007.1 [a]AF122915.1 [b]KJ082064.1 [b]AY590626.1
Pan-TTSuV UTR 1	qPCR	Untranslated region (Conserved)	5'cgaatggctgag tttatgcc3'	5'gataggcccctt gactccg3'	95	[a]KR054745.1 [ab]KR131718.1 [b]HM633251.1 [b]KR054745.1
TTSuV 1- UTR 2	Gel-based	Untranslated region (Conserved)	5'gcggtcaaaatg gcggaag3'	5'ggacttgagct cccgaccaa3'	124	[a]EU564163.1 [a]JF451574.1 [b]EU006509.1 [b]JX872390.1
TTSuV 1- ORF2	Gel-based	ORF2 (Variable)	5'agtcaagcttttg ccggaacactggg aggaag3'	5'acgtctcgagc cagccatcgtcgc cgat3'	235	[a]JX535326.1 [a]HM633254.1 [b]HM633254.1 [b]JX535326.1
TTSuV 1- ORF3	Gel-based	ORF3 (Variable)	5'gcgacgatggct gtttggaggtgaaat accaaccc3'	5'acgtctcgagg cgtttcttttgtttttt at3'	477	[a]HM633244.1 [a]HM633254.1 [b]HM633254.1 [b]JX535326.1

[a] Top 2 nucleotide BLAST results obtained from the sequenced PCR amplicons two swine samples
[b]Top 2 nucleotide BLAST hits obtained from the sequenced PCR amplicons of two human samples

Genome cloning and sequencing

The TTSuV1 genome was amplified from the bone marrow of a swine diagnostic case. Two opposing primers (5'-GACAATTAATTTATGCAAAGTAGGA-3' and 5'-GACAATTAA TTTGCATAAACTCCGC-3') with flanking Ase-I sites were used to amplify the entire circular genome, which was then cloned into the pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA). The genome was sequenced and deposited in GenBank (KT037083).

Permissiveness of human PBMCs to TTSuV1

To ensure they were PCR negative, human peripheral blood mononuclear lymphocytes (PBMCs) were tested by the pan human and TTSuV PCR's as described above. Cells were cultured at $5x10^5$ cells/ ml in 10% DMEM with 8 µg/ml of anti-CD3 antibody (Tonbo Biosciences, San Diego, CA), 1µg/ml of phytohemagglutinin (PHA-M) (Life Technologies, Grand Island, NY, USA) and human rIL-2 (10U/ml) (Tonbo Biosciences) for 48hr. The 2877bp genome of TTSuV1 was excised from the shuttle vector by AseI restriction digestion, purified by gel extraction and re-circularized by ligation (T4 DNA ligase, New England Biolabs, Ipswich, MA). The cultured PBMCs were transfected with the circularized genome at 1ug/wellTransIT-2020 (Mirus Bio, Madison, WI), following the manufacturer's instructions. One replicate of cells was maintained as the untransfected negative control. Plates were incubated for 48hr at 37°C in a CO_2 incubator. The rescued virus culture was used to infect new human PBMC cultures, as described above and serially passaged three times.

Viral replication was assessed using an indirect immunofluorescence assay (IFA). Briefly, the adherent and non-adherent PBMCs were washed in Hank's Balanced Salt Solution (HBSS) (Mediatech/Corning, Manassas, VA) and fixed in ice cold, acetone: methanol (1:1). Fixed cells were stained with a rabbit polyclonal anti-ORF1 TTSuV1b antibody (27) as primary antibody at a 1:100 dilution for 2hr at 37°C, followed by a 1:50 dilution of anti-rabbit FITC (KPL, Gaithersburg, MD) conjugate, as secondary antibody, for 45mins. Stained slides were from each serial passage examined by confocal microscopy (Zeiss Laser scanning confocal microscope, NDSU Core imaging facility).

Effect of TTSuV1 infection on human PBMC proliferation

Five replicates each of plated PBMCs were either transfected, as described above, with the circularized TTSuV1 genome, an unrelated linearized plasmid DNA (pcDNA™3.1/V5-His TOPO®, Life Technologies, Grand Island, NY) or served as untransfected negative controls, while growth media alone was used to control for background fluorescence. All cells were stimulated 1µg/ml of phytohemagglutinin (PHA-M) (Life Technologies, Grand Island, NY) for 48 hours. PHA-M is a non-specific mitogen which stimulates lymphocytes by binding to their receptors and causes clonal expansion. Lymphocyte proliferation in response to the mitogen stimulation was measured using the Alamar-blue reagent (AbDSerotec/Bio-Rad, Raleigh, NC), according to the manufacturer's instructions. Briefly, 10ul of Alamar-blue reagent was added to 100ul of cells and incubated further for 4hr. The data obtained was analyzed by a Student's T-test to determine statistical significance.

Expression and purification of HIS-tagged TTSuV-ORF2 protein

The TTV-ORF-2 gene was amplified from cloned TTSuV1 genome and shuttled into the pET-28a bacterial expression vector (EMD, Millipore, Billerica, MA) in conjunction with a 6X HIS tag at both the N and C termini. Primers 5'-GTCAAGCTTTGCCGGAACACTGGGAGGAAG-3' and 5'-ACGTCTCGAGCCAGCCATCGTCGCCGATAGTC-3' with HindIII and XhoI restriction sites respectively, were used to amplify the gene which was later cloned into a plasmid. The plasmid was transformed into a bacterial expression vector (BL21 DE3, Life Technologies, Grand Island, NY) and induced over-night by the addition of 1mM IPTG at 37°C. The over-expressed ORF2 protein was purified by affinity chromatography (His-Spin Protein Miniprep kit, Zymo Research, Irvine, CA), following the manufacturer's instructions.

ELISA for the detection of anti-TTSuV antibodies

Plates (High Bind Microplate, Corning®, Corning, NY) were coated with 50µl of a 1:100,000 dilution of the purified TTSuV1 ORF2 the antigen. Coated plates were blocked with 2% BSA and 2% normal sheep serum in a commercial blocking buffer (General block, Immunochemistry technologies, Bloomington, MN) for 2hr at 37ºC, incubated with 1:50 of the human or swine sera for 2hr at 37ºC, in duplicate, followed by the respective anti-species HRPO-conjugate (KPL, Gaithersburg, MD), at a 1:5000 dilution for 45mins at 37ºC, and incubation with the substrate (TMB, KPL, Gaithersburg, MD). The reaction was stopped with a 1M HCl solution after 2min. Plates were read at 450nm on an ELISA plate reader (Elx800 reader, BioTek Instruments, Inc., Winooski, VT). All samples were run in duplicate and the mean optical density (OD) values were used for analysis. The cutoff value for the human sera (0.125) was calculated as the lowest quartile value of the data set +/- two standard deviations. The cutoff value for the swine samples (0.16) is the mean OD value of the negative control serum sample.

Results

Detection of TTSuV DNA in the human and swine sera

To determine whether TTSuV DNA can be detected in human sera and vice versa, we examined a total of 60 sera samples. Forty of the samples were from healthy humans while 20 were from a high-health, swine herd. All sera were examined by 4 PCRs targeting various regions of the TTSuV genome (Table 2) and a human TTV-specific PCR. Detection of TTSuV1 and 2 DNA by the real-time quantitative pan-TTV PCR, showed that 17 of the 20 swine samples tested were positive. Surprisingly, 32 of the 40 human samples tested were positive for TTSuV DNA by this assay (Table 3). To further ensure the accuracy of our results, the samples were tested by a previously validated gel-based PCR (TTSuV1 UTR2) (5) which targeted a different

region of the conserved UTR. Twenty-seven of the 40 human samples also tested positive on the second PCR confirming the presence of TTSuV1 DNA in the human sera. Twelve of the 20 swine samples tested positive. To further validate our results, the samples were tested with PCRs targeting the more variable protein coding regions for TTSuV1 ORF2 and 3. With the TTSuV1 ORF2-specific PCR, 8 of the 40 human samples and 6 of the 20 swine samples were positive. Two of the human samples and 11 of the swine samples were positive by the TTSuV1ORF3-specific PCR (Table 3). To validate the specificity of the assays, swine TTV PCR product sequences, specific to the UTR, ORF2 or ORF3 respectively, were subjected to a nucleotide BLAST analysis (Table 2). All no-template controls were negative in each PCR run.

Detection of huTTV DNA in the human and swine sera

To detect the presence of hu TTV DNA, the human and swine sera were examined by a pan human TTV-specific PCR (Table 2). As expected, 85% of the human sera were positive for huTTV DNA. A nucleotide BLAST analysis of sequenced amplicons returned accession numbers KJ082064.1 and AY590626.1, both of which were huTTV sequences from China and Venezuela respectively. Of the 20 swine sera samples, 16 tested positive for huTTV DNA (Table 3). Accession numbers AB041007 and AF122915, consisting of huTTV sequences, were the top hits for the sequenced amplicons (Table 2). The human and swine sequences obtained were genetically distinct. In general, swine and human TTV genomes share less than 50% sequence identity (28). All no-template controls were negative in each PCR run.

Table 3: PCR detection of human and swine TTVs in human and swine serum

Human sera					
Sample	**huTTV-PCR**	**TTSuV-UTR1**	**TTSuV1-UTR2**	**TTSuV1-ORF2**	**TTSuV1-ORF3**
1	+		+		
2	+		+		
3	+	+			
4	+		+		
5	+	+			
6	+	+	+		
7	+				
8	+	+	+	+	+
9	+	+	+		
10	+	+		+	
11	+				
12	+	+	+		
13	+	+	+	+	+
14	+	+	+		
15	+	+	+		
16	+	+	+	+	
17	+	+			
18	+	+	+		
19	+	+	+		
20	+	+	+		
21		+	+	+	
22		+	+		
23		+			
24		+			
25	+	+	+		
26	+		+		
27	+	+			
28	+	+			
29	+	+			
30		+	+		
31	+	+			
32	+	+	+	+	
33	+	+	+	+	
34	+	+	+		
35	+	+	+	+	
36	+	+	+		
37	+	+	+		
38	+		+		
39		+	+		
40	+				

Table 3: PCR detection of human and swine TTVs in human and swine serum (continued)

Swine sera					
Sample	**huTTV-PCR**	**TTSuV-UTR1**	**TTSuV1-UTR2**	**TTSuV1-ORF2**	**TTSuV1-ORF3**
1	+	+		+	+
2	+		+	+	
3	+	+			
4	+	+			
5	+	+			
6	+	+	+		
7	+	+	+		+
8	+	+	+		+
9	+	+	+		+
10	+	+	+		+
11	+		+		
12		+	+		+
13	+	+	+		+
14	+	+		+	
15		+		+	+
16		+	+		+
17		+	+	+	
18	+				
19	+	+	+	+	+
20	+	+			+

Infectivity of TTSuV1 for human PBMCs

To determine whether a TTSuV1 can produce sustained infections of human immune cells, virus culture derived by transfection of the TTSuV1 genome in human was serially passaged three times in human PBMCs. Bright-green nuclear fluorescence indicative of TTSuV1 replication was evident in all the three serial passages tested. The negative controls did not show TTSuV1-specific staining but virus-specific nuclear staining was detected in both monocytes and lymphocytes, indicating that the cell types present in PBMCs support TTV replication and that TTSuVs can replicate in human PBMCs (Figure 5). Prior to transfection, the PBMCs were negative when assessed by the pan human and TTSuV PCRs.

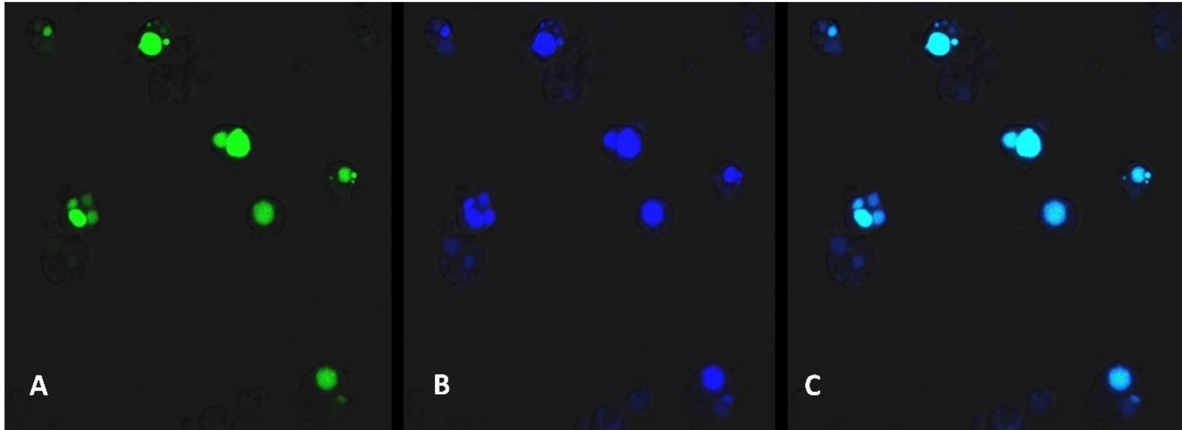

Figure 5: Indirect Immuno-Fluorescence Assay [IFA] detection of TTSuV1 replication in human PBMCs. Recombinant TTSuV1culture, rescued by transfection of human PBMCs with the circularized viral genome, was passaged three times in human PBMCs. A representative image from the third passage is depicted. A – Bright-green, nuclear fluorescence in human PBMCs infected with TTSuV1 and stained with an anti-TTSuV1 ORF1- specific antibody, B – Blue, nuclear counter-staining with DAPI, C – Overlay image of A and B showing the nuclear localization of the replicating TTSuV1.Untransfected negative controls did not show specific fluorescence [image not shown].

Lymphocyte proliferation responses in infected immune cells

To determine whether the infection of human PBMCs with TTSuVs can affect immune function, the ability of the infected cells to respond to non-specific stimulation by a mitogen was assessed using a non-radioactive, dye based assay (29). When stimulated with PHA, the mean fluorescence intensity (MFI) of infected cells was lower than that of the uninfected control cells, indicating that TTSuV1 infection diminished the capacity of the PBMCs to respond to immune stimulation (Figure 6). The difference was statistically significant with p= 0.001. The differences in the proliferative capacity of cells transfected with an unrelated DNA (plasmid control) =-was not statistically significant in comparison to the uninfected control cells (p=0.2), although slightly reduced. The difference between the cells infected with TTSuV1 and cells transfected with the unrelated DNA was highly significant at p=0.0002.

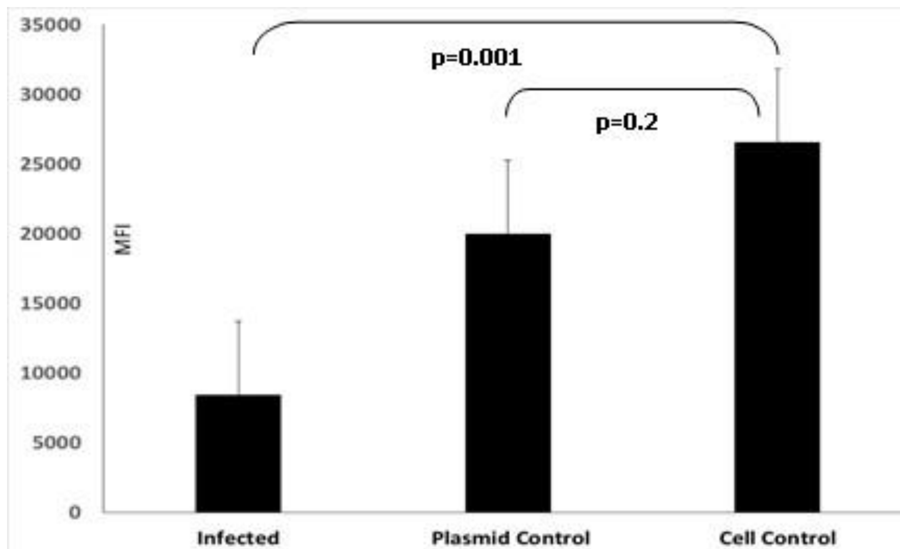

Figure 6: Ability of TTSuV1 infected human PBMCs to proliferate in response to mitogens. Five replicates each of human PBMC's were either infected with TTSuV1, an unrelated DNA control or remained as uninfected cell controls. Bars represent the average mean fluorescence intensity [MFI] values of the five replicates after subtraction of the mean background fluorescence of the media controls. * - significantly different from the cell control as assessed by a Student's T-test.

Antibody responses to TTSuV1 ORF2

To prepare capture antigen for the detection of TTSuV1-specific antibodies, the TTSuV1 ORF2 protein was expressed in a bacterial expression system. The recombinant protein was obtained in a soluble form at the expected molecular weight of approximately 10kDa. Affinity purification led to the separation of a single protein, as determined by SDS-PAGE analysis (Figure 7). The purified protein also showed specific reactivity to a commercial anti-HIS tag antibody by Western blotting and rabbit hyper-immune anti-ORF2 antibody (data not shown). To determine whether activeTTSuV1 infection indicated by sero-conversion occurs in healthy individuals who are PCR positive, the human and swine sera were examined by a TTSuV1-ORF2 specific ELISA, using the purified TTSuV1 ORF2 protein as the capture antigen. Low to moderate (OD values ranging from approximately 0.15 to 0.45) antibody responses, which were above the cutoff value of 0.125 (calculated as the lowest quartile value +/- two standard deviations) were detected in 16 of the 40 human sera tested, indicating that sero-conversion to

TTSuV occurred in human beings. Among the swine samples tested, except for two animals with low titers, all other animals had high antibody levels against the TTSuV1-ORF2 protein (Figure 7), indicating that that antibody responses to the ORF2 protein was mounted in natural infections of swine. All ELISA-positive samples were also TTSuV PCR positive except for swine sample 18.

Figure 7: Expression of the TTSuV1 ORF2. TTSuV1 ORF2 protein purified by HIS-tag affinity purification from lysates of transformed *E.coli* BL21 DE3 cells. Coomassie blue stained, SDS-PAGE gel showing the protein ladder in the left lane and purified TTSuV1-ORF2 [approximately 10kDa] in the right lane.

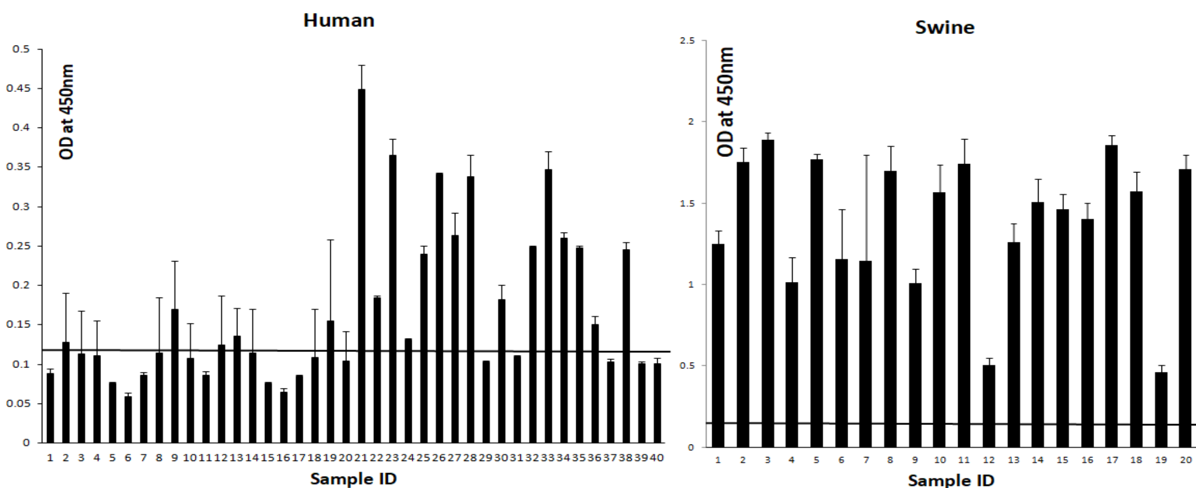

Figure 8: ELISA for the detection of antibody responses to TTSuV1: a) Antibody responses to the TTSuV1 ORF2 protein in human sera (N=40). b) Antibody responses to the TTSuV1 ORF2 protein in swine sera (N=20). The mean optical density values of duplicate values are depicted. The cutoff value for the human sera (0.125) was calculated as the lowest quartile value of the data set +/- two standard deviations. The cutoff value for the swine samples (0.16) is the mean value of a negative control sample which was obtained after screening a panel of field swine sera.

Discussion

With the recently published evidence for the pathogenicity of TTSuVs in swine (10, 11), the detection of TTSuVs in pork products (24), human drugs and veterinary vaccines (21) and its ubiquitous presence in the environment (16, 20), understanding whether TTSuVs can infect human beings and whether TTVs, in general, can cross species barriers has become both important and essential.

High levels of genetic diversity is common in TTVs (30).The detection huTTV DNA in 85% of the human sera tested in this study is consistent with prevalence estimates of between 5-90% in other studies (3, 4), depending on the sensitivity of the assay used. Unexpectedly, in this study, only 3 out of the 40 human samples tested negative for TTSuV DNA on all the assays used (Table 3). To rule out a sample-source bias, samples were purchased from two different vendors, while ensuring that all samples were from individual donors. To ensure validity, samples were also tested with PCRs targeting four different regions of the TTSuV genome. The pan huTTV (26) and pan TTSuV real-time PCR assays used were highly sensitive, with a detection limit of 1.5 copies per reaction, which is comparable to other qPCRs for the detection of TTVs (31). As previously described in details (5), the gel-based PCR for the detection of TTSuV1 was less sensitive at 1000 copies per reaction. Overall, detection was consistent between the two assays, considering that TTSuV2 was not detected by the gel-based assay, the sensitivity was lower in the gel-based assay and that different regions of the UTR were targeted by the two assays. The amplicons of the two PCRs targeting the TTSuV UTRs were highly conserved; with an average percentage similarity of over 90% when aligned with other TTsuV1 or TTSuV2 sequences. The finding that fewer samples were positive with PCRs targeting the TTSuV1 ORF2 and ORF3 is consistent with the fact that they are more variable. Human fecal

matter was previously shown to contain TTSuVs DNA (23), and its presence can be attributed to the contamination of water supplies with swine manure (19). It is likely that environmental or feed contamination, rather than proximity to swine, plays a significant role in the exposure to TTSuVs in humans. While our findings call for a more extensive characterization of the animal to human transmission of TTSuVs involving a larger sample size, sequential sampling and incorporating pork consumption as a variable, this study provides important initial evidence for the potential zoonosis of TTSuVs.

Currently, TTVs are classified in a species-specific manner, with this report being the first to present data supporting a possible cross-species transmission. Although the genomes of TTVs from various animal species, including humans, are similarly organized, each species-specific TTV is genetically distinct. The genomes differ from each other, and human TTVs by more than 50% sequence identity (28). Therefore, the data presented does not warrant a reclassification of TTVs.

Of the 20 swine samples examined in this study, only one was negative for TTSuVs. The high prevalence of TTSuVs in this sample set compared to our previously published rate of approximately 55% (5) is likely due to the fact that the samples were collected from multiparous, adult sows, while the population estimation was carried out in production animals which were likely to be younger (6). Surprisingly, despite being derived from a “closed” herd with no exposure to outside animals, a majority of the swine samples in our study were positive for huTTV DNA (Table 3). While exact estimates of the extent of contamination of water sources within the U.S with huTTV DNA are not available, extensive contamination has been reported in other parts of the world (16, 18). Moreover, huTTV DNA has been previously detected in buffalo milk (32).

The presence of DNA in fecal matter could represent a merely transient passage through a non-definitive host. However, the presence of TTSuV DNA in human serum could be indicative of viremia. Since recent findings support a possible role for TTSuVs as the primary or secondary etiological agents of viral infections in swine (10, 11) the question of whether TTSuVs can replicate in humans is important from a public health perspective. While the exact cell types which support TTV replication have not been identified, it has been suggested that T lymphocytes and PBMCs can support replication (33). Our findings that human PBMCs are able to support serial infections with TTSuV1, not only confirm that PBMCs can support viral replication, but also show that PBMCs are a possible site of viral replication in cross-species TTV infections. Additionally, our finding that the ability to respond to immune stimuli is diminished in TTSuV1-infected PBMCs provides preliminary evidence that TTVs could be immuno-suppressive. However, since we did not perform a cell viability staining assay to demonstrate the health of the infected cells, the lower proliferation responses to PHA could also be attribute due to death of the PBMCs caused by TTSuV1 transfection. In addition, we did not perform any time-point optimizations for optimal readings by Alamar-blue assay and so, these results could have been recorded after saturation. Therefore, we recommend that future studies involving such assays should perform such optimizations for better results.

Widespread detection of antibody responses to TTSuVs in swine and huTTVs in humans has been previously described (34, 35). While antigenic cross-reactivity between human and swine TTVs has not been studied extensively, a human genogroup 1-specific anti-serum did not cross-react with the TTSuV1 ORF1 antigen (34). The TTSuV1 ORF2 protein is about 53% similar between the TTSuV1a and 1b subtypes, while it is about 40% similar to the TTSuV2 ORF2 proteins, and about 20% similar to its counterpart in human TTVs. Therefore, the

detection of antibody responses to the TTSuV1 ORF2 protein in 16 of the 40 human sera tested further substantiates our hypothesis that TTSuVs can be potentially zoonotic. While exact information about when the individuals in this study were infected is not available, patterns of sero-conversions and sero-reversions, where PCR-positive individuals lose detectable serum antibodies in subsequent samplings, have been detected in TTV-infected individuals (36). Similarly, in infections of swine with the closely related porcine circoviruses, we have detected a waxing and waning pattern of viremia, probably in response to host immunity, especially in older animals in which chronic infection has been established (37). Therefore, a combination of PCR and antibody detection is likely to provide accurate evidence for active viremia as well as previous exposure for TTVs.

In conclusion, our findings are the first to support the possibility that TTV infections can be zoonotic or reverse zoonotic. With the abundance of epidemiological data linking TTVs to various human diseases, the possibility of opportunistic pathogenicity cannot be ignored. While healthy humans will most likely be able to clear TTSuV1 infection, more detailed studies are required to determine if TTSuVs or TTVs from other mammalian species can establish infections or alter immune functions in immuno-compromised individuals, especially because the inverse correlation between TTV viral loads and the immune status of the individual is well-established (14, 15). Further research is required to determine the significance of these initial findings in the context of host immunity or pathology and is the focus of our future research.

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CHAPTER THREE: IMMUNE GENE REGULATION IN EXPERIMENTAL SWINE INFECTIONS WITH SWINE TORQUE-TENO VIRUS 1 (TTSuV1)

Abstract

Torque-teno viruses (TTVs) are small non-enveloped DNA viruses with high prevalence in both human and swine populations. The infections are usually acquired very early in life and they persist over a lifetime of the host. Several epidemiological studies have linked human TTVs to disease causation but its role as a pathogen remains unknown. Also, swine TTVs (TTSuVs) were shown to cause lesions in experimentally infected pigs and exacerbate coinfections in pigs. However, their role in coinfections is not fully understood since there is no available *in-vivo* model to demonstrate possible TTV pathogenesis. Here, we studied how TTSuVs could regulate the host immune responses, using a swine model. A total of 27 piglets were used; 18 were infected with a TTSuV1 culture, while 9 were used as controls. Our results showed a possible delayed antibody response and minimal viremia. While all adaptive immune genes studied here were insignificantly regulated, most innate immune genes were only transiently regulated. Significant upregulation of DHX36 suggests that cytoplasmic viral sensing could be through the interferon-inducing pathway. Also, the significant upregulation of PD-1, a regulatory cytokine, could suggest possible inhibition of cell-mediated immune responses, leading to TTSuV1 persistence. However, future studies to further understand role of TTVs in adaptive immune responses are still required.

Introduction

Torque-teno viruses (TTVs) are recently isolated very small non-enveloped DNA viruses (1), and are highly prevalent in over 90% of both healthy human and swine populations (2, 3). Both human TTV and swine TTV (TTSuV) infections are acquired very early in life and viremia

persists over a life time (4-6). Also, both these TTVs have been reported to have zoonotic or reverse zoonotic capabilities (7-9). Furthermore, while many epidemiological studies linking human TTVs to disease causation (10-13), its role as a sole causative agent of disease remains to be studied. Meanwhile, TTSuVs were shown to cause lesions in experimentally infected pigs, and also exacerbate coinfections in pigs (14-16), further necessitating the study of these viruses.

While coinfections of TTVs and other pathogens has been extensively reported (2, 11, 16, 17), the role of TTSuVs in coinfection systems is not fully understood. In some cases, TTV viremia was found to be increased (11, 18), and yet in others, TTV improved replication of other pathogens (2). Since TTVs have been reported to establish chronic infections in both humans and swine (5, 6, 19, 20), they could be immune-suppressive, and result in thriving of the co-infecting pathogens. Like other chronic pathogens, a few studies have demonstrated a resemblance in the modulation of the host immunity and immune suppression. One study showed suppression of IFNs (21), while another showed short-lived response to IFN treatments (4).

In-vitro studies have shown how major TTSuV1 proteins modulate the host immune responses. A recent study demonstrated that human TTV encodes miRNAs which interfere with interferon expression (22). Although TTSuV1 ORF1 elicits an early anti-viral response, it was also reported to upregulate immune-suppressive genes (21). ORF2 has been reported to diminish NF-kB pathways (23) and also downregulate IFN-β expression (21). However, ORF3 was involved in minimizing adaptive immune responses by inhibiting IL4 and IL13 expression (24). However, no study has fully described how TTVs regulate the host immune responses *in-vivo*. This was the major goal of this study.

The main setback, however, in understanding the pathogenicity and immunobiology of TTVs has been the lack of both *in-vitro* and *in-vivo* model systems. Our team has previously

developed a unique *in-vitro* culture system, which used a modified reverse genetics approach to produce more viable TTSuV1 viral particles. As a secondary goal, we also wanted to determine if these recombinant viral particles would replicate in the swine host as the wildtype virus and establish a chronic infection. The use of swine in xenotransplantation and other biological uses in human medicine, makes the use of swine as the best model to study TTVs (25). Furthermore, several genetically-engineered swine models to study other viral pathogens already exist (26), and the swine genome is fully sequenced, immunological studies would be simplified. Therefore, in this study, we examine the role of TTSuV1 in immunoregulation, using a swine model.

Materials and methods

Cell culture and viral culture preparation

Swine testis (ST) cells (ATCC CRL-1746) were used to propagate the TTSuV1 viral particles (RVPs). ST cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin, and 100μg/ml streptomycin, as previously described (27).

The virus culture was prepared using our modified *in-vitro* culture system. Briefly, the TTSuV1 DNA genome (2877bp) was excised from the shuttle vector by AseI restriction digestion, purified by gel extraction and re-circularized by ligation (T4 DNA ligase, New England Biolabs, Ipswich, MA). ST cell-lines were grown to a 70% confluence in 25cm^3 flask, and then transfected with 2ug of circularized TTSuV1 genome, 1ug TTSuv1 ORF1, 1ug TTSuV1 ORF2 and 1ug of TTSuV1 ORF3, using TransIT-2020 (Mirus Bio, Madison, WI), following the manufacturer's instructions. To validate the production of RVPs in the flasks, 100ul of the DNA-TransIT complex solution was added to a chamber-slide well for detection by immunofluorescence (IFA). The flask and slide were incubated for 72hr at 37°C in a CO_2

incubator. Recombinant virus production was assessed using IFA on a slide. To rescue the recombinant viral particles, the flasks were frozen at -80°C and treated with three freeze and thaw cycles to release the particles into the supernatant. The rescued virus culture was clarified by centrifugation at 300xg for 10min to remove cell debris, and the virus titer determined by TCID50 method. The virus culture was stored at -80°C until further use.

Animal experimental design

Twenty-seven (27) 2-3week old piglets of both sexes, delivered from TTSuV1 antigen-negative sows from North Dakota State University swine barn, were divided into two groups. In one group, eighteen (18) piglets were infected with $1x10^6$ TCID50/ml RVP culture (2ml intranasally and 2ml subcutaneously), while nine (9) were injected with 1X PBS and served as the negative controls. Whole blood for PBMC preparations and serum were collected weekly. Six pigs (4 treatment and 2 control) were sacrificed at day 14, 21, 28 and 36 respectively, and different tissues and serum collected. All animals were handled in accordance to the Institutional Animal Care and Use Committee (IACUC) of South Dakota State University, with oversight by the North Dakota State University's IACUC and Institutional Biosafety Committee (IBC).

Viral DNA extraction and detection by probe-based qPCR

To determine viral replication in the study animals, we used a probe-based qPCR assay. Briefly, we extracted total DNA from 200µl of each individual pig sera, using a QIAamp DNA mini kit (Qiagen, Valencia, CA); and then, 25ng of template DNA was mixed with QuantiTest probe mix (Qiagen), primers (Forward: GCTGACCAGGAAAATCCCAAAGTA and Reverse: TAAGTCAATCTGGAATACATC) and probe (5'-CAL-FluorOrange 560-AATATGGAACACACAGGGTACA-BHQ1-3'), cycled 35 times in a iCycler CFX96 Touch Real Time PCR Detection system (Biorad, Hercules, CA) at Ta = 60°C. No-template controls

were included in the assay as negative controls, while TTSuV1 plasmid DNA was used as the positive control. Viral quantification was based on Ct values (the higher the Ct value, the lower the viral titer). All samples with Ct<32 were considered positive for TTSuV1.

TTSuV1 ORF1 protein expression and purification for ELISA

Based on a previous study by Huang *et al.* 2011 (28), a truncated N-terminal region (aa position 1-166) of the TTSuV1 ORF1 protein (GenBank accession number: KT037083), was amplified by PCR, and later cloned in frame into pET28a bacterial expression plasmid, using BamHI and XhoI restriction sites (Forward: 5'-AGTCggatccGCTCCTGCTCGCCGATGGA-3' and Reverse: 5'-AGTCctcgagGTATCCGTATTGGTCCTCG-3'); this produced a C-terminally His-tagged protein. The recombinant plasmid construct was confirmed by diagnostic restriction digestion and sequencing. For expression, the plasmid was transformed into BL21 (DE3) cells (Thermofisher Scientific, Waltham, MA) following manufacturer's instructions. A starter culture of freshly transformed BL21 cells was grown in 100ml of LB media containing 50mg/ml Kanamycin overnight at 37°C. 10ml of the overnight culture was then used to inoculate 1000ml of LB Kanamycin media for expression culture. When the absorbance of the expression culture was 0.4 (at OD600), expression was induced with 1mM β-D-1-thiogalactopyranoside (IPTG) and incubated further at 37°C for 6hr. After 6hr post-induction, the bacterial cell pellet was collected by centrifugation at 6000xg for 15min. Since the recombinant TTSuV1 ORF1 protein was insoluble and expressed within bacterial inclusion bodies, purification was done under denaturing conditions. Briefly, the pellet was resuspended in binding buffer (8M Urea, 10mM Imidazole, 100mM sodium phosphate monobasic, 10mM TRIS pH=8.0) and sonicated on ice. The cell lysate was centrifuged, and the supernatant incubated with 2ml of Ni-NTA agarose beads (Qiagen) for 1hr with rotation at room temperature and later put into a gravity-flow

column. The agarose beads were then washed 5x with 10ml of wash buffer (50mM Imidazole, 100mM sodium phosphate monobasic, 10mM TRIS, pH=6.3). The protein was eluted using 5ml of Elution buffer (250mM imidazole, 100mM sodium phosphate monobasic, 10mM TRIS, pH=4.5). The purified protein was then precipitated out of solution using 9 volumes of absolute ethanol, and then centrifuged at 10000xg for 30min at 4°C to collect the protein pellet. The pellet was air-dried and then resuspended with 1x Tris-Glycine-SDS Running Buffer (Boston BioProducts). The purified protein was quantified using Pierce BCA protein assay kit (Thermosfisher Scientific), and then analyzed by western blotting. The purified protein was stored at -80°C till further use.

Western blotting

To check the purity and integrity of the expressed TTSuV1 ORF1 protein, a western blot assay was performed. Briefly, a discontinuous SDS-PAGE was used to resolve the proteins with a 12% polyacrylamide gel in Mini-Protean Electrophoresis System (Bio-Rad, Hercules, CA). Before transfer, the PVDF membrane was activated in 50% methanol, and then incubated, together with the gel, in transfer buffer (Boston BioProducts). A Trans-Blot SD semi-dry transfer system (Bio-Rad, Hercules, CA) was used for transfer, for 30 minutes at 10V. After the transfer, the membrane was rinsed in 1X PBST, and then blocked for 1hr with 2% skimmed milk in 1X PBST, on a shaker at room temperature. A monoclonal Anti-His tag antibody was used, at dilution of 1:250 in 1X PBST, to stain the membrane overnight at 4°C. The membrane was then washed 4x with 1X PBST for 5min. HRPO-labeled goat anti-mouse IgG antibody (KPL, Gaithersburg, MD) was used as a secondary antibody at dilution of 1:2000 for 1hr. Membrane was then washed 4x, and developed using Chloro-naphthol substrate (Thermofisher Scientific).

Indirect TTSuV1 ORF1-specific ELISA

To detect antibody responses to the TTSuV1 RVP *in-vivo*, we used the above expressed TTSuV1 ORF1 protein as the capture antigen in an ELISA. Briefly, ELISA plates (High Bind Microplate, Corning®, Corning, NY) were coated with 50µl of a 1:100,000 dilution of the purified TTSuV1 ORF1/ORF2 protein in carbonate-bicarbonate buffer (pH 9.6), overnight and room temperature. The coated plates were then blocked with a commercial blocking solution (Immunochemistry technologies, Bloomington, MN) for 1hr at 37°C on a rotor shaker. The plates were washed 3x for 5min, and then incubated with 1:50 of the swine sera, in duplicate, for 2hr at 37 °C with shaking. After washing 3x for 5min, the plates were incubated with HRPO-conjugated goat anti-swine IgG antibody (KPL), at a 1:5000 dilution for 45min at 37°C. This was followed by incubation with TMB substrate (KPL) for 3min, and the reaction stopped using 1M HCl solution. Plates were then read at an absorbance of 450nm in an ELISA plate-reader (Elx800 reader, BioTek Instruments, Inc., Winooski, VT). Two biological replicates were used, with three technical replicates in each run (total 6 values). Both Positive and Negative controls were used in quadruplicates, and the final ELISA value was calculated as S/P value, meaning it is a ratio of the mean OD value of a sample to the mean OD value of the positive control. The cut-off was determined as mean OD of the negative control. Also, to validate our results, we ran the samples on a previously described TTSuV1 ORF2 assay (7). Statistical significance between OD values of infected and control animals was determined by student T-test.

Lymphocyte proliferation assays

To evaluate TTSuV1-specific T-cell responses, peripheral blood mononuclear cells (PBMCs) from 5 RVP-infected and 3 uninfected control animals were selected, such that samples from the same pigs were available for each time timepoint tested (i.e. 6x8=48 samples),

The cells were stimulated with inactivated virus particles (RVP), ORF1 protein and ORF2 protein, respectively. Briefly, PBMCs from each pig were purified from heparinized venous blood using Ficoll-Hypaque density gradient centrifugation (Sigma, St. Louis, MO, USA). The cells were then resuspended in 1X RPMI 1640 media, supplemented with 10% Fetal Bovine Serum and 100U/mL Pen-Strep, at a concentration of 2×10^5cells/ml.

For stimulation, cells from each timepoint were seeded into 96-well cell culture plates (2×10^4 cells/well) in triplicates, in the presence of recombinant viral proteins (20 ug/ml of ORF1 or ORF2); and 25µl of inactivated TTSuV1-RVP culture/well (10^5 TCID50/ml). The TTSuV1 RVP was inactivated by heat treatment at 90°C for 15min. For controls, 5ug/ml of phytohemagglutinin (PHA-M, Life Technologies, Grand Island, NY) were used as positive control, while unstimulated cells were used as negative control. The plates were then incubated at 37°C in a CO_2 incubator for 72hr. After incubation, cell proliferation was assessed by a colorimetric Alamar blue reagent (AbDSerotec/Bio-Rad, Raleigh, NC), following manufacturer's instructions. Stimulation indices (SI), for each animal at each specific time point, were calculated to show proliferation [(Stimulation Index = Fluorescent Intensity of stimulated cells/ Fluorescent Intensity of unstimulated cells)x100]. At each timepoint, average SI values for the 5 infected (3 technical replicates each) and 3 control animals (3 technical replicates each) was calculated, and the difference between the average SI values was then analyzed using student's T-test for statistical significance. To validate the cell-mediated responses observed, the stimulated cells were also collected for RNA extraction and later checked for adaptive immune regulation by analyzing the expression of IL10, IL13, IL4 and IFN-γ mRNA, as listed in Table 4.

Viral RNA extraction and cDNA synthesis

Since TTSuV1 RVPs did not replicate well in the pigs, based on the qPCR viral replication data, unstimulated PBMCs from 3 infected pigs (which were viremic at DPI28 and 36) and 3 control pigs (with no viremia) for each timepoint (i.e. 6x6=36 samples), were tested. Briefly, $1x10^6$ PBMCs collected from the animals at DPI 0,7,14,21,28 and 36 were used for RNA extraction with the Qiagen RNeasy mini kit (Qiagen), following manufacturer's instructions. The extracted RNA was quantified, purity verified using the 260/280nm ratio, and also checked for integrity on agarose gel. In addition, the RNA was treated with DNAse enzymes to remove any DNA contaminations, and later assessed by PCR amplification of GAPDH gene, using the RNA as the template. Then, 1µg of the purified RNA was used to make cDNA using iScript cDNA synthesis kit (Biorad, Hercules, CA), following manufacturer's instructions. The synthesized cDNA was quantified by spectrophotometry and immediately stored at -80°C, until it was required.

Differential expression of immune genes

The differential expression of immune genes between infected animals (three) and control animals (three) was determined using the standard ΔΔCt method, as previously described (29). A panel of 21 genes was used in this assay, including 3 housekeeping gene (Table 4 and Figure 4). In addition to previously used primers (21, 24, 29), we designed other gene primers (Table 5), and all primers were optimized for efficiency and specificity as previously described (21, 24, 29). At every timepoint, each individual animal sample was separately run three times (3 biological replicates), including two technical replicates in each run (total of 6 values/animal sample). Average values of all infected animals and control animals were then used to calculate the fold changes at each time point, using the ΔΔCt method. The qPCR cycling conditions were;

95°C denaturing step for 3min, and 40 cycles of 95°C for 15sec and 60°C for 40sec, using iTaq Universal SYBR green super-mix (Biorad). An average of the 3 housekeeping genes was used to calculate the fold changes since standard deviations between HKG replicates was non-significant. Also, A fold change of 2 was considered significant, as previously described (21, 24, 29). Flow diagram illustration of how the immune genes are related is included in Chapter 1 (Figure 4).

Table 4: List of differential expression of immune genes

Functional Category	**Genes**
Innate	IFN-α, IFN-β, RSAD2, STING, IRF3
Inflammation	IL1β, IL6, TNFα, TRAIL, IL18
Regulatory	SOCS-1, PD-1, PD-L2
Viral DNA sensors	DAI-ZBP1, TLR9, LRRFP1, APOBEC3, cGAS, DHX36, IFI16
Adaptive	IL10, IL13, IL4
Housekeeping	GAPDH, HPRT, TBP-1

Table 5: List of primers for immune genes testing with qPCR

Gene	Primers	References
IFI16	F- ggcagctgagatctgtaat R- gcttccatatatgaatcagg	https://www.ncbi.nlm.nih.gov/nuccore/XM_013996900.1
RSAD2 viperin	F- cgagtctaaccggcagatgag R- ccaggatggacttggatgga	ARS porcine database http://199.133.11.115/fmi/webd#PINdb
APOBEC3	F- cgcttggtcacagagctgaagc R- gtagcacaagtaggtcttcctc	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3126140/
IL18	F- cgtgtttgaggatatgcctgatt R- tggttactgccagacctctagtga	ARS porcine database http://199.133.11.115/fmi/webd#PINdb
DHX36	F- cagcttcaaagaccccttt R- tggtcacttttagtatcctttg	https://www.ncbi.nlm.nih.gov/nuccore/456753274
STING (TMEM173)	F- catcaagggccgggtgta agattctccggcaccttcg R- gcgtactccaggacacag gctgttccatgccactgat	http://www.sciencedirect.com/science/article/pii/S0161589015000425 ARS porcine database http://199.133.11.115/fmi/webd#PINdb
LRRFIP1	F- gaaatactatgggctggatt R- gccgatgtgtttcttctg	ARS porcine database http://199.133.11.115/fmi/webd#PINdb
cGAS	F- tacccaagcatgccaaggaa R - gtaagtaacactgacaactctt	http://www.sciencedirect.com/science/article/pii/S0161589015000425
PDL2	F- gggcagtatcgctgcttca R- tctttattttcttgtaggaagctttgact	ARS porcine database http://199.133.11.115/fmi/webd#PINdb
IRF3	F- acgctacaccctctggttct R- gacaaccttgaccatcacca	http://www.sciencedirect.com/science/article/pii/S0161589015000425

Results

Viral replication in infected animals

In this study, we infected 18 piglets with TTSuV1 RVP while 9 were used as negative controls. To detect viral replication, we designed and optimized a TTSuV1 specific probe-based qPCR to only detect our strain of TTSuV1. Upon infection, infected pigs did not show viral replication until DPI21 (Table 6). 1/14 pigs on DPI21, 3/10 pigs on DPI28 and 3/6 pigs on DPI36 showed viremia respectively. No viremia was detected in all negative pigs over the study period (Table 6). The mean Ct values ranged between 31.2 (DPI21) and approximately 28 for DPI28 and 36.

Table 6: Viral replication of TTSuV1 in pigs. DPI - days post-infection

	DPI0	DPI7	DPI14	DPI21	DPI28	DPI36
Negative pigs	0/9	0/9	0/9	0/7	0/5	0/3
Mean Ct value (StdDev)	-	-	-	-	-	-
TTSuV1-RVP infected pigs	0/18	0/18	0/18	1/14	3/10	3/6
Mean Ct value (StdDev)	-	-	-	31.15 (+/-0)	28.10 (+/- 1.57)	28.23 (+/- 1.37)

Antibody responses to TTSuV1 RVP infection

We used TTSuV1 ORF1specific ELISA to detect production of antibodies to TTSuV1 RVPs. All piglets (27/27) showed high antibody titers by DPI0. No specific antibody responses to viral infection were observed in the infected group, as the antibody titers reduced over time. Surprisingly, after Day14, negative pigs showed slightly high antibody titers, compared to the infected pigs, peaking at Day28; and later declining by Day36 (Figure 9). The same trend was observed with TTSuV1 ORF2 specific ELISA; however, there is a late response at DPI36 (Figure 9b). The antibody levels were not statistically different between infected and control animals at all time points (p>0.05), as determined by student T-test.

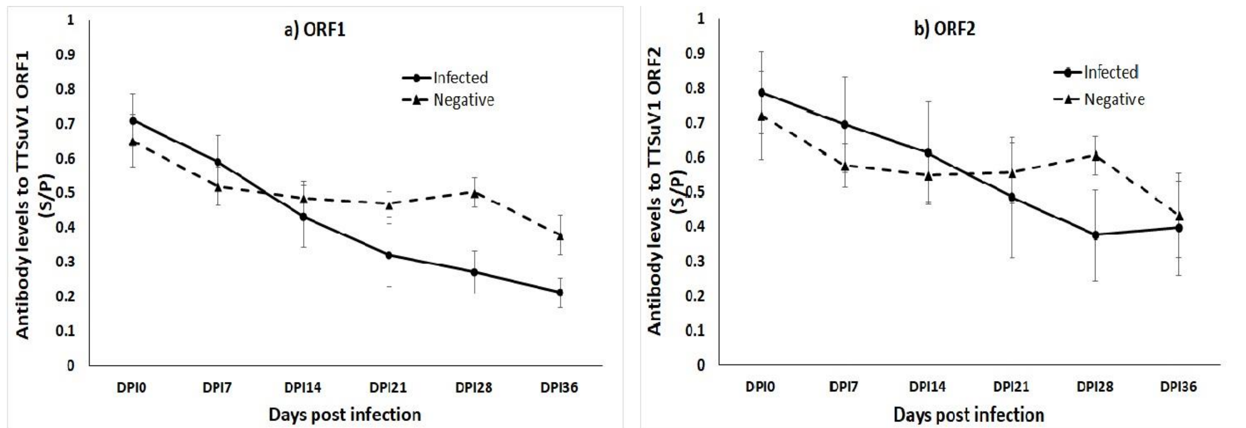

Figure 9: ELISA Antibody responses in infected animals (solid line) compared to control animals (dot line), with time. (a) Antibody titers to TTSuV1 ORF1 protein; (b) Antibody titers to TTSuV1 ORF2 protein. Each time point shows a mean of titer values from all animals (six values each). No statistical difference between antibody levels of infected and control animals (Student T-test p>0.05) at all time points.

Response to recall antigens

To check for cell-mediated immune responses, we did lymphocyte proliferation assays. No significant differences in cell proliferation were observed between treatments (infected and control animals) upon stimulation with the different antigens, including the non-specific mitogens (Figure 10). Although we observed some response to PHA on Day21, no recall responses were observed for ORF1 and ORF2 proteins, in infected animals. In addition, no significant differences were observed in regulation of adaptive immune genes (IL10, IL13, IL4 and IFN-γ), between infected and control pigs (data not shown).

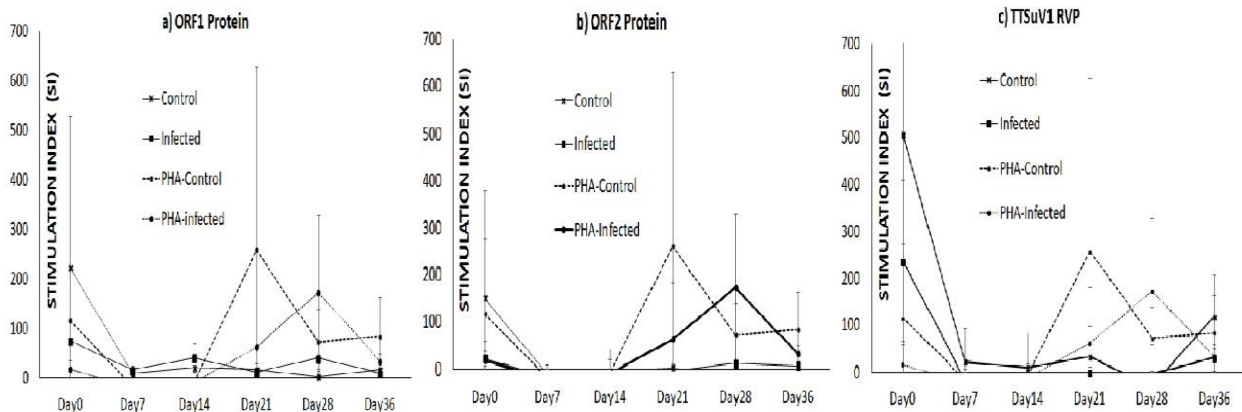

Figure 10: Lymphocyte proliferation assay. Stimulation of PBMCs with different TTSuV1 proteins (a) TTSuV1 ORF1; (b) TTSuV1 ORF2; and (c) Inactivated TTSuV1 RVP. Each data point, at each timepoint, represents an average of 3 replicates of five infected pigs or three control pigs, respectively. No statistical difference observed between infected and control animals (Student T-test, $p>0.05$) at all time points. Stimulation Index = (Fluorescent Intensity of stimulated cells/ Fluorescent Intensity of unstimulated cells)x100. Adaptive immune regulation was insignificant for all tested genes (data not shown).

Expression of type-I interferons and interferon-induced innate genes

Early detections of viral infections involves the expression of type-I interferons, IFN-α and IFN-β (30). Type-I interferons were briefly upregulated by DPI7, especially IFN-β, before they are insignificantly downregulated on DPI14 (Figure 11a). After that, they are only slightly upregulated until DPI36. RSAD2, a viperin protein, is one the interferon-stimulated effector

genes during viral infection. Here, although insignificantly regulated, RSAD2 was slightly upregulated, only showing a negative trend on DPI21 (Figure 11a). Also, IRF3 was upregulated significantly on DPI36.

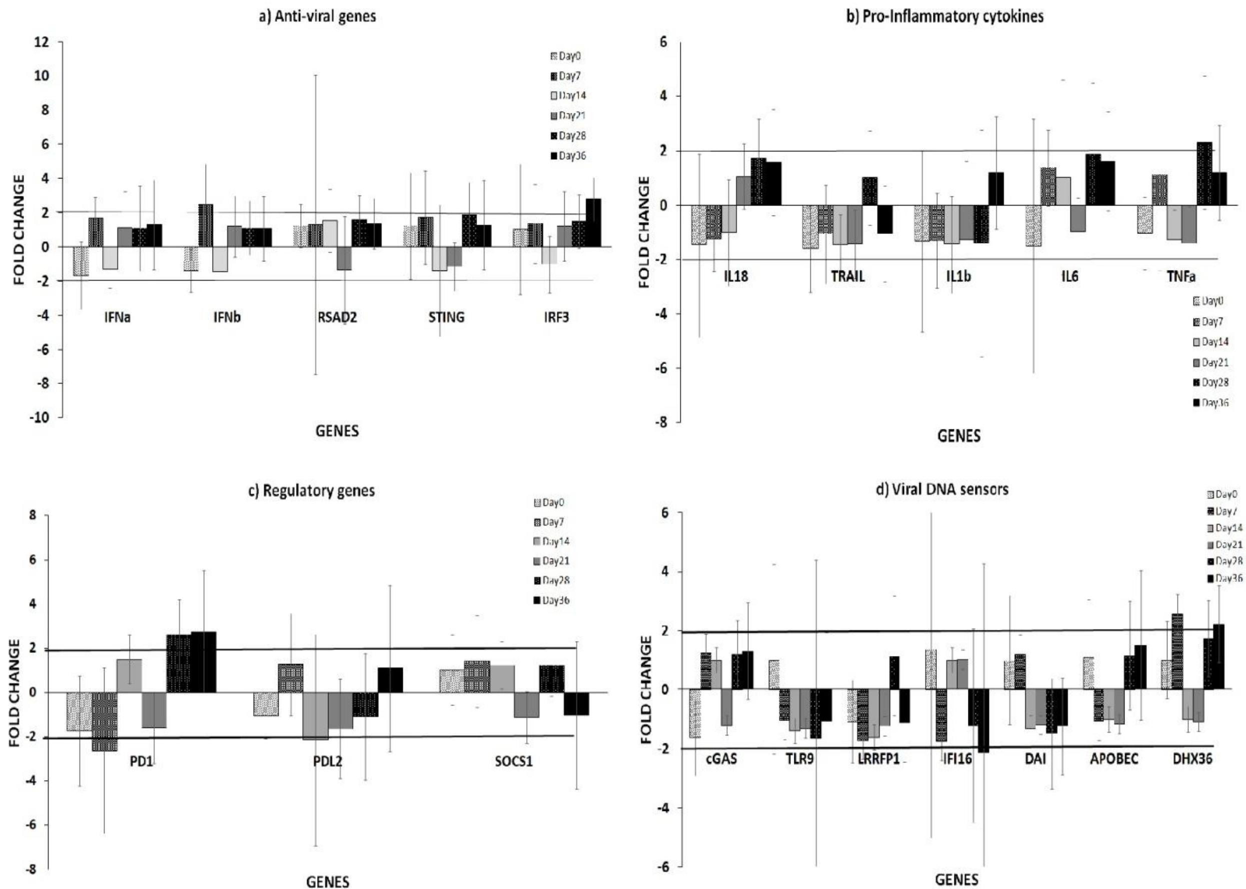

Figure 11: Differential expression of immune genes: a) Anti-viral genes; b) Pro-inflammatory cytokines; c) Immune regulatory genes; and d) Viral DNA sensors. Each bar represents average fold changes of three biological replicates and 2 technical replicates (6 values per animal), from 3 infected and 3 uninfected pigs over 6 time points, calculated by ΔΔCt method. Infected pigs were selected based on qPCR and viremia at DPI28 and DPI36. Fold change ≥2 is considered significant.

Expression of pro-inflammatory cytokine genes

Pro-inflammatory cytokines are very important in stimulating both innate and adaptive mechanisms in macrophages and neutrophils. Although no significant regulation was observed in all pro-inflammatory cytokines, most showed a downward trend until DPI14 (IL18, TRAIL and

IL1β) (Figure 11b). An upregulation trend was observed, but with no significance, in most genes on DPI 28, except for TNFα (Figure 11b).

Expression of regulatory immune genes

Both PD-1 and PDL2 were significantly downregulated at DPI7 and DPI14 respectively (Figure 11c), although PD-1 was later significantly upregulated at DPI28 and DPI36. Expression of SOCS at all time points was non-significant (Figure11c).

Expression of cytosolic viral DNA sensors

The innate immune system uses certain genome-encoded receptors, called pattern recognition receptors (PRRs), to sense non-self molecules and then activate the appropriate immune response (30). Since TTVs have a DNA genome, we used a panel of viral DNA sensors to demonstrate immune regulation by these viruses. Although most interferon-inducing DNA sensors showed an insignificant regulation, DHX36 was significantly upregulated on DPI7 and DPI36 (Figure 11d). Similarly, endosomal DNA sensing through TLR9 showed an insignificant downward regulation from DPI7 to DPI36 (Figure 11d).

Discussion

The similarities in immunological, physiological and anatomical systems between humans and pigs make swine organs the most common xenotransplantation tools and their use as biomedical models for human diseases. However, with the ubiquitous nature of TTVs, several studies have showed their contaminations in human vaccines, drugs, food, and blood supplies (31). Recent experimental studies have demonstrated that infection with tissue/bone marrow lysates from TTSuV1 positive pigs, the naïve pigs develop lesions (15); coupled with their continued isolation in coinfections with other pathogens which needs to be elucidated (2, 16, 32). Therefore, the availability of an *in-vivo* model system for TTV infections is critical. Furthermore,

several *in-vitro* studies demonstrated that TTV infections could modulate the host immunity through suppression of both innate anti-viral and cell-mediated responses (21, 22, 24).

In this study, infecting 3-week old piglets with TTSuV1 RVP ($1x10^6$ TCID50/ml) resulted in a delayed viremia, detected at DPI21 (Table 6). This could be attributed to the high TTSuV1 antibody titers detected in the piglets at DPI0 (Figure 9), which could have cleared off the replicating viruses. The sows which birthed the piglets were only screened by a TTSuV1-specific PCR, and not past TTSuV1 exposure by antibody assays. It is possible that maternal antibodies could have conferred protection in the early days of infection (33, 34). Another reason could be that the piglets received very low doses of the RVP culture, which led to low viral replication. Indeed, this could also be confirmed by the antibody assay (Figure 9), as there were no observable antibody responses to the viral proteins. The late viral replication peaks on DPI28 could explain the late ORF2 antibody response on DPI36 (Figure 9b); however, this was not observed for ORF1. In a previous vaccination study involving TTSuVk2a (35), a similar delayed antibody response to ORF1 and ORF2 proteins was observed in pigs, taking 6 weeks post vaccination. Since our study lasted only 5 weeks, this could explain the undetectable antibody response to the RVPs.

The most important anti-viral cytokines during early detections of viral infections are the type-I interferons, IFN-α and IFN-β (30). Here, we observed a transient expression of IFN-β at DPI7 (Figure 11a), and downregulation at DPI14 for both IFN-α and IFN-β (not significant). Our results are consistent with previous *in-vitro* studies that showed upregulation of IFN-β, and not IFN-α, in early anti-viral responses (21, 24). Another study also reported that human TTVs encode a miRNA which inhibits type-I interferon expression (19). Although our results are non-significant, the regulation trends align with these previous reports. Interferon-inducible proteins

are very important effector proteins in inhibiting viral infections. Viperins, like RSAD2, are well studied and known as interferon-inducible proteins, via the JAK-STAT pathways, and affect early viral replication (36). However, in our study, RSAD2 was readily regulated in correlation to interferon expression profile (Figure 11a). This suggests viral blocking of downstream interferon signaling pathways for this protein could be possible, similar to previous reports regarding other interferon-inducible genes (21). Meanwhile, like other chronic viruses which cause direct viperin expression (37), TTVs could be exhibiting similar behaviors, and further studies are still required to explain this phenomenon.

STING has been highly reported to be central in the regulation of interferon signaling for most DNA sensors (30, 38). When activated, STING leads to phosphorylation of IRF3, which in turn results in downstream regulation of type-I interferons (38). Here, although upward regulation was seen, no significant regulation was seen with STING; but at DPI36, we observed a significant upregulation of IRF3 (Figure 11a). This result shows viral sensing for interferon production could only be through the cytosolic IFN-inducing sensors, but not via the STING-TBK-IRF3 pathway as all DNA sensors involved in this pathway were not significantly regulated (Figure 11d). However, TTV DNA could be sensed through DHX36, another IFN-inducing cytosolic sensor, which uses the MyD88 pathway to upregulates IFN-α via the IRF7 activation (39). Like RIG-I, DHX36 are DEAD/H-box helicases which sense viral DNA in the cytoplasm, specifically CpG DNA (39). Indeed, DHX36 was significantly upregulated at DPI7 and DPI36 (Figure 11d). However, IFN-α was barely regulated (Figure 11a); and so, further studies to explain this discrepancy and DHX36's role in innate responses to TTVs are required. Further still, endosomal viral sensing via TLR9 was also ruled out as the gene showed a downward regulation at all timepoints, although not significant (Figure 11d).

The regulation of pro-inflammatory cytokines by TTVs has also been studied. One study noted downregulation of these cytokines (23) , while the other contradicted the findings (21). These cytokines are important in stimulating both innate and adaptive mechanisms in macrophages and neutrophils; Also, IL18 helps in IFN-γ production by NK cells. Here, although regulation was not significant, almost all genes showed a downward trend (Figure 11b), and only TNF-α was significantly upregulated at DPI36. However, our study also showed upward regulation (not significant) for IL6, as previously reported (23). Since most genes were insignificantly regulated, we also concur with previous studies that TTVs largely invade the inflammasome-dependent recognition pathway (24). In addition, a DNA restriction factor, IFI16, has been shown to activate the inflammasome (39), and we observed a significant downregulation at DPI36 (Figure 11d). However, further *in-vivo* studies to elucidate this hypothesis will be critical. In chronic infections, upregulation of PD-1 or its ligands, and SOCS-1 is common (30, 40), as they negatively regulate IL12, hence resulting in decreased T-cell responses. In addition, previous studies demonstrated that blocking PD-1 signaling also diminishes expression of SOCS-1 in HCV infection, another known chronic virus (41). Here, although these genes were initially downregulated, PD-1 was later significantly upregulated at DPI28 onward (Figure 11c), but no significant regulation was observed with SOCS-1. This is consistent with previous *in-vitro* studies done with TTSuV1 ORF1 protein (21). However, it is possible that PD-1 uses other regulatory pathways to control T-cell activation, as we did not see a correlation in expression of its ligand, PD-L2. A possible reason could be that PD-1 preferentially binds more to PD-L1, another PD-1 ligand, which has been previously showed to have more interaction capacity compared to PD-L2 (43), hence T-cell inactivation during TTSuV infections could be through PD-1 and PD-L1 interaction.

Cell-mediated immunity is another important arm of the host immunity in fighting viral infections. Recognition of antigens results in clonal expansion and activation of effector T-cells to clear the infection. Here, we checked for lymphocyte recall responses by activation with TTSuV1 viral proteins respectively. However, our study did not observe any significant proliferation in previously infected pigs, compared to the controls (Figure 10). Although, adaptive immune genes were not significantly regulated (data not showed), this is typical of chronic viruses to dampen cell-mediated responses (42). Previous *in-vitro* studies have however shown possible regulation of the adaptive immunity by TTVs (21, 23), through the downregulation of IL4, IL13 or NF-kB respectively. In addition, due to long storage in -80C (almost 2 years), it is also possible that the PBMCs were no longer viable. We therefore suggest a repetition of these assays for better and reliable conclusions.

In conclusion, our study is the first to demonstrate possible role of TTVs in *in-vivo* immune regulation, using a swine model. Based on our results and discussion, we propose a TTSuV1 pathogenesis and persistence model (Figure 12). Our model shows that the host innate immunity senses TTSuVs through the IFN-inducing cytosolic sensors, mainly DHX36, leading to type-1 interferon activation via the MyD88 pathway (solid lines). However, TTSuVs seem to evade other cytosolic sensors which use the STING-TBK1-IRF3 pathway to activate type-I interferon transcription (dotted lines), a central pathway in sensing of most DNA viruses. In addition, TTVs also evade the endosomal sensing through the TLR9. In addition, our model proposes that TTVs could establish a chronic state through the possible upregulation of molecules responsible for tolerance and immune escape, like PD-1 which is known to diminish T-cell responses. Therefore, PD-1 upregulation leads to deactivation of T-cell response, hence the persistence of TTV infection. However, due to possible long storage of the PBMCs at -80ºC,

the cells seemed non-responsive to antigen stimulation, and so future studies to demonstrate cell-mediated immune responses to TTVs are still required. We also suggest that future studies utilize a combination of mRNA transcript and protein expression for better results. Also, since the pig genome has been entirely sequenced, employing next-gene sequencing techniques to further elucidate this hypothesis would be critical.

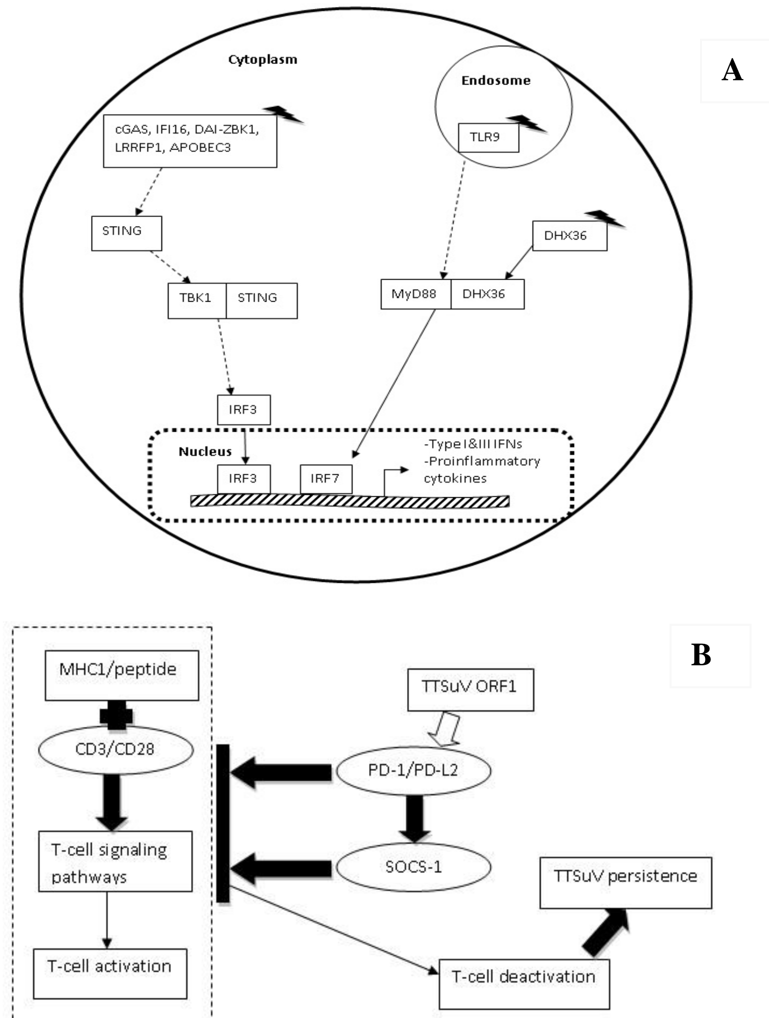

Figure 12: Proposed model for TTSuV1 pathogenesis in host. A. Chart proposes that TTSuV1 DNA sensing is through DHX36, a cytosolic sensor, leading to type-1 IFNs via the MyD88 pathway (solid lines). Other cytosolic viral sensing pathways, via STING-TBK1-IRF3, and endosomal sensing (TLR9) are not regulated by TTSuV1 (dotted lines). B. Flowchart proposes that TTSuV1 persistence could be through upregulation of PD-1 and SOCS-1 proteins. Dotted box represents normal T-cell activation through the MHCI/peptide presentation to T-cells (CD3/CD28 signaling) by antigen presenting cells; however, this activity could be downregulated by TTSuV1 upregulation of PD-1, as observed in this study.

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CHAPTER FOUR: SYNERGISTIC REPLICATION OF SWINE TORQUE TENO VIRUS 1 AND PORCINE CIRCOVIRUS STRAIN 1

Abstract

Torque-teno viruses (TTVs) are small non-enveloped circular single-stranded DNA viruses which infect several vertebrates. Previous studies have demonstrated that titers of TTVs increase in coinfections with other viruses, such as Swine influenza and Porcine circovirus 2 infections. It was also observed that clinical manifestations of the coinfecting pathogen could be exacerbated by presence of TTVs. Currently, the molecular mechanisms of these inter-viral interactions are unknown. Here, we investigated possible molecular mechanisms responsible the synergistic replication relationship between Porcine circovirus 1 (PCV 1) and TTSuVs. By using *in-vitro* co-infection assays, we demonstrated that either virus replicates more in co-infection systems compared to single infections, respectively. The ability of the PCV1 and TTSuV1 replicase proteins to physically bind to the TTSuV1 UTR region was assessed by EMSA, while the interaction of the PCV1 replicase proteins with a major TTSuV1 promoter was assessed by a reporter gene assay. Strong promoter activity was detected for the putative major promoter of TTSuV1 by the reporter gene assay. However, the PCV1 and TTSuV1 replicase proteins could be binding with more affinity to the UTR, hence blocking binding of cellular transcription factors which leads to low promoter activity. Physical interaction assays showed no interaction but need further optimizations. Results from this study provide further insight into the mechanisms involved in TTSuV1 mediated pathogenesis of viral coinfections.

Introduction

Torque-teno viruses (TTVs) are very small non-enveloped DNA viruses and are so ubiquitous in nature. Since their discovery in a post-transfusion hepatitis patient (1), several

diverse TTVs have been isolated in several vertebrate hosts (2-4); with possible zoonotic transmission also reported (3, 5, 6). In addition, they have been detected in the environment, water sources, human drugs, vaccine and blood product as contaminants (7-9). Intriguingly, the role of TTVs in disease causation is still not fully understood, as they have been isolated in both healthy and diseased hosts; with over 90% prevalence reported in both healthy humans and swine (10, 11). Several epidemiological studies have continuously associated TTVs to human diseases, like cancers, hepatitis, autoimmune diseases, among others (12-15), but no clear link between infection and clinical disease has been demonstrated yet. In contrast, experimental studies done in pigs reported that swine TTVs (TTSuVs) cause lesions in several organs, including kidneys, liver, heart, and lungs (16, 17), and this demonstrates a possible etiological role in swine diseases.

Furthermore, TTVs have been continuously isolated or detected in conjunction with other disease pathogens, both in humans and swine (10, 13, 18). Again, the role of TTVs in coinfections is not well understood; but a synergistic relationship has been reported. In coinfection studies, it was shown that TTV viral titers were greatly increased, compared to cases with TTV infection alone (13). Furthermore, TTSuV has been greatly associated with other swine infections, like porcine respiratory and reproductive syndrome virus (PRRSV) and porcine circoviruses (PCV2), even exacerbating their progress (10, 19-21).

Since TTSuVs have been increasingly isolated with PCV2 infections (10, 22, 23), we hypothesized that these viruses could be using their replicase proteins interchangeably, as helper proteins for their own replication, hence improving viral titers. TTSuVs and PCVs have very similar genome organization, both are single-stranded DNA viruses, and they both replicate by rolling-circle mechanism (24, 25). The replicase protein of PCVs has been well studied and

characterized, and it has been demonstrated to bind to the stem-loop of the viral genome to initiate replication (26). Anelloviruses are known to have conserved stem-loop sequences as origins of replications. The replicase protein binds to this sequence, creates a nick in the DNA to initiate replication (24, 26). However, the molecular mechanisms responsible for the inter-viral interactions between TTSuVs and PCVs are still are not well understood; and this was the goal of this study.

Currently, TTVs are propagated through backbreaking cell culture methods, as they do not replicate to higher titers in cell culture. The lack of a good understanding about their biology or how they establish infections is largely attributed to the lack of a good permissive cell line; as TTVs do not replicate to higher titers *in-vitro*. Several attempts to grow the TTVs *in-vitro* have been reported. Some recent studies have cloned the complete viral genome into shuttle plasmids (infectious clones) (27-30); the circularized genome is then transfected into mammalian cells to produce replicative viral particles (RVPs). However, although viral mRNA and proteins were produced, low viral replication was observed, and no serial transmission was demonstrated.

Our lab has previously developed a novel *in-vitro* culture system to grow recombinant swine TTV1 (TTSuV1-RVP). Our system involved use of modified reverse genetic tools to produce TTSuV viral particles. In this study, we utilized this novel *in-vitro* cell culture system to study and characterize molecular mechanisms responsible for inter-viral interactions between TTSuVs and PCVs.

Materials and methods

Cell culture and media used

All mammalian cells used in this study i.e. Swine testis (ST) cells (ATCC CRL-1746), porcine kidney (PK-15N) cells and African monkey kidney epithelium (Vero) cells (ATCC

CCL-81), were grown and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin, and 100μg/ml streptomycin, as previously described (31). All reagents and cells used were previously tested and were negative for TTSuV1 and PCV1.

TTSuV1 genome cloning and expression of viral proteins

The TTSuV1 genome was amplified by PCR from a diagnostic swine bone marrow (10). Two primers with flanking AseI sites (Forward 5'-GACAATTAATTTATGCAAAGTAGGA-3' and Reverse 5'-GACAATTAATTTGCATAAACTCCGC-3') were used to amplify the entire circular genome, which was then cloned into the pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA). The genome was sequenced and deposited in GenBank (KT037083) (5). Our team has also previously cloned, and validated the protein expression of the individual TTSuV1 ORF1, ORF2, and ORF3 by immunofluorescence assay (IFA) (32, 33). These same plasmid constructs were used for co-transfection this study. Here, we cloned and validated the expression of PCV1 replicase (ORF1) protein. Briefly, a PCR product for PCV1 replicase was amplified using Forward 5'-CTGAggatgcGCCACCATGCCCAGCAAGAAGAATG-3' and Reverse 5'-AGCTctcgagGTAATTTATTTCATATGGAAA-3'. It was then directionally cloned into a pcDNAV5-HisA mammalian expression vector (Thermofisher, Grand Island, NY), using BamHI and XhoI restriction sites. The integrity of the recombinant plasmid constructs was verified by restriction digestion and sequencing. All expressed proteins resulted in production of C-terminally V5-tagged fusion proteins. Protein expression for the individual constructs was verified by transfection in ST cells (32, 33), using Lipofectamine® LTX reagent (Thermofisher, Grand Island, NY), following the manufacturer's instructions. After 48hrs, protein expression was validated by IFA staining, using specific antibodies.

Immunofluorescence assay (IFA)

This technique was used to assess the production of both the TTSuV1 RVP and expression of its individual viral proteins in mammalian cells. Briefly, the transfected adherent cells were washed in Hanks Balanced Salt Solution (HBSS) (Mediatech/Corning, Manassas, VA) and fixed in ice cold Acetone:Methanol (1:1). The individual TTSuV1 viral proteins were previously validated for protein expression in mammalian cells using IFA, with a monoclonal anti-V5 tag antibody, as specific antibodies are not yet available (32, 33). We used anti-V5 antibody since the proteins were expressed as C-terminally V5-tagged fusion proteins. A rabbit polyclonal anti-TTSuV1 ORF1 antibody (provided by Dr. X.J. Meng, Virginia Tech) (35) at a 1:100 dilution, and a 1:500 dilution of the anti-V5 tag antibody (ThermoFisher, Grand Island, NY) was used to detect the TTSuV1 capsid (ORF1) protein. Swine polyclonal anti-PCV1 serum at a dilution of 1:100 was used to detect PCV1 replicase protein expression. To detect recombinant TTSuV1, the fixed cells were stained with a mixture of the rabbit polyclonal anti-TTSuV1 ORF1 antibody (1:100) and commercial anti-V5 tag antibody (1:500) for 2hr at 37°C, followed by a mixture of anti-rabbit and anti-mouse FITC (KPL, Gaithersburg, MD) at 1:50 for 45 mins (Figure 15). For the staining of TTSuV1 TCID50 plates, we designed and synthesized a N-terminal TTSuV1 ORF1 peptide (RWRRRLGRRRRRYRK, Position 6-20), which was then used to inoculate rabbits, collect and purify antisera (ProMab Biotechnologies, Richmond, CA) to use as primary antibodies to TTSuV1.

Rescue of recombinant TTSuV1 by co-transfection

Our novel *in-vitro* propagation system uses reverse genetics to produce improved titers of recombinant TTSuV1 particles. The system involves modifications to previously reported attempts to produce replicative TTV particles by use of infectious clones (27, 29). In these

studies, although viral particles were successfully produced, successive passaging of the virus led to low viral titers. We hypothesize that concurrently providing individual TTSuV1 proteins, together with the viral genome, would improve viral titers. Briefly, the entire TTSuV1 genome DNA (2877bp) was excised from the shuttle vector by AseI restriction digestion, purified by gel extraction and re-circularized by ligation (T4 DNA ligase, New England Biolabs, Ipswich, MA). ST cell-lines were grown to a 70% confluence in $25cm^3$ flask, and then transfected with 2µg of circularized TTSuV1 genome, 1µg TTSuV1 ORF1, 1µg TTSuV1 ORF2 and 1µg of TTSuV1 ORF3, using TransIT-2020 (Mirus Bio, Madison, WI), following the manufacturer's instructions. To validate the production of recombinant TTSuV1 particles in the flasks, 100ul of the DNA-TransIT complex solution was added to a chamber-slide well for detection by IFA. The flask and slide were incubated for 72hr at 37°C in a CO_2 incubator. Production of viral particles was assessed using IFA. To rescue the recombinant virus, the flasks were frozen at -80°C and subjected to three freeze-and-thaw cycles to release the viral particles into the supernatant. The rescued virus culture was clarified by centrifugation at 300xg for 10min to remove cell debris, and the supernatant was stored at -80°C until further use.

Transmission electron microscopy

To confirm the successful assembly and production of recombinant TTSuV1 particles created by our novel reverse-genetics approach, the harvested virus culture was further centrifuged at 13000xg for 10min in a microtube. A sample (50µl) was used to coat a 300-mesh carbon coated palladium grid for 10min. The coated grid was then negatively stained with 2% phosphotungstic acid (PTA) (36), and examined by a JEOL JEM-100CX II transmission electron microscope (Figure 15, lower panel).

Preparation of PCV1 culture

The PCV1 viral culture used in this study was prepared from persistently infected PK-15 cells. Briefly, PCV1-infected PK-15 cells (Source??) were grown in a $25cm^3$ flask to 100% confluency, and then frozen at -80°C for 3 cycles of freeze-and-thaw to release the virus. The virus culture was clarified by centrifugation at 300xg for 10min. The collected PCV1 viral particles were then quantified by TCID50 method in 8-well slides, and IFA was done using PCV1-specific polyclonal antisera. The $1x10^5$ TCID50/ml PCV1 culture was then later diluted to $1x10^3$ TCID50/ml before use in coinfection studies.

***In-vitro* coinfection of TTSuV1 and PCV1**

To test the hypothesis that TTSuV1 and PCV1 could have a synergistic relationship in coinfection systems, we performed an *in-vitro* coinfection experiment. Here, ST and PK-15N cells were separately grown in 6-well plates to a confluence of 60-70%, and then overlaid with 1ml of PCV1 culture ($1x10^3$ TCID50/ml) and incubated for 6hr at 37°C in a CO_2 incubator. The cells were then washed 5x to remove the PCV1 culture, and then overlaid with 1ml of TTSuV1 culture ($1x10^3$ TCID50/ml). After 24hr, the cells were washed 5x to remove TTSuV1 culture and then, 2ml of 2%DMEM media added to the cells. The cells were incubated further for 72hr at 37°C in a CO_2 incubator. The experiment was done in triplicate. As controls, single virus infection experiments (i.e. TTSuV1 only and PCV1 only) were included. Also, as negative controls, uninfected cells were included. After the 72hr incubation, the plates were frozen, and viral cultures were collected as previously described above. Cultures were stored at -80°C until further use.

Viral titers of both viruses were quantified by TCID50 method. Here, TCID50s were performed in 96-well plates using four technical replicates for each dilution. After 48hr of

incubation, the TCID50 plates were fixed and stained, using either anti-TTSuV1-ORF1 peptide-specific polyclonal rabbit antibody (dilution of 1:100) for TTSuV1 infection or PCV1-specific polyclonal swine antibody (dilution of 1:100) for PCV1 infection, as the primary antibody respectively. Results were read by microscopy and TCID50 calculations were done using the Reed and Muench method (34) (Figure 18).

Quantification of viral particles using qPCR

To further validate and test our hypothesis that TTSuV1 and PCV1 could have a synergistic relationship in coinfection systems, we used qPCR assays to quantify the produced viral particles in coinfection cultures obtained in the above section. Briefly, we used a previously validated TTSuV1 probe-based qPCR (Chapter 3) to quantify the TTSuV1 viral particles. However, for PCV1, we designed and validated a SYBr-green based qPCR assay, using Forward 5'-ATGCCAAGCAAGAAAAGCGGCCC-3' and Reverse 5'-CAAACCTTCCTCTCCGCAAACA-3'. TTSuV1 genome shuttle plasmid DNA and PCV1 ORF1 plasmid DNA (both described in above section) were used as positive controls respectively, while no-template controls were used as negative controls in both assays. Also, uninfected cells tested negative for both viruses. All statistical differences between treatments were determined using a Students' T-test ($p<0.05$) (Figure 16).

Rescue of recombinant TTSuV1 in presence of PCV1 replicase (ORF1) protein

To further check the hypothesis that TTVs could use external viral proteins, from other viruses (like PCV1), to enhance their own replication, we repeated the co-transfection procedure as described above, with modification. Briefly, ST cell-lines were grown to a 70% confluence in $25cm^3$ flask, and then transfected with 2µg of circularized TTSuV1 genome, 1ug TTSuV1 ORF1, 1µg TTSuV1 ORF2, 1µg of TTSuV1 ORF3, and 1µg of PCV1 replicase plasmid DNA,

using TransIT-2020 (Mirus Bio), following the manufacturer's instructions. Again, 100ul of the DNA-TransIT complex solution was added to a chamber-slide well for detection by IFA to validate viral particle production. After 72hr of incubation, the recombinant viral particles were rescued as previously described. The experiment was done in duplicate, and another coinfection treatment with no PCV1 plasmid was used as a control. The viral particles from the treatment in the presence or without the presence of PCV1 replicase protein was quantified by TCID50 assay, calculated using Reed and Muench method (34). Four technical replicates of each TCID50 dilution were used for the calculations. Statistical difference between treatments was determined using a Student T-test ($p<0.05$).

Luciferase reporter assay

To determine if the PCV1 ORF1 protein binds to the TTSuV1 UTR region containing the putative promoter site, we used the luciferase reporter assay. Briefly, the untranslated region (UTR) of the TTSuV1 genome (566bp) was cloned into pGL3 basic (Promega, Madison, WI), a promoter-less reporter gene system (For primer: CGATgctagcAATCTATGGCCGAGCATGGG and Rev primer: ATGCaagcttTCCGCTCAGCTGCTCCTGC), as previously described (37, 38). The cloned region covered -432 to +130 around the TATA promoter region (Figure 13). Table 7 shows all possible transcription factors predicted to bind to this same sequence (PROMO database, http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). The validated construct was labeled pGL3UTR (pGL3 basic containing TTSuV1 UTR). Vero cells were then co-transfected, using TransIT-2020, with *pGL3UTR+TTSuV1 ORF1* (pGL3UTR + TTSuV1 ORF1 – a putative TTSuV1 replicase protein, as experiment positive control), *pGL3UTR+PCV1 ORF1* (pGL3UTR + PCV1 ORF1 – a PCV1 replicase protein, to test hypothesis), *pGL3UTR* as a baseline control, while *pGL3 Basic* (no promoter) as negative

control and *pGL3UTR+emptyV5 plasmid* as transfection control were also used. Five replicates of each treatment were used. After 72hr of incubation at 37°C, the luciferase activity was assessed using the Bright-Glo kit system (Promega), following manufacturer's instructions. Luminescence was detected using the Synergy luminometer machine (Core biology lab, NDSU). All the obtained results were normalized and analyzed using a Student T-test to test statistical differences between treatments.

Table 7: List of possible human transcription factors predicted to bind to TTSuV1 UTR DNA (566bp) sequence).

Class	**Transcription factor**
Cys2His2 zinc finger domain	Sp1, TFIID
Cys4 zinc finger of nuclear receptor type	RXR-α
Leucine zipper factors (bZIP)	c-Fos, c-Jun, C/EBPα, AP-1
NF-1 class	NF-1
TATA-binding proteins	TBP
Tryptophan clusters	GABP, GABP-α, PEA3
HMGI(Y) class	HMG I(Y)
Homeo domain	POU2F1, E2F-1

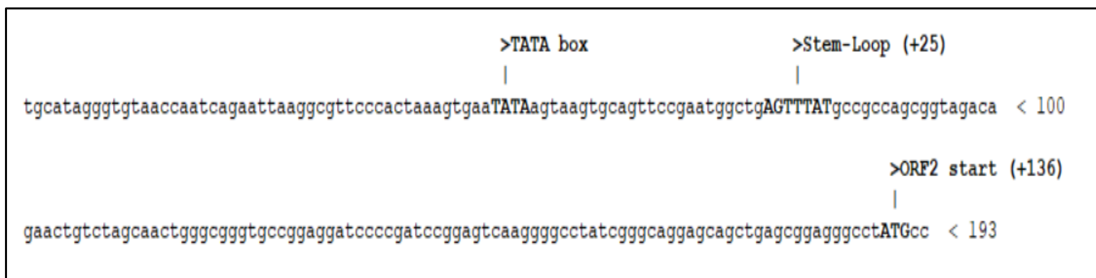

Figure 13: Sequence showing entire cloned TTSuV1 UTR (566bp) with putative TATA promoter and stem-loop (GenBank accession no. KT037083).

Electrophoretic mobility shift assay (EMSA)

To further understand the molecular interaction between the PCV1 ORF1 (replicase) protein and the TTSuV1 UTR region, we used EMSA – a protein-DNA interaction assay. TTSuV1 ORF1 protein was used as a positive control. Briefly, a short sequence of the TTSuV1 UTR (210bp), with a putative stem-loop as the origin of replication (-76 to +130) (Figure 13),

was amplified by PCR (For primer: TGATTGGACGGGAGCTCAAGTC and Rev primer: TCCGCTCAGCTGCTCCTGC), and Biotin-labeled using a Pierce 3' End DNA labeling kit (Thermofisher Scientific, Cat#89819) following manufacturer's instructions. Meanwhile, PCV1 replicase and TTSuV1 ORF1 proteins were expressed in Vero cells respectively. 72hr post-transfection, the crude proteins were respectively extracted using the M-PER mammalian protein extraction buffer (Thermofisher Scientific, Cat#78501) following manufacturer's instructions. The extracted crude proteins were immediately aliquoted and stored at -80ºC until further required.

To check the physical protein-DNA interaction, the LightShift Chemiluminescence EMSA kit (Thermofisher Scientific, Cat#20148) was used. Briefly, 2µl of Biotin-labeled DNA was mixed with 5µl of crude protein extract (TTSuV1 ORF1 – as positive control, or PCV1 replicase protein), in presence of 1µl of NP buffer, 1µl of glycerol and 1µl of 1µg/µl Poly (dA.dT) in a 20µl reaction. Poly (dA.dT) was used as a binding competitor in the reaction since the UTR amplicon has high GC content. The reaction was then incubated at room temperature for 30min. Refer to Figure 14.

Native PAGE, blotting and chemiluminescence staining

All EMSA reaction products were separated on 8% Native polyacrylamide gel at 100V for 1.5hr, including PCV1 replicase+TTV DNA and TTSuV1 ORF1+TTV DNA. TTV UTR DNA only was included as a negative control. The resolved complexes in gel were immediately transferred onto a nylon membrane, using a semi-dry transfer system at 15V for 45min. The membrane was then cross-linked before staining. Then, a Chemiluminescence staining kit (from EMSA kit above) was used, following manufacturer's instructions. Briefly, the membrane was incubated in 20ml of blocking buffer with gentle shaking for 15min, and then incubated in

Streptavidin-HRPO conjugate (1:300) for more 15min. The membrane was then washed 4x, and then incubated in 30ml of substrate equilibration buffer for 5min. Finally, the membrane was incubated in 10ml of Enhancer-Peroxide (1:1) solution for 5min. For visualization, the stained blot was exposed in the FluorChem FC2 Imaging system, with a CCD camera (Alpha Innotech). Refer to Figure 14.

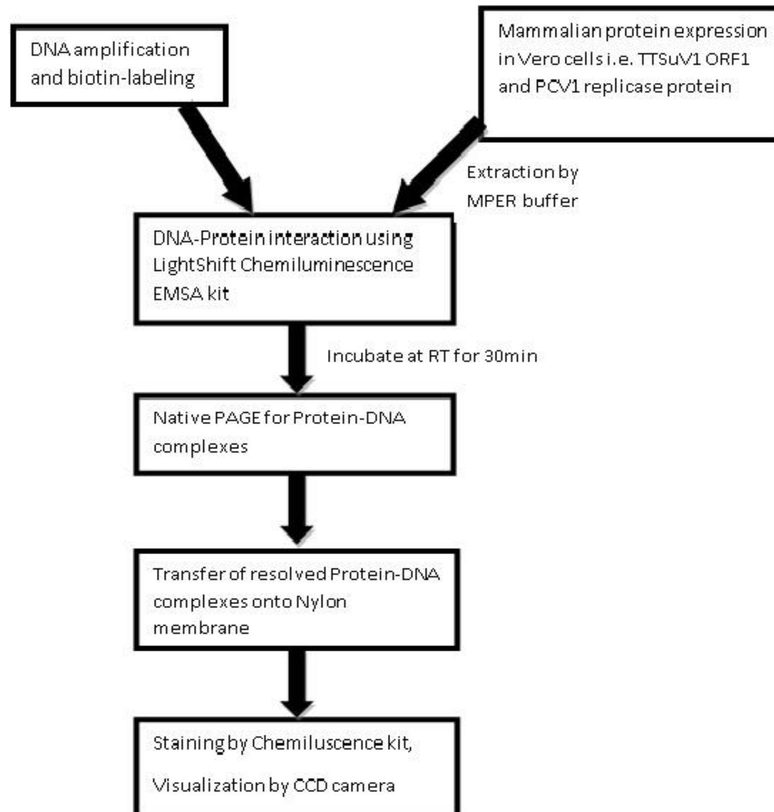

Figure 14: Scheme to show flow of EMSA protein-DNA interaction experiments

Results

Recombinant TTSuV1 production in mammalian cells

Co-transfection of TTVSuV1 genome, and its individual protein expression plasmids in ST cells resulted in production of viral particles, localized in the nucleus (Figure 15A), using IFA staining. Our team has also found that transfection with TTSuV1 infectious clones alone resulted in less viral titers compared to our co-transfection system (ongoing work, unpublished).

To further confirm ultra-structure of the produced recombinant TTSuV1 particles, negative staining for electron microscopy revealed icosahedral/spherical viral particles of about 20-35nm in diameter, after first passage in ST cells (Figure 15, lower panel). Expression of PCV1 replicase protein in Vero cells resulted in specific localized bright green fluorescence within the nucleus of transfected cells (Figure 15B). All negative or untransfected cells did not show green fluorescence.

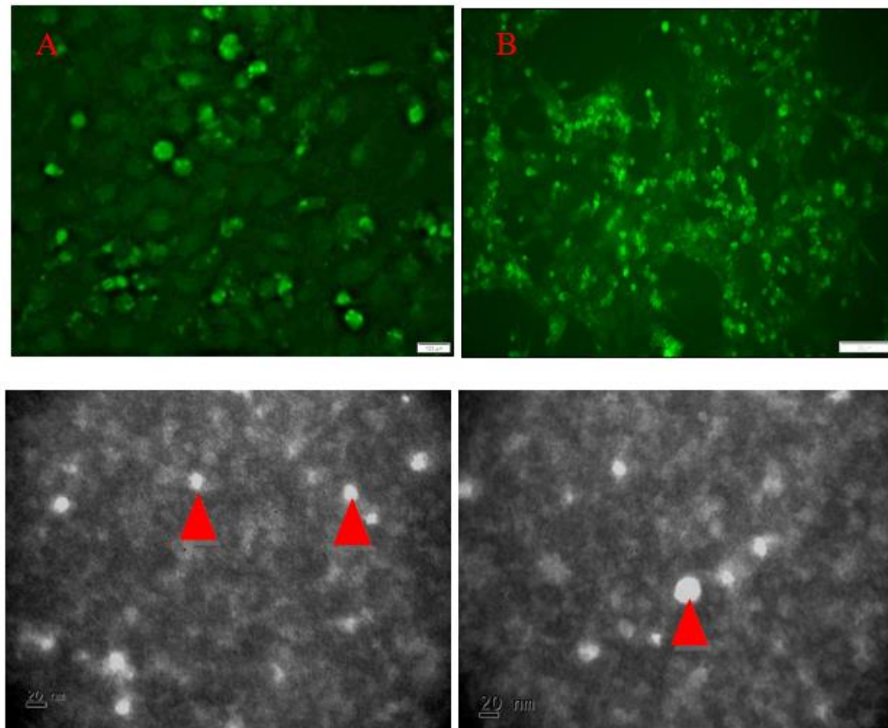

Figure 15: Top panel – Immunofluorescence assay (IFA) staining, with FTIC-labeled antibody (Magnification=20x), A) Recombinant TTSuV1 particles expressed in ST Cells and B) PCV1 ORF1 protein expressed in PK15 Cells. Lower panel – Electron micrographs of TTSuV1 viral particles after first passage in ST cells (Red arrows, Scale bar = 20nm).

Coinfection with TTSuV1 and PCV1 increase viral titers of either virus *in-vitro*

To determine whether there is a synergistic relationship between TTSuV1 and PCV1 during coinfections, we performed an *in-vitro* coinfection assay with TTSuV1 and PCV1, and then quantified both viruses by both TCID50 and qPCR methods. By TCID50 method (34), we observed increased viral titers when both viruses were infecting cells, compared to when was

used singly. TTSuV1 viral titers were significantly higher when co-cultured with PCV1 compared to when TTSuV1 was grown by itself, both in ST cells (p<0.05) and PK15 cells (p<0.01) (Figure 16). Although not significant, PCV1 titers also showed similar results. Surprisingly, quantification of viral particles by qPCR showed contradictory results. For all treatments, viral titers in single virus infection were significantly higher compared to when both viruses were co-cultured, both in ST and PK15 cells (Figure 16).

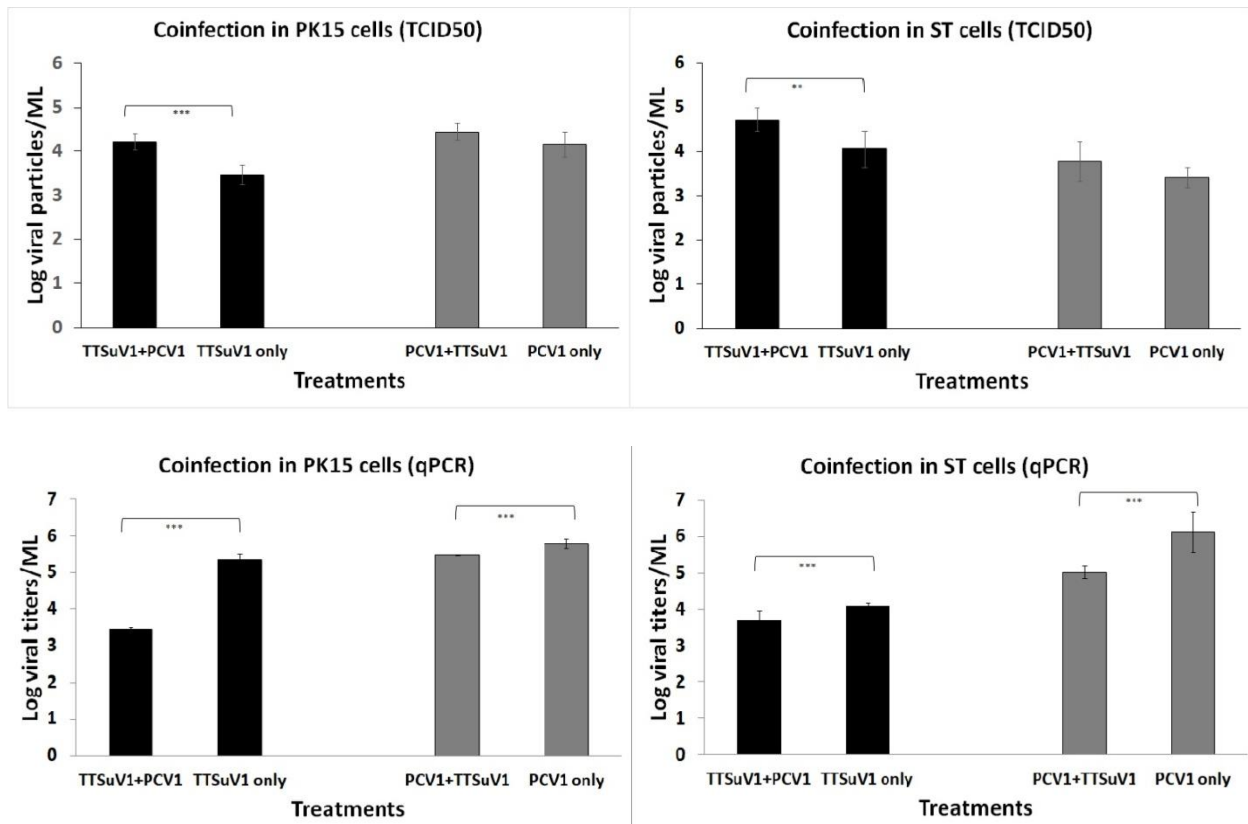

Figure 16: Viral titer quantification for in-vitro coinfection. Top panel – Quantification by TCID50 method. Each bar represents an average of 3 biological replicates of N=4. Bottom panel – Quantification by qPCR. Black bars represent TTSuV1 titers while Grey bars represent PCV1 titers. Each represents an average of 2 biological replicates of N=2. Statistical differences observed at ***p<0.01 and **p<0.05 by Student T-test.

Increased TTSuV1 RVP production in presence of PCV1 replicase protein

To further determine if PCV1 replicase protein could be responsible for the increased TTSuV1 titers in coinfection systems with PCV1, we rescued the recombinant TTSuV1 virus in

presence of or without over-expressed PCV1 replicase protein, and after 72hr post-infection, we collected the viral culture and measured the viral titers produced in either treatments by TCID50 method (34). The production of the TTSuV1 RVPs in presence of PCV1 replicase protein resulted in significantly more viral particles, a log higher compared to the control treatment where no PCV1 replicase was used (Figure 17). This difference in viral particle produced between both treatments represents approximately a percentage increase of 1133.9%.

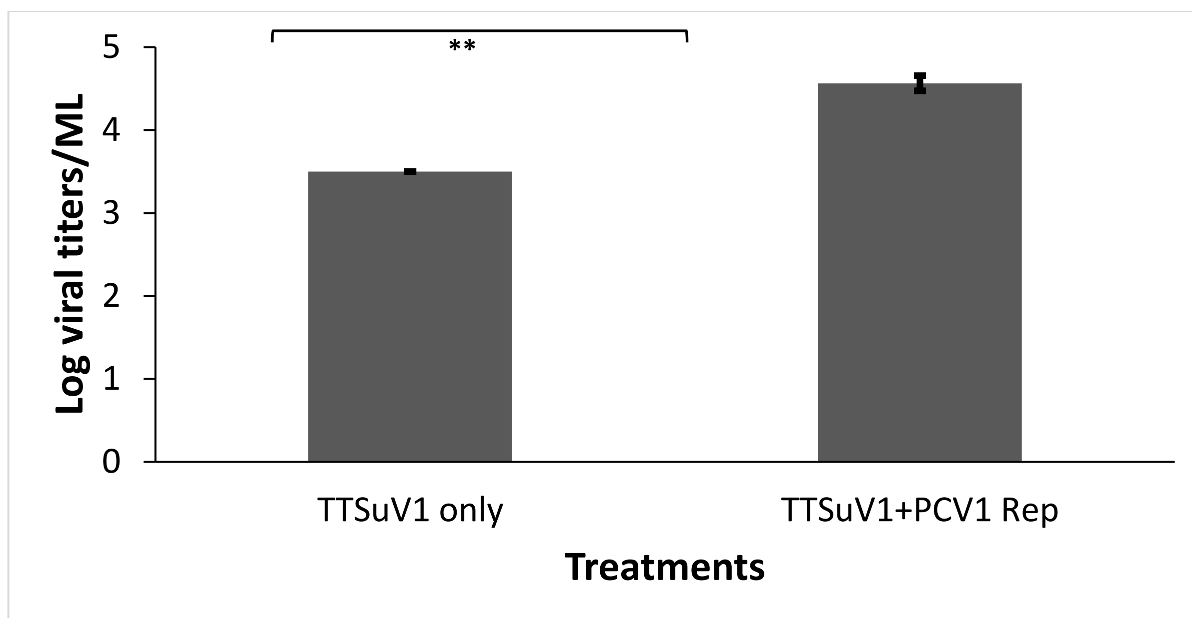

Figure 17: Rescue of the TTSuV1 RVPs in presence of PCV1 Replicase protein in ST cells. Two biological replicates of N=4, **p ≤0.05, as determined by a Student T-test. No standard deviation seen between the 2 biological replicates for TTSuV1 only.

Influence of PCV1 replicase protein on the promoter activity of the TTSuV1 UTR

To test whether the PCV1 replicase protein binds to the TTSuV1 UTR region, which contains the putative promoter site, we used a reporter assay. We cloned the TTSuV1 UTR sequence (566bp) upstream of the luciferase gene in the promoter-less pGL3 Basic plasmid (named pGL3UTR), and we observed an increased mean luciferase activity compared to the pGL3 Basic control (Figure 18), as previously reported in human TTVs (37, 38). However, to determine the effect of PCV1 replicase protein on the promoter activity of the TTSuV1 UTR, we

concurrently expressed the pGL3UTR plasmid in presence of either PCV1 replicase proteins or TTSuV1 ORF1 (positive control). Although TTSuV1 ORF1 gene encodes the capsid protein, human TTV ORF1 was reported to contain sequence motifs responsible for replication in anelloviruses (39), and similar motifs were observed in TTSuV1 ORF1; hence it is used as a positive control for these experiments. Here, we observed that the promoter activity was greatly decreased or blocked, compared to absence of either proteins (pGL3UTR) (Figure 18).

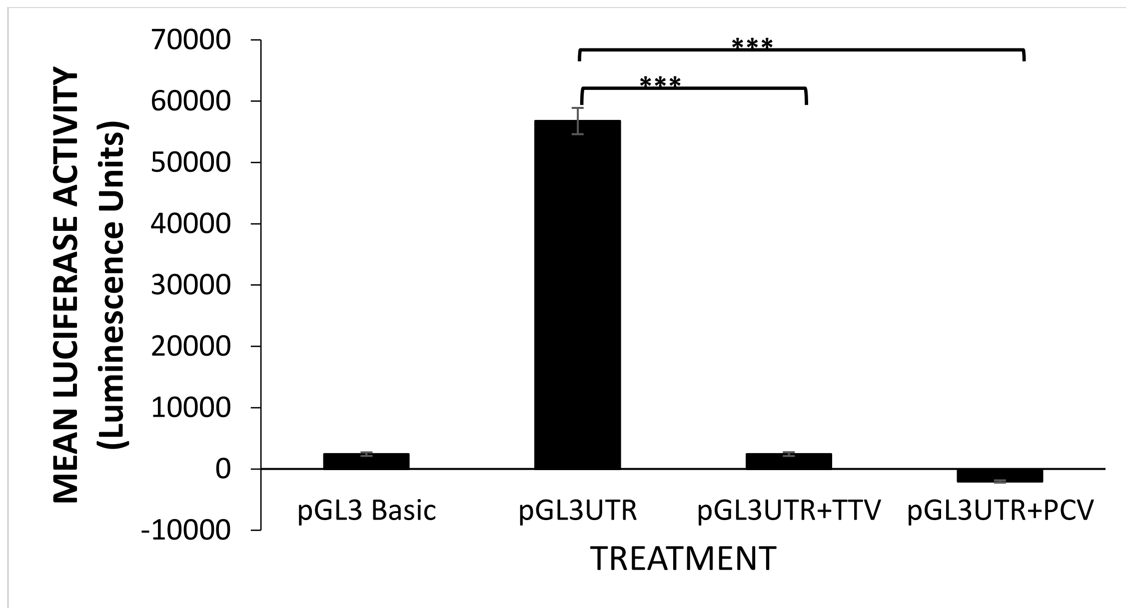

Figure 18: Luciferase Reporter gene assay. When pGL3UTR was co-transfected with plasmids expressing either the PCV1 or TTSuV1 replicase proteins, decreased or blocked activity was observed. Promoter-less pGL3Basic was used as experiment negative control, while empty pCDNA-V5 plasmids was used to obtain the baseline values, which were then subtracted from values of the treatments (N=3). ***p<0.01 = significantly different as determined by Student T-test.

Physical interaction between TTSuV1 UTR DNA and PCV1 replicase protein

To further determine if the PCV1 replicase protein physically interacts with the TTSuV1 UTR DNA, we use an *in-vitro* protein-DNA interaction assay (EMSA). The putative stem-loop UTR DNA sequence (210bp) was successfully amplified and purified on a 1.5% agarose gel (Figure 19: Left panel), and later biotin-labeled. However, when the labeled-DNA sequence was

mixed with a crude protein extract of PCV1 replicase, no physical interaction between the PCV1 replicase protein and the putative stem-loop UTR DNA was detected, since no shift was observed after the interaction reaction (PCV1Rep+TTV DNA lane), compared to the UTR DNA only control (Figure 19: Right panel). Surprisingly, our positive control did not show any interaction as well (TTSuV1 ORF1+TTV DNA). We expected that if the protein and DNA do interact, a shift band or protein-DNA complex, with high molecular weight would appear higher compared to the unbound DNA (refer to Kit control, Figure 19: Right panel). In our experiment, no protein-DNA complexes were observed.

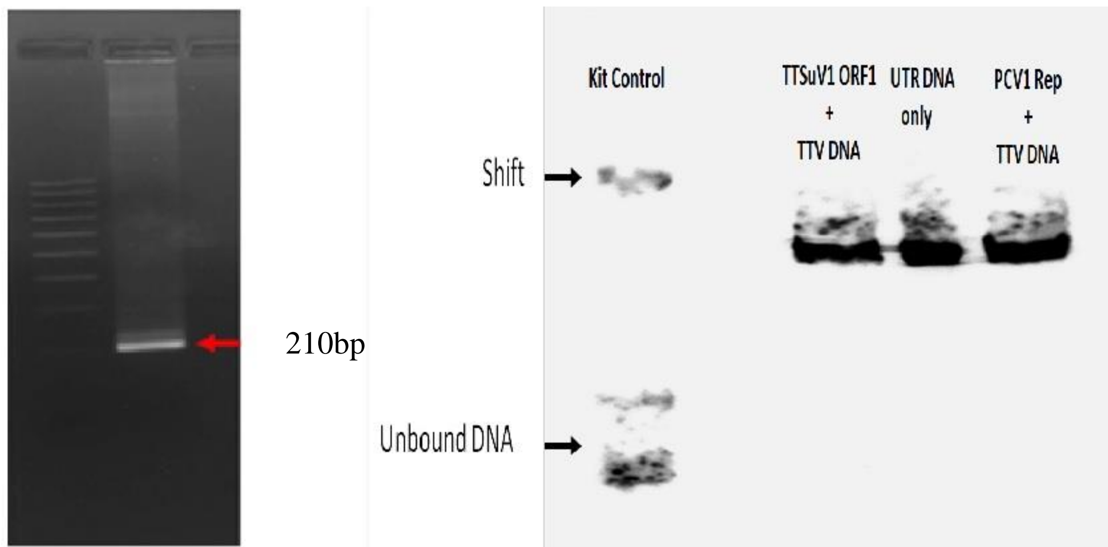

Figure 19: Left panel - Biotin-labeled TTSuV1 UTR DNA, a 210bp sequence with putative stem-loop. Right panel – EMSA blot, physical interaction of the PCV1 replicase protein with the TTSuV1 UTR DNA was assessed and the complexes with the biotin-labelled UTR DNA were detected by chemiluminescence blot staining.

Discussion

TTVs were first isolated in 1997 in a post-transfusion hepatitis patient (1). Although human TTVs have not been clearly shown to be primary pathogens, swine TTVs (TTSuVs) have been reported as possible disease agents (17, 21, 40), and their cross-species transmission (3, 5, 6) further poses greater threat to global human and animal health. Further still, several previous

studies in humans and swine have reported detection of TTVs in coinfection cases, with a synergistic relationship. There are increased viral loads of TTVs which correlate with increased severity of other viral pathogens, like PRRSV and PCV2 infections in swine (10, 20, 21), or Epstein-Barr virus and HIV in humans (13, 41). However, the biological mechanisms for these inter-viral interactions have not been fully understood, and this was the goal of this study. Porcine circoviruses are very similar to TTVs and have been well studied and characterized. In this study, we concurrently infected mammalian cells with TTSuV1 and a non-pathogenic PCV1 to study if these viruses could be using their replicase proteins interchangeably for their own replication, hence improving viral titers. We also used our novel *in-vitro* cell culture system to rescue recombinant TTSuV1 viral particle in presence of PCV1 replicase protein. We further studied the molecular mechanisms which could be responsible for these inter-viral interactions.

Previously, TTVs have been propagated through tedious methods, using infectious clones to transfect cell-lines and produce replicative viral particles, but no or few viral titers were detected on secondary viral transmission (27, 29). In this study, we used a novel and modified reverse genetics-based *in-vitro* propagation system to produce TTSuV1 replicative particles, and to study their role in inter-viral interactions. After one serial passage, the viral particles could be seen localized within the cell nuclei after FITC staining (Figure 15A), and the ultra-structure of the viral particles was obtained by electron microscopy (Figure 15, lower panel). In contrast to other TTV studies utilizing reverse genetics (27-29), by providing individual proteins in our system, this approach could be reducing the replication and transcription burden on the viral genome by providing individual proteins, rather than when the TTSuV1 infectious clone is used alone. Similar approaches have also been used for production of viable human papilloma virus (HPV) particles (42, 43). With this method, over a thousand-fold infectious HPV particles were

produced compared to traditional methods. Although not much is known about the individual TTSuV1 proteins, transfecting them transiently with the viral genome could be helping with production of TTSuV1 viral particles. TTV ORF1 gene has been reported to encode for the capsid protein (2, 44); and as this is expressed, it could be packaging the TTSuV1 genome to make viable viral particles. In addition to this, previous bioinformatics studies identified replication motifs as those seen in the replicase proteins of other anelloviruses or PCVs (26), hence could be binding to the regulatory sites of viral genome to initiate or promote replication. Furthermore, TTSuV ORF2 and ORF3 proteins are early non-structural proteins and have been reported to help with genome replication and expression (29, 45, 46). Our approach facilitates an easier way of producing TTSuV1 virus-like particles, which could be used to further understand the biology of TTVs, their pathogenesis and role in coinfections. We therefore used this same propagation system to study role of TTSuVs in inter-viral coinfection systems with PCV1.

In this study, we hypothesized that TTSuVs and PCVs could be interchangeably using their replication-associated viral proteins to enhance the replication of the other virus. Our study is the first to demonstrate such *in-vitro* coinfection interaction between TTSuVs and PCVs. In addition, while most previous studies generally used PCR to quantify or detect TTSuVs, our study used both qPCR and TCID50/IFA assays. We showed that when TTSuV1 was co-cultured with PCV1, viral titers of either virus were increased when using infectivity-based detection assays (TCID50/IFA), and not nucleic acid-based assays (qpCR) (Figure 16). A significant increase in TTSuV1 particles was observed in the coinfection system, in both ST and PK15N cell-lines. TCID50 method quantifies the infectious titer of the virus, and since TTSuVs show no cytopathic effects, IFA was used to detect the produced viral antigens at each dilution. Similar results were obtained with PCV1 titers although they were non-significant. These results further

demonstrate a possible synergistic relationship between these two viruses. Several studies have previously shown that presence of TTSuVs in coinfections could increase replication of other coinfecting pathogens *in vivo*, including PCVs (10, 22, 23, 47). TTSuVs and PCVs are very similar; they both have single-stranded DNA, identical genome organization and same replication mechanisms (24, 25, 48). It could be possible that these viruses do share their replication factors. In coinfection studies of adeno-associated virus 2 (AAV2) and its helper viruses, it was suggested that the AAV2 uses the herpes simplex virus 1 (HSV1) encoded polymerases – a helper virus, instead of host cell DNA polymerases (49). Similarly, PCV1 could be utilizing TTSuV1 encoded replicases/polymerases for its own replication, and vice versa. Surprisingly, when we used nucleic acid-based detection (qPCR) to quantify the viral particles, we observed contradictory results. Significantly higher viral titers were seen in single infection compared to TTSuV1-PCV1 coinfections, in both cell-lines. As reported in other studies, a possible reason for this discrepancy could be because qPCR is highly more sensitive compared to IFA (50, 51). Detection by IFA relies on successful production of viral proteins in cells, hence showing true infection and replication of the virus. In contrast, qPCR detects any viral DNA present in the system, which may not be due to infection. However, isolation of viral RNA would be the better approach, as this could represent amount of the replicating virus (49). As the virus replicates, mRNA transcripts are expressed, and these could be converted to cDNA and used to estimate viral titers.

PCVs have been well studied and function of their respective proteins characterized. It has previously been shown that PCV1 replicase proteins bind to the origin of replication to initiate viral replication (Figure 1) (26). To further understand this TTSuV1-PCV1 *in-vitro* synergistic interaction, we hypothesized that using the PCV1 replicase in rescuing TTSuV1 viral

particles would lead to increased viral titers. Indeed, when we rescued TTSuV1 in presence of over-expressed PCV1 replicase protein, we observed more viral particles were produced compared to when TTSuV1 was rescued without PCV1 replicase. These results further elucidate on the mechanisms involved in the TTSuV-PCV1 interaction. They also indicate that the increase in TTSuV1 titers is not only dependent on replication of PCV1 in the system, but only the replicase protein. Similar results have been reported in AVV2 coinfections with another helper virus (human bocavirus 1-HBoV1), where they found that only a few genes were necessary to promote AVV2 replication (53) However, our study considered only one PCV1 gene (replicase), and so further studies involving other expressed proteins would be required.

To further elucidate on the molecular mechanisms for these inter-viral interactions between TTSuV1 and PCV1, we used both a luciferase reporter assay and EMSA. Cloning of the TTSuV1 UTR sequence upstream of the promoter-less luciferase plasmid showed that the TTSuV1 UTR (pGL3UTR) has strong promoter activity (Figure 18). Our results were similar to previous reports which showed that the human TTV UTR sequence has promoter activity (37, 38). However, our study is the first to report such activity for TTSuV1 UTR. This is not surprising since all TTVs are known to have a conserved UTR, with replication and transcription regulatory sequences (2). Interestingly, when the pGL3UTR was expressed in presence of PCV1 replicase protein, the promoter activity was reduced significantly. The same result was observed with the TTSuV1 ORF1. A possible reason for this result could be because we used a full-length *Rep* gene for expression of replicase protein. A previous study on PCV1 transcription by Mankertz and Hillenbrand (2002) showed a full-length replicase (*Rep*) protein expression could repress its own transcription initiation, whereas the *Rep'* gene did not (52). However, although this result could mean direct or indirect interaction between the PCV1 replicase protein with the

TTSuV1 UTR sequence, it does not confirm physical interaction. To achieve this, we did a protein-DNA interaction assay (EMSA) to check for physical interaction, but no interactions were observed between the UTR DNA sequence with either proteins (Figure 19, Right panel). This was surprising since we expected positive interaction, as the replicase proteins of other anelloviruses have been shown to bind specifically to the stem-loop sequence (26), using this same method. Taken together, both these results could mean that these proteins could indirectly be binding to and inhibiting normal function of cellular transcription factors which would bind to the promoter region (Table 7), hence the observed diminished promoter activity of the TTSuV1 UTR. Indeed, some of these factors have been shown to be important in driving transcription in human TTVs, like the Sp1 (38). EMSA is a very tedious procedure and requires understanding the stereochemistries of both the protein and DNA for better results; for example, further analysis of the protein polarity, composition and structure could demonstrate how DNA binding occurs. Therefore, further optimizations of all binding reaction co-factors could be required. In addition, most EMSA reactions also uses very short DNA sequences and it could be that the selected region of the UTR does not physically interact with the PCV1 replicase protein.

In conclusion, our study demonstrated the production of more TTSuV1 particles using our co-transfection approach (Figure 15). This approach provides a novel method of propagating and studying TTVs, both *in-vitro* and *in-vivo*. We also showed a possible synergistic relationship between TTSuVs and other viral pathogens in coinfections systems, and this could be through sharing of replication and transcription factors among coinfecting viruses. Although our study did not find any physical interaction between the PCV1 replicase protein with the putative stem-loop region of the TTSuV1 genome, further molecular studies to explain this interaction are still

required. Understanding these inter-viral interactions could help with designing a better cell culture system for TTVs and better control measures against virulent coinfecting viruses.

Acknowledgements

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CHAPTER FIVE: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Torque-teno viruses (TTVs) are recently isolated very small non-enveloped DNA viruses and are highly prevalent in over 90% of both healthy and diseased human and swine populations. However, no or few studies had demonstrated zoonotic transmission of TTSuVs to humans. Our findings are the first to demonstrate the possibility that TTV infections can be zoonotic, as TTSuVs were detected in human sera. In addition, low antibodies to TTSuVs were detected, demonstrating that these viruses could cause true infection. TTSuVs have been documented to contaminate in several food, drug and water sources for human consumption. Although human TTVs have not been shown to cause disease yet, swine TTVs (TTSuVs) have been singly shown as possible pathogens in swine. In addition, they have also been reported to worsen manifestation of symptoms for other pathogens in case of coinfections. With this zoonotic evidence, revisions in both drug/vaccine and food safety regulations could be required, as these viruses could potentially contribute to disease causation in humans, as shown by the suppression of lymphoproliferation. In addition, there could be implications in the use of swine organs for transplantation for fear of infection, and that these viruses could affect the administration of immunosuppressive drugs.

Both human TTV and swine TTV (TTSuV) infections are acquired very early in life and viremia persists over a lifetime. However, no study has demonstrated how TTVs could regulate the host immunity. TTVs' role in disease causation is still debatable, with many epidemiology studies suggesting they could be pathogens, or be co-factors in coinfection systems. Understanding how TTVs regulate and establish chronic infections is critical. Although our cells were old and possibly non-responsive to antigen stimulation, our *in-vivo* work further illustrated the possible role of TTVs in host immune regulation. We also proposed possible host immune

pathways involved in TTSuV pathogenesis. Our results showed possible viral sensing through interferon-inducing cytosolic sensors, mainly DHX36. Also, our results further elucidated on previous reports that TTSuVs are chronic, and this could be through upregulation of a regulatory gene (PD-1) responsible for suppressing interferon expression, hence causing chronic infections. However, we could not make any conclusions about the regulation of the host adaptive immunity.

Several epidemiological and experimental studies have showed that many viral diseases are associated with TTVs, especially PMWS and PRDC causing pathogens. However, their role in coinfections with other pathogens remains to be determined. Here, we demonstrated possible role of TTSuVs in coinfection systems, using *in-vitro* assays. Presence of PCV1 and TTSuV1 in cells showed that these viruses could have a synergistic interaction, as both their titers increased in coinfection, compared to the respective single infections. These results could have implications in natural coinfection systems of TTSuVs with other pathogens, probably through exchanging replication-related proteins (replicases) and leading to thriving of other viruses. Further still, we suggest a novel technique to propagate TTSuVs by using modified reverse genetics procedures. TTSuVs have initially been cultured through tedious procedures leading to low viral titers; however, our system resulted in more viable TTSuV1 viral particles. Future *in-vitro* or *in-vivo* studies could utilize this system to produce TTSuV particles for use in studying viral pathogenesis and replication mechanisms. However, more ultra-purification of the recombinant viral particles would be required for *in-vivo* studies, identify particles with a packaged genome, and not empty capsids. We also demonstrated that replication of other viruses may not be the sole requirement to help TTSuV replication, but specific viral proteins (like PCV1 replicase) could be helping with its replication.

In conclusion, we suggest that future studies should focus on using freshly isolated PBMCs to explain how TTSuVs could regulate host adaptive immunity; and this remains a goal of our team. We also suggest that future studies utilize a combination of mRNA transcript and protein expression for better results. Also, the recent isolation of TTVs in rodents could also be utilized as a cheaper *in-vivo* model to study immune-regulation by TTVs, another area our team is studying. In addition, future studies should focus on identifying similar proteins with other viruses involved in coinfections with TTSuVs. Although our molecular characterization does not fully explain this interaction, further studies to elucidate this phenomenon remain the goal of our team, as this could also help with designing better propagation systems for TTVs.