A cultivable primate calicivirus causes enteric infections in gnotobiotic piglets

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

Yue Duan, B.S.

Graduate Program in Food Science and Technology

The Ohio State University

2013

Master's Examination Committee:

Dr. Jianrong Li, Advisor

Dr. Steven Krakowka

Dr. Ahmed Yousef
Copyrighted by

Yue Duan

2013
Abstract

Many enteric caliciviruses cause acute gastroenteritis in humans and animals. Examples of these include human norovirus (NoV), human sapovirus, and the newly discovered recoviruses. It is estimated that human NoV accounts for over 95% of nonbacterial acute gastroenteritis worldwide. In order to develop effective preventive measures and antiviral strategies, there is an urgent need to understand the pathogenesis and immunology of human NoV and other enteric caliciviruses. However, research efforts on enteric caliciviruses have been severely hampered primarily because many of these cannot be propagated in vitro. Tulane virus (TV), a newly discovered enteric primate calicivirus recovered from the feces of rhesus macaques, is cultivable and recognizes histo-blood group antigens (HBGAs) similar to human NoV. TV is now recognized as an improved surrogate for the study human NoV. The objectives of this study are to determine (i) Whether the pathogenesis of TV can be recapitulated in gnotobiotic piglets, (ii) Whether TV can be adapted to pig macrophage cell lines, (iii) Whether macrophage cell-adapted TV has altered virulence in piglets, and (iv) Whether TV can be passed repeatedly in piglets.
To determine the pathogenesis of TV in gnotobiotic piglets, twenty-eight newborn gnotobiotic piglets were orally inoculated with $2 \times 10^8$ PFU of TV and euthanized at days post-inoculation (PID) 3, 4, 5, 6, and 10. Twelve of the twenty-eight (43%) inoculated piglets developed mild diarrhea. Infectious virus was detected in stools on PIDs 1-5, but was undetectable beyond PID 6. Viral RNA was detectable through PID 1 to 10, and viral RNA level remained high in the feces for 10 days. Viral RNA was detected in the intestinal contents and intestine tissue segments of the duodenum, jejunum, and ileum on PIDs 3, 4, 5, 6, and 10, but infectious virus was detected only on PIDs 4 and 5. Piglets developed TV-neutralizing antibodies by PID 6. Immunofluorescent staining results showed that TV antigens were present in villus tip (epithelial) cells and cells within the lamina propria of the duodenum and jejunum only. Histologic examination demonstrated mild villous atrophy (villi shortening and blunting), increased numbers of mononuclear inflammatory cells, and occasional neutrophils and macrophage monocytes undergoing necrosis/apoptosis in duodenal and proximal jejunal tissues of the infected gnotobiotic pigs. These results demonstrated that TV caused mild enteritis, viral shedding, and intestinal histologic lesions similar to those described for TV in primates and human NoV in humans.

To determine whether TV has a tropism for macrophages, a continuous swine macrophage cell line (3D/4) was infected with TV at multiplicity of infection (MOI) of 1. It was found that TV caused typical cytopathic effects (CPE) and replicated efficiently in pig macrophages although a slight decrease in TV titer was observed from passages 1 to
5. To determine whether macrophage-adapted TV had altered virulence *in vivo*, $10^7$ PFU of TV was inoculated into newborn gnotobiotic piglets. It was found that the macrophage cell-adapted TV caused low levels of viral and RNA shedding in feces but did not cause any histologic changes or TV antigen expression in the intestine, suggesting that the macrophage cell-adapted TV had reduced viral virulence in pigs. To determine whether TV can be passed in piglets, fecal samples were collected from TV infected piglets, and used for oral inoculation of naive piglets. Although histologic changes and TV antigens were not detectable in the intestine, fecal infiltrates containing $10^4$ PFU of TV from TV-infected piglets can lead to viral shedding at level of $10^{2.5}$ PFU/g feces for at least 4 days in gnotobiotic piglets. Thus, it is promising to adapt TV in piglets by a serial of *in vivo* passage.

Collectively, it was concluded that (i) TV, the only cultivable primate calicivirus, caused symptoms of enteritis and intestinal histologic lesions in gnotobiotic pigs and, (ii) TV has a tropism for porcine macrophages. These findings suggested that TV infection of gnotobiotic pigs can be used as a novel model to study the replication and pathogenesis of enteric caliciviruses *in vivo*. 
Acknowledgments

First I would like to thank my advisor Dr. Jianrong Li for his guidance in the past two years. I sincerely appreciate him for giving me such a great opportunity to advance my education in The Ohio State University, for teaching me how to conduct high-standard research and think critically, and for the time and support he gave me. I would like to specially thank Erin DiCaprio and Dr. Yuanmei Ma for their help with proofreading my thesis. I would also like to thank all the other members in Dr. Li’s research group: ElBashir Araud, Dr. Hui Cai, Fangfei Lou, Ana Puriganto, Jing Sun, Rongzhang Wang, Dr. Yongwei Wei, Ben Yeap, Yu Zhang, and Yang Zhu. They have been so great to me, and always glad to share the knowledge and ready to help. They have made my time in the lab so unforgettable. I would like to thank Dr. Krakowka and Dr. Yousef for being on my committee, their time and help is greatly appreciated. I would like to thank Judy Dubena, Susan Ringler, Heather Strange and Dr. Tracey Papenfuss for their help and support, I couldn’t have done this without them. I would also like to thank the other students working in the food virology laboratory for their help and friendship throughout my studies.

I would like to thank my parents for their love, support, and confidence in me. I would like to thank my dear friends for great times together and all the love and support they give me throughout the precious two years. Thanks for your love.
Vita

2007 .................................................. Xing Zhi High School, Shanghai, China

2011 .................................................. B.S. Biotechnology, Shanghai Jiao Tong
    University, China

2011 to present ................................. Graduate Research Associate, Department
    of Food Science and Technology, The Ohio
    State University

Fields of Study

Major Field: Food Science and Technology
# TABLE OF CONTENTS

ABSTRACT ...................................................................................................................................................................... ii

ACKNOWLEDGMENTS ...................................................................................................................................................... v

VITA ............................................................................................................................................................................. vi

LIST OF TABLES ............................................................................................................................................................ xiii

LIST OF FIGURES .......................................................................................................................................................... xv

CHAPTER 1 LITERATURE REVIEW ............................................................................................................................... 1

1.1 The *Caliciviridae* ................................................................................................................................................... 1

1.1.1. Morphology and physicochemical properties of caliciviruses ................................................................. 2

1.1.2. Genome organization of caliciviruses ........................................................................................................... 4

1.1.3. Proteins of caliciviruses ............................................................................................................................... 6

1.1.4. Classification of caliciviruses ......................................................................................................................... 7

1.1.5. Caliciviruses related to human health ........................................................................................................... 9

1.2. Human norovirus, the most important calicivirus ............................................................................................ 10

1.2.1. Introduction to human norovirus .................................................................................................................. 10

1.2.2. Epidemiology of human norovirus .............................................................................................................. 12
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2.3.</td>
<td>Human norovirus, foodborne illnesses and health impacts</td>
</tr>
<tr>
<td>1.2.4.</td>
<td>Animal caliciviruses as surrogates for the study of human norovirus</td>
</tr>
<tr>
<td>1.2.4.1</td>
<td>Murine norovirus (MNV-1)</td>
</tr>
<tr>
<td>1.2.4.2</td>
<td>Porcine sapovirus</td>
</tr>
<tr>
<td>1.2.4.3</td>
<td>Feline calicivirus (FCV)</td>
</tr>
<tr>
<td>1.2.4.4</td>
<td>Tulane virus (TV)</td>
</tr>
<tr>
<td>1.3.</td>
<td>Tulane virus</td>
</tr>
<tr>
<td>1.3.1</td>
<td>Discovery of Tulane virus</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Taxonomy of <em>Recovirus</em></td>
</tr>
<tr>
<td>1.3.3</td>
<td>Virion structure of Tulane virus</td>
</tr>
<tr>
<td>1.3.4</td>
<td>Genome structure of Tulane virus</td>
</tr>
<tr>
<td>1.3.5</td>
<td>Proteins of Tulane virus</td>
</tr>
<tr>
<td>1.3.5.1</td>
<td>Nonstructural proteins</td>
</tr>
<tr>
<td>1.3.5.2</td>
<td>Structural proteins</td>
</tr>
<tr>
<td>1.3.6</td>
<td>Replication cycle of Tulane virus</td>
</tr>
<tr>
<td>1.3.7</td>
<td>Receptors of Tulane virus</td>
</tr>
<tr>
<td>1.3.7.1</td>
<td>Histo-blood group antigens are the cellular receptors for TV</td>
</tr>
<tr>
<td>1.3.7.2</td>
<td>TV-HBGA interaction</td>
</tr>
</tbody>
</table>
1.3.7.3 Comparison between TV- and human norovirus-HBGA interaction .................................................................................................................. 39

1.3.8 Pathogenesis of Tulane virus ........................................................................................................... 41

1.3.8.1 TV infection in juvenile rhesus macaques ................................................................. 41

1.3.8.2 Potential zoonotic transmission of TV ........................................................................ 44

1.3.8.3 Comparison of TV and norovirus pathogenesis ......................................................... 45

1.3.8.3.1 Human norovirus ........................................................................................................ 45

1.3.8.3.2 Murine norovirus ........................................................................................................ 48

1.3.8.3.3 Comparison of TV, MNV, and human norovirus ................................................. 49

1.3.9 Immunology and host susceptibility of Tulane virus and human norovirus .................................................................................................................. 51

1.3.9.1 Immune response caused by TV infection ................................................................. 51

1.3.9.2 Immune responses to human norovirus in healthy people ................................. 54

1.3.9.3 Immune responses to human norovirus in immunocompromised population .................................................................................................................. 56

1.3.10 Animal model for enteric caliciviruses .................................................................................. 58

1.3.10.1 Non-human primate models for human norovirus ............................................. 58

1.3.10.2 Non-human primate models for Tulane virus .................................................... 60

1.3.10.3 Gnotobiotic animal models ....................................................................................... 61
CHAPTER 2 PATHOGENESIS OF TULANE VIRUS IN GNOTOBIOTIC PIGS ...... 66

2.1. Abstract ............................................................................................................... 66

2.2. Introduction ......................................................................................................... 67

2.3. Materials and methods ...................................................................................... 71

2.3.1. Cell culture and virus inoculum preparation ............................................... 71

2.3.2. Delivery of gnotobiotic piglets ................................................................. 72

2.3.3. Animal challenge studies .......................................................................... 73

2.3.4. Enumeration of infectious TV by plaque assay ........................................ 74

2.3.5. Quantification of viral genomic RNA by real-time RT-PCR ................... 74

2.3.6. Virus neutralization assay ........................................................................ 75

2.3.7. Immunofluorescence microscopy.............................................................. 76

2.3.8. Histological examination .......................................................................... 77

2.3.9. Statistical analysis .................................................................................... 77

2.4. Results ................................................................................................................. 77

2.4.1. TV infection caused mild diarrhea in gnotobiotic pigs ......................... 77

2.4.2. Fecal shedding of infectious TV was observed in inoculated pigs .......... 79

2.4.3. TV RNA in feces ...................................................................................... 81

2.4.4. Infectious TV in intestinal contents and tissues ....................................... 83
2.4.5. TV viral RNA was detected in intestinal contents and tissues ............... 85

2.4.6. Virus-neutralizing antibody in serum ....................................................... 87

2.4.7. Gross pathologic findings ...................................................................... 89

2.4.8. Histologic findings ................................................................................. 91

2.4.9. Detection of TV antigen-positive cells in the intestine by IFA ............. 96

2.5. Discussion ........................................................................................................ 98

CHAPTER 3 ADAPTION OF TULANE VIRUS TO A SWINE MACROPHAGE CELL LINE AND PASSAGE OF TULANE VIRUS IN GNOTOBIOTIC PIGLETS ........ 105

3.1. Abstract .......................................................................................................... 105

3.2. Introduction .................................................................................................... 106

3.3. Material and methods ................................................................................... 108

3.3.1. Cell cultured virus stock preparation ..................................................... 108

3.3.2. Adaption of TV in pig macrophage cells ............................................. 108

3.3.3. Inoculum preparation using animal samples ......................................... 109

3.3.4. Animal challenge studies ....................................................................... 110

3.3.5. Enumeration of infectious TV by plaque assay ..................................... 111

3.3.6. Quantification of viral genomic RNA by real-time RT PCR ............... 112

3.3.7. Immunofluorescence microscopy ........................................................... 113

3.3.8. Histological examination ........................................................................ 114
3.3.9. Statistical analysis .................................................................................. 114

3.4. Results ............................................................................................................... 114

3.4.1. TV replicates efficiently in pig macrophage cells................................. 115

3.4.2. Pathogenesis of TV grown in macrophage cells ................................. 116

3.4.3. *In vivo* passage of TV grown in LLC-MK2 cells ......................... 122

3.5. Discussion ......................................................................................................... 132

CHAPTER 4 CONCLUSIONS ......................................................................................... 135

LIST OF REFERENCES ................................................................................................ 137
LIST OF TABLES

Table 1. Members of Caliciviridae family ................................................................. 2

Table 2. Comparison between the HBGA receptor interaction of TV and human
norovirus ............................................................................................................ 41

Table 3. Comparison of the pathogenesis of three caliciviruses ......................... 50

Table 4. Prevalence of TV antibodies in rhesus macaques grouped by age .......... 53

Table 5. Characteristics of norovirus gastroenteritis in immunocompetent versus
immunocompromised humans ........................................................................... 57

Table 6. Comparison of human norovirus and several animal calicivirus research
surrogates .......................................................................................................... 65

Table 7. Diarrhea, fecal virus shedding, and viral RNA detection in TV-infected
gnotobiotic pigs ............................................................................................... 78

Table 8. Duration of diarrhea, fecal virus shedding and viral RNA detection in TV-
infected gnotobiotic pigs ................................................................................. 80

Table 9. Summary of histologic changes in intestinal tissue ............................... 95

Table 10. Summary of antigen detection in intestines by immunofluorescent assay ...... 98

Table 11. Viral titer of in vitro passaged TV in 3D/4 cells .................................... 116
Table 12. Histologic Changes and Viral Detection in Stool Samples and Different Segments of Intestine of TV Inoculated Pigs .................................................. 121

Table 13. Histologic Changes and Viral Detection in Stool Samples and Different Segments of Intestine of TV Inoculated Pigs .................................................. 131
LIST OF FIGURES

Figure 1. Morphology of human norovirus, a typical Calicivirus, examined by transmission electron microscopy at neutral pH ................................................ 3

Figure 2. Comparison of genome organization of several members of Caliciviridae .... 5

Figure 3. Classification of Caliciviridae based on phylogenetic analysis of the complete capsid sequence of calicivirus strains......................................................... 8

Figure 4. Genomic organization of human norovirus.................................................... 11

Figure 5. Number of human norovirus outbreaks confirmed by the CDC in the United States from 1994 to 2006 by setting and genotype ........................................ 14

Figure 6. Change in relative frequencies of globally circulating GII.4 variants associated with sporadic gastroenteritis in children during the last decade ............... 16

Figure 7. Top pathogens contributing to domestically acquired foodborne illnesses and deaths from 2000 to 2008 .................................................................. 17

Figure 8. Unrooted phylogenetic tree based on amino acid sequence alignment of calicivirus VP1 ........................................................................................................ 22

Figure 9. Classification of recoviruses base on phylogenetic analysis and comparison to several norovirus strains................................................................. 24

Figure 10. Transmission electron microscope pictures of Tulane virus ..................... 25
Figure 28. Detection of (A) infectious virus and (B) viral RNA in fecal samples of TV P0 inoculated piglets ................................................................. 123

Figure 29. Detection of (A) infectious virus and (B) viral RNA in intestinal contents of TV P0 inoculated piglets ................................................................. 124

Figure 30. Histologic changes in tissue segments of piglets inoculated with TV P0 or TV P1 ................................................................................ 125

Figure 31. Antigen detection in tissue segments of piglets inoculated with TV P0 or TV P1 by indirect immunofluorescent assay (IFA) ............................. 126

Figure 32. Detection of (A) infectious virus and (B) viral RNA in fecal samples of TV P1 inoculated piglets ................................................................. 128

Figure 33. Detection of (A) infectious virus and (B) viral RNA in intestinal contents of TV P1 inoculated piglets ................................................................. 130
ABBREVIATIONS

ANOVA – Analysis of variance
ASC – Antibody-secreting cells
BSA – Bovine serum albumin
CDC – Centers for Disease Control and Prevention
CPE – Cytopathic effect
EBHSV – European Brown hare syndrome virus
ELISA – Enzyme-linked immunosorbent assay
FBS – Fetal bovine serum
FCV – Feline calicivirus
FDA – Food and Drug Administration
HBGA – Histo-blood group antigen
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, a zwitterionic organic chemical buffering agent
HuNoV – Human Norovirus
IFA – Immunofluorescent assay
ILACUC – Institutional Laboratory Animal Care and Use Committee
LLC-MK2 – A monkey kidney cell line
LLC-PK – A renal epithelial cell line derived from porcine kidneys
MEM – Minimal eagle medium
MNV – Murine norovirus
MOI – Multiplicity of infection
NGS – Normal goat serum
NoV – Norovirus
OPTI-MEM – a modification of Eagle's Minimum Essential Medium
ORF – Open reading frame
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PFU – Plaque forming units
PI – Post infection/inoculation
PID – Day post infection/inoculation
PPB – Potassium Phosphate Buffer
RAG2 – Recombination-activating gene 2
RAW 264.7 – A mouse macrophage cell line
RdRp – RNA-dependent RNA polymerase
RHDV – rabbit hemorrhagic disease virus
RNA – Ribonucleic acid
RNAi – RNA interference
RT – Reverse Transcription
RT-qPCR – Reverse transcriptase- quantitative polymerase chain reaction
SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA – Small interfering RNA
SMSV – San Miguel sea lion virus
STAT-1 – Signal transducer and activator of transcription 1
TNPRC – Tulane National Primate Research Center
TV – Tulane virus
UTR – Untranslated region
VESV – vesicular exanthema of swine virus
VLP – Virus-like particle
VSV – Vesicular stomatitis virus
CHAPTER 1
LITERATURE REVIEW

1.1 The Caliciviridae

The Caliciviridae is a family of non-enveloped viruses with single stranded positive sense RNA genomes. There are six genera in this family including Vesivirus [members include feline calicivirus (FCV), vesicular exanthema of swine virus (VESV), and San Miguel sealion virus (SMSV)], Lagovirus [rabbit hemorrhagic disease virus (RHDV) and European Brown hare syndrome virus (EBHSV)], Norovirus (members include human norovirus, porcine norovirus, bovine norovirus, and murine norovirus), Sapovirus (members include human sapovirus and porcine sapovirus), Becovirus [members include bovine enteropathogenic caliciviruses (Newbury agent-1 and Nebraska)], and Recovirus (members include primate enteric caliciviruses such as Tulane virus). Many members in the Caliciviridae family are human and animal pathogens, causing diseases ranging from acute gastroenteritis (human norovirus and human sapovirus) to hemorrhagic or lethal infections (murine norovirus, feline calicivirus, and rabbit hemorrhagic disease virus). Though caliciviruses can infect human and animals, there is no direct evidence of any zoonotic transmission to date (Green 2007).
Table 1. Members of Caliciviridae family (adapted from Farkas et al. 2008 and Clarke and Lambden 1997)

<table>
<thead>
<tr>
<th>Genus</th>
<th>Prototypical virus strain</th>
<th>Genome length (nt)</th>
<th>Cell culture</th>
<th>Natural Host</th>
<th>Disease caused in host</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Norovirus</strong></td>
<td>Norwalk</td>
<td>7654</td>
<td>No</td>
<td>Humans</td>
<td>Acute gastroenteritis in humans, occasional fever</td>
</tr>
<tr>
<td></td>
<td>MNV-1</td>
<td>7382</td>
<td>Yes</td>
<td>Mice</td>
<td>Systemic even fatal infection in immune deficient mice</td>
</tr>
<tr>
<td><strong>Vesivirus</strong></td>
<td>FCV-CF168</td>
<td>7677</td>
<td>Yes</td>
<td>Cats</td>
<td>Upper respiratory tract infection in cats</td>
</tr>
<tr>
<td></td>
<td>SMSV-1</td>
<td>8284</td>
<td>Yes</td>
<td>Marine animals</td>
<td>Vesicles on the flippers and abortion in marine animals</td>
</tr>
<tr>
<td><strong>Sapovirus</strong></td>
<td>Manchester</td>
<td>7431</td>
<td>No</td>
<td>Humans</td>
<td>Gastroenteritis in humans, mainly in children</td>
</tr>
<tr>
<td><strong>Lagovirus</strong></td>
<td>RHDV-FRG</td>
<td>7437</td>
<td>No</td>
<td>Rabbits</td>
<td>Fever and fatal hemorrhagic liver disease in rabbits</td>
</tr>
<tr>
<td><strong>Becovirus</strong></td>
<td>Newbury-1</td>
<td>7454</td>
<td>No</td>
<td>Cattle</td>
<td>Non-hemorrhagic enteritis, mild diarrhea in calves</td>
</tr>
<tr>
<td><strong>Recovirus</strong></td>
<td>Tulane</td>
<td>6714</td>
<td>Yes</td>
<td>Rhesus monkey</td>
<td>Mild diarrhea and fever in rhesus macaques</td>
</tr>
</tbody>
</table>

1.1.1. Morphology and physicochemical properties of caliciviruses

The virions of the *Caliciviridae* are round in shape when visualized under electron microscopy and small in size with average diameters ranging from 20-35 nm. The virions are non-enveloped and the structure of the virion consists of only two capsid proteins (the major capsid protein, VP1; and the minor capsid protein, VP2) and the naked genomic RNA. The three dimensional structure of the viruses in the family *Caliciviridae* is composed of multiple copies of monomers of the major capsid protein, VP1. The capsid
usually exhibits icosahedral symmetry with cup-shaped depressions on the capsid surface (Clarke and Lambden 2000; Green 2007).

The structure of the capsid of the virus particles are formed by dimers of the viral capsid protein VP1. The number of copies of VP1 in the capsid varies between viruses, and it is known that the capsid of human NoV is composed of 90 dimers (180 copies) of VP1. There are two domains in VP1 of human NoV, the shell (S) domain and the protruding (P) domain. The P domain is further divided into P1 and P2 subdomains (Chen et al. 2006). The P2 domain is located on the external surface of the capsid and plays a vital role in the attachment to cellular receptors and antigenicity of the viruses.

Figure 1. Morphology of human norovirus, a typical Calicivirus, examined by transmission electron microscopy at neutral pH (adapted from Rohayem et al. 2010)
The small size and icosahedral symmetry of the capsid paired with the lack of a lipid envelope contribute to the fact that the virions of caliciviruses are highly stable in the environment and resistant to disinfectants, as well as thermal and non-thermal processing. Researchers have found that the caliciviruses are highly resistant to chlorine treatment, heat treatment, acidic conditions, organic solvents, and high pressure processing (Koopmans and Duizer 2004; Rzezutka and Cook 2004; Hirneisen and Kniel 2013; Cannon et al. 2006; Li et al. 2012; Lou et al. 2011; Green, Chanock, and Kapikian 2001). The high stability of these viruses in combination with the low infectious dose required to cause disease contribute to the high prevalence of outbreaks attributed to members of the family Caliciviridae.

1.1.2. Genome organization of caliciviruses

The genome of viruses in the family Caliciviridae is composed of RNA which is single-stranded and positive-sensed. The size of the genome is approximately 7.5kb on average, ranging from 6.7kb (Tulane virus) to 8.2kb (SMSV) (Table 1). The genome of caliciviruses consists of two or three open reading frames (ORFs) depending on the genus: Sapovirus, Lagovirus, and Becovirus have two ORFs, while Norovirus, Vesivirus, and Recovirus have three (Figure 2).
Figure 2. Comparison of genome organization of several members of Caliciviridae
(adapted from Rohayem et al. 2010)

The presence of a viral protein genome-linked (VPg) 5’ terminus of the genome is one of the distinguishing characteristics of Caliciviridae. ORF 1 encodes nonstructural proteins and the capsid protein VP1 for those genera that only have two ORFs. The nonstructural (NS) proteins include N-terminal protein (NS 1-2), NTPase (NS 3), 3A-like protein (NS 4), VPg (NS 5), viral protease (NS 6), and RNA-dependent RNA polymerase (RdRp, NS 7) (Rohayem et al. 2010; Green 2007). In Sapovirus, Lagovirus, and Becovirus, which only have two ORFs, the viral capsid protein VP1 is also encoded within ORF1, whereas Norovirus, Vesivirus, and Recovirus have a separate ORF2 encoding VP1. All six genera have another ORF to encode structural viral protein VP2.
At the 3’ terminus of the genome there is an untranslated region followed by a poly (A) tail. It should be pointed out that an additional ORF encoding a putative small basic protein has been reported for sapovirus genogroups I, IV, and V, which distinguishes this genus from other members of the *Caliciviridae* (Clarke and Lambden 2000; Hansman, Takeda et al. 2005).

1.1.3. **Proteins of caliciviruses**

The nonstructural proteins of caliciviruses are encoded within ORF1. A large polyprotein precursor encoded by ORF1 is further cleaved into at least six nonstructural post translation proteins (Figure 2). These nonstructural proteins are involved in the replication of the virus. The N-terminal protein and 3A-like protein are involved in the formation of replication complex, and they are also responsible for inhibiting host protein expression and trafficking. The viral RNA dependent RNA polymerase (RdRp) and VPg are essential for the transcription and replication of the antigenomic RNA and subsequently genomic RNA, as well as the transcription and replication of the subgenomic RNA used for the translation of the structural proteins during replication. The viral protease is essential as it cleaves the nonstructural proteins as well as VP1 (if encoded in ORF1) from the ORF1-encoded polyprotein precursor (Belliot et al. 2003; Green 2007).

The structural proteins, VP1 and VP2, compose the viral capsid of caliciviruses. The major capsid protein VP1 ranges from 445-702 amino acid in length, with an average molecular weight of approximately 60 kDa. VP1 is very important for the virus for the
following reasons. First, it is the main component of the viral capsid, which maintains the stability of the virus particle (Jiang et al. 1992). Second, it can bind to host cell receptors and mediate viral entry (Farkas, Cross et al. 2010; Green 2007; Hutson et al. 2003; Tan et al. 2003; Hardy 2005). Moreover, it determines the antigenicity and strain specificity of the virus (Katayama et al. 2002; Prasad et al. 1999; Rohayem et al. 2010; Hansman, Natori et al. 2005; Hansman, Takeda et al. 2005). VP2 is the small basic structural protein encoded by ORF 2 or ORF 3. The VP2 is usually made of 106-268 amino acids, and the average molecular weight is about 20 kDa. VP2 can increase the stability of VP1 and protect VP1 from disassembly and protease degradation. Moreover, it plays a role in RNA packaging and regulation of the synthesis of VP1 proteins. It has also been reported that the expression of VP2 is essential for the generation of complete virions (Bertolotti-Ciarlet et al. 2003; Glass et al. 2000; Wei et al. 2008).

1.1.4. Classification of caliciviruses

For lack of a cell culture system to cultivate many of the caliciviruses, serological tests cannot be performed with most members of Caliciviridae. For this reason, the classification of Caliciviridae is based on the phylogenetic analysis of viral genes that encode the structural and nonstructural proteins. To date there are six genera in the Caliciviridae family, including both human and non-human pathogens (Figure 3). The genera Norovirus and Sapovirus include both human and animal virulent strains, whereas the other four genera only include animal virulent viruses.
The genera *Norovirus* and *Sapovirus* have been further classified into genogroups. Noroviruses are clustered into five genogroups based on the phylogenetic analysis of the complete VP1 sequence. The human noroviruses belong to genogroups (G) I, II, and IV.
Animal noroviruses have been found to infect pigs, cattle, lions, dogs, and mice (Oliver et al. 2003; Martella et al. 2007; Wobus, Thackray, and Virgin 2006; Park et al. 2007; Martella et al. 2008; Mauroy et al. 2008; Wang et al. 2005). Two genogroups contain only animal viruses, the GIII genogroup (bovine norovirus) and the GV genogroup (murine norovirus), additionally there are also animal noroviruses in the GII (bovine and porcine noroviruses) and GIV (feline and canine noroviruses) genogroups. *Sapovirus* also contains five genogroups. The strains that infect humans belong to the GI, II, IV, and V genogroups, however the major strains circulating in the human population are classified in the GI and GII genogroups. Genogroup III contains sapovirus strains that exclusively infect animals (Rohayem et al. 2010; Green 2007; Green et al. 2000).

### 1.1.5. Caliciviruses related to human health

Viruses in the family *Caliciviridae* can infect a wide range of hosts including humans, primates, cattle, mink, swine, cats, dogs, and rabbits. Moreover they can cause a broad spectrum of disease in humans and animals including acute gastroenteritis, hemorrhagic disease, respiratory tract infection, and even lethal systemic infection. Only viruses within the *Norovirus* and *Sapovirus* genera have been shown to cause disease in humans, and there is no evidence to support the zoonotic transmission of animal caliciviruses to humans. However, the study of some animal caliciviruses which are closely related these human pathogens can serve as models to understand the molecular biology and pathogenesis of the human caliciviruses.
1.2. Human norovirus, the most important calicivirus

1.2.1. Introduction to human norovirus

In 1968, an outbreak of acute gastroenteritis occurred in Norwalk, Ohio causing nausea and vomiting in over 50% of the students and teachers at Bronson Elementary School. In 1972, Kapikian et al. visualized small round structured viral particles using electron microscopy in patient stool samples from the outbreak and determined the virus to be the etiologic agent of the disease (Kapikian et al. 1972). The virus, first termed Norwalk virus, was later identified as the causative agent of numerous outbreaks with similar clinical manifestations. Later renamed norovirus, the virus is also referred to as the food poisoning virus, small round-structure virus (SRSV), Hawaii virus, and Snow Mountain virus. The disease caused by this agent is often called winter vomiting disease, viral gastroenteritis, non-bacterial gastroenteritis, and the stomach flu (Appleton 1987; Adler and Zickl 1969; Koopmans and Duizer 2004). In 2002, the International Committee on the Taxonomy of Viruses officially approved the name norovirus (genus Norovirus).

Human norovirus belongs to the genus *Norovirus* in the *Caliciviridae* family. The size of the human norovirus particle is 28-35 nm in diameter, and it has the typical small round shape of caliciviruses with icosahedral symmetry. Like other caliciviruses, human norovirus has a single-stranded positive-sensed RNA genome. The viral genome is 7.5–7.7 kb in length and contains three open reading frames (ORFs) (Figure 4). ORF1
encodes a large polyprotein that is post-translationally cleaved into six nonstructural proteins that are involved in viral replication as described before. ORF2 encodes the major structural protein VP1 that forms the capsid, and ORF3 encodes a minor structural protein VP2. The viral capsid contains 180 copies of the VP1 protein and 1-2 copies of VP2. It has been found that \textit{in vitro} expression of the VP1 gene leads to the spontaneous formation of virus-like particles (VLPs), which are antigenically and structurally identical to the capsid of human norovirus but lack the genome component. Histo-blood group antigens (HBGAs) have been identified as the cellular receptors of human norovirus through outbreak investigation and human volunteer studies (Green, Chanock, and Kapikian 2001; Green 2007; Jiang et al. 1992; Li et al. 2012; Rohayem et al. 2010; Tan and Jiang 2005; Hennessy et al. 2003; Hutson et al. 2002; Lindesmith et al. 2005; Rockx et al. 2005).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{genomic_organization.png}
\caption{Genomic organization of human norovirus}
\end{figure}

(adapted from Rohayem et al. 2010)
Human norovirus infection causes acute gastroenteritis, which is characterized by watery diarrhea and projectile vomiting. Other symptoms include nausea, abdominal cramping, fever, chills, headache, and dehydration. Generally speaking, the clinical manifestation of human norovirus infection is usually mild with an average mortality rate of only 0.003%, but the social and economic burden is quite severe (CDC 2011). Norovirus infection typically leads to a self-limited illness with a short duration of 2-3 days in healthy individuals. Prolonged disease and more severe complications including volume depletion and electrolyte disturbances, have been observed in immunocompromised individuals (Koopmans 2008; Koopmans and Duizer 2004; Lopman et al. 2004; Rockx et al. 2002).

1.2.2. Epidemiology of human norovirus

The primary transmission mode of human norovirus is the fecal–oral route, though airborne transmission has also been reported (Atmar and Estes 2006; Koopmans and Duizer 2004). Transmission through infectious vomit or feces either by direct contamination (through hand and/or mouth contact) or by indirect contamination (through contaminated foods or water) leads to rapid transmission of the virus in closed settings (Marks et al. 2003). Recent studies have also found that certain genotypes are more likely to be associated with specific routes of transmission. For example, the GII.4 strains are more commonly spread through person-to-person transmission, while GI strains are more frequently associated with shellfish-related outbreaks (Le Guyader et al. 2006; Siebenga et al. 2007)
Human norovirus is shed in the stool and vomit of infected persons at high levels throughout the infection. The shedding starts during the incubation period and persists even after clinical symptoms are fully resolved. Asymptomatic viral shedding has also been reported. According to Atmar et al., one third of persons infected with norovirus shed virus prior to onset of illness, and peak fecal virus shedding may occur after gastroenteritis symptoms have completely dissipated (Atmar et al. 2008). It has been estimated that a person infected with human norovirus could shed up to 100 billion virus particles per gram of feces (CDC 2011). Moreover, the duration of viral shedding is longer in the elderly, children, and immunocompromised persons (Rockx et al. 2002).

The infectious dose of human norovirus is low, with less than 10 virus particles being sufficient to cause the disease in human (Teunis et al. 2008; Koopmans and Duizer 2004). A recent study using human volunteers and mathematical modeling estimated the average probability of a single human norovirus particle causing infection to be 0.5. That is, when an individual is exposed to one viral particle there is a 50% chance that infection will occur (Teunis et al. 2008). The low infectious dose escalates the transmission of the virus following point source outbreaks (Patterson, Hutchings, and Palmer 1993; Lo et al. 1994). Hence, human norovirus outbreaks usually occur in places where a large number of people have close contact with each other, such as restaurants, schools, cruise ships, hotels, swimming pools, hospitals, daycare centers, nursing homes, and even sport stadiums (Goodgame 2006; Rockx et al. 2002; Becker et al. 2000; Estes, Prasad, and
Atmar 2006; Marks et al. 2003). As shown in Figure 5, of the 660 reported cases of norovirus-associated outbreaks which were laboratory-confirmed by the CDC from 1994 to 2006, 35.4% (234 cases) were associated with long-term care facilities, 31.1% (205 cases) were associated with restaurants or social events, 31.1% (205 cases) were associated with vacations including cruise ships, and 13.0% (86 cases) were associated with schools or community (Zheng et al. 2010; Hall and Aron 2011).

![Figure 5. Number of human norovirus outbreaks confirmed by the CDC in the United States from 1994 to 2006 by setting and genotype](adapted from Hall and Aron 2011)
Human norovirus causes infections throughout the year, but in terms of climatic season there is a strong association with winter, which explains its former name of “winter vomiting disease” (Lopman et al. 2009). It has been shown that the cold weather and dry conditions in the winter favor the survival of norovirus in the environment.

Genogroup II noroviruses are the most prevalent strains responsible for infection and illness and account for 96% of all sporadic infections worldwide during the past 15 years. Among these, GII.4 is the most prevalent genotype, followed by the GII.3 and GII.6. As shown in Figure 6, GII.4 is most notable genogroup in the global emergence of new human norovirus variants and the emergence of novel GII.4 variants results has resulted in dramatic increases in outbreaks (Hoa Tran et al. 2013). It is believed that the emergence of new norovirus variants results from an increase in population immunity against circulating strains, a similar phenomenon which is seen with influenza viruses (Lindesmith et al. 2008; Siebenga et al. 2009).
1.2.3. Human norovirus, foodborne illnesses and health impacts

Foodborne illness caused by human norovirus is becoming an increasingly important problem in the US and worldwide. It is estimated that human norovirus causes over 95% of non-bacterial gastroenteritis (Li et al. 2012). From 2000 to 2008, noroviruses accounted for the vast majority, approximately 58%, of domestically acquired foodborne illnesses (CDC 2013). In 2009 and 2010, human norovirus was the leading cause of all confirmed, single-etiologic outbreaks, accounting for 42% of all of these outbreaks (CDC 2013).
According to the latest outbreak statistics, foodborne viruses remain the major causative agent of foodborne illnesses (Fox 2011; CDC 2013).

Figure 7. Top pathogens contributing to domestically acquired foodborne illnesses and deaths from 2000 to 2008 (adapted from CDC, 2013)

It is estimated that roughly 48 million (1 of every 6) people acquire a foodborne illness each year in the United States, resulting in 128,000 hospitalizations and 3,000 deaths (CDC 2011). It is also estimated that human norovirus causes a total of 21 million cases of acute gastroenteritis from all causes each year in the United States, and the number of cases are known to increase by roughly 50% when a new strain emerges. Of the 21 million estimated annual cases of human norovirus infection approximately 800
result in death, most frequently among elderly persons, young children, and immunocompromised patients (CDC 2013, 2013). This number may actually be underestimated due to underreporting of cases, as most individuals with acute gastroenteritis will not seek medical care and norovirus is not a reportable disease which requires notification of a diagnosis to the CDC. Additionally, a number of norovirus cases may go undiagnosed due to the lack of reliable detection methods.

1.2.4. Animal caliciviruses as surrogates for the study of human norovirus

As the most prevalent foodborne virus, human norovirus has significant impact on both food safety and public health. However, research on human norovirus has been hampered because it cannot be cultivated in vitro and lacks a small animal model. In order to understand the replication, pathogenesis, and environmental stability of human norovirus, several animal caliciviruses that are closely related to human norovirus have been used as research surrogates. These viruses share structural, genetic, antigenic, or pathogenic properties with human norovirus and can serve as models to estimate how human norovirus may interact with its host and the environment.

1.2.4.1. Murine norovirus (MNV-1)

Murine norovirus (MNV-1) belongs to the GV genogroup of the genus Norovirus and is the only cultivatable virus in this genus to date. It was isolated in 2003 from signal transducer and activator of transcription (STAT-1) defective mice suffering from a lethal systemic infection (Karst et al. 2003). The particle size of MNV-1 is 28-35nm in diameter
with a 7.3 kb genome of with the prototypical gene organization for caliciviruses. MNV-1 is able to replicate in murine macrophage cell line (RAW 264.7), murine BV-2 cell line, and other cell lines (Cox, Cao, and Lu 2009; Wobus et al. 2004). MNV-1 resembles human norovirus in terms of viral capsid structure, viral particle size, and genetic organization. However, unlike human norovirus which uses the HBGAs as its cellular receptors, MNV-1 is known to attach to sialic acid receptors on the cell surface (Taube et al. 2009). Moreover, MNV-1 causes systemic infections in immune deficient mice, hence the symptoms and pathogenesis of MNV-1 differs from human norovirus which causes acute gastroenteritis. Though MNV-1 has been extensively used as research surrogate for human norovirus, the differences in the cellular receptors and the pathogenesis of MNV-1 and human norovirus limit comparisons that can be made.

**1.2.4.2. Porcine sapovirus**

Porcine sapovirus belongs to the GIII genogroup of the genus *Sapovirus*. Porcine sapovirus was isolated from the stool of swine exhibiting the symptoms of gastroenteritis in 1980 (Saif et al. 1980). The size of porcine sapovirus is about 27-40nm in diameter, with a 7.3 kb genome exhibiting the typical genetic organization of viruses in this genus with two ORFs. Porcine sapovirus was adapted to cell culture and grows in porcine kidney cells (LLC-PK) supplemented with intestinal content fluid filtrate. The cellular receptor for porcine sapovirus is not known. However, since porcine sapovirus can cause enteritis in its host, it has been proposed as a good model to study the pathogenesis of human norovirus.
1.2.4.3. Feline calicivirus (FCV)

FCV is a member of the genus *Vesivirus*. FCV was isolated in the 1950’s, it infects domestic and wild cat species, causing upper respiratory tract infections (Radford et al. 2007). FCV has been adapted to cell culture and can grow in feline kidney cells. FCV has been found to be unstable at low pHs, utilizes sialic acid as its cellular receptor, and is not an enteric pathogen. The use of FCV as surrogate for human norovirus study has diminished in recent years.

1.2.4.4. Tulane virus (TV)

Tulane virus (TV) is a newly discovered member of *Caliciviridae* in the genus of *Recovirus*. TV was originally isolated from the stool of rhesus macaques showing symptoms of gastroenteritis in 2008 (Farkas et al. 2008). The size of TV is 27-40nm in diameter, with a 6.7 kb genome of typical calicivirus genetic organization. TV has been adapted to cell culture and grows in rhesus macaque kidney cell line (LLC-MK2). TV not only resembles human norovirus in its genetics, morphology, and stability, it also utilizes HBGAs receptors as human norovirus does, and it causes enteric disease in its host, a non-human primate (Farkas, Cross et al. 2010; Farkas et al. 2008; Hirneisen and Kniel 2013; Sestak et al. 2012). These properties make TV a promising surrogate for the study of the general biology, pathogenesis, immunology, and antiviral strategies for human norovirus.
1.3. Tulane virus

1.3.1 Discovery of Tulane virus

Tulane virus, also known as monkey calicivirus or rhesus enteric calicivirus, was discovered in 2008 (Farkas et al. 2008). It is the prototype virus of the newly proposed genus *Recovirus* in the *Caliciviridae* family. It was first isolated in the stool samples from nine rhesus macaques in Tulane National Primate Research Center both with and without clinical symptoms of diarrhea. TV was later successfully adapted to tissue culture in LLC-MK2 rhesus macaque kidney cell line, and was found to cause significant cytopathic effects (CPE) in cells after infection (Farkas et al. 2008). Subsequently, the genome of TV was cloned and sequenced. It was found that the full-length genome of TV is 6714 nt in length (Farkas et al. 2008).

Phylogenetic analyses were conducted on the TV viral nonstructural protein, NTPase, protease, polymerase, and capsid protein VP1 genes. The results showed that TV was consistently placed on the branch rooting with norovirus (Figure 8). Using the mean distances between TV and other genera, it was suggested that TV was most closely related to *Norovirus*, compared to other genera. However, the distance between TV and the noroviruses was longer than those between the established genera in the family, and it was proposed that TV should belong to a new genus of *Caliciviridae*. Based on these findings, TV was placed under a newly proposed genus, the *Recovirus* (*rhesus enteric Calicivirus*), and is the prototype virus of this genus. More recovirus strains have been isolated recently, however the TV is the most widely used strain for research purposes.
1.3.2 Taxonomy of Recovirus

Since the discovery of the prototype TV, more viruses have been isolated and classified into the Recovirus genus. A calicivirus prevalence study was conducted in the Tulane National Primate Research Center (TNPRC) in which 500 stool samples were collected from randomly selected juvenile (less than 3-year-old) rhesus macaques during 2008, and 57 samples were found to be positive for recovirus as detected by PCR (Farkas, Cross et al. 2010). These recovirus sequences exhibited 61-90% nucleotide and 62-90% amino acid homology with the prototype TV. Based on phylogenetic analysis, these 57 strains were divided into two genogroups and four genotypes (Figure 9).
Genogroup I (GI) of recovirus contains three genotypes, whereas GII only has one. Of the 57 recovirus isolates recovered from the 2008 TNPRC study, 15 strains were classified into GI.1, 11 into GI.2, 25 into GI.3, and 6 into GII.1. The mean intergenogroup distances between GI and GII recoviruses are comparable to those between GI and GII noroviruses. Moreover, the mean distances between genotypes within GI were similar to those between GI or GII norovirus genotypes. Such results demonstrate that the genetic diversity, which is reflected in the genotype organization, of recoviruses closely resembles the those of human noroviruses.
Figure 9. Classification of recoviruses based on phylogenetic analysis and comparison to several norovirus strains (adapted from Farkas et al. 2010)
1.3.3 Virion structure of Tulane virus

After CsCl density and plaque purification, a clear peak with mean density of approximately 1.372 g/ml was revealed, which contained purified TV particles. When examined under transmission electron microscope, small round virions with cup-like protrusions were visualized (Figure 10). The diameter of the TV particle is 34 to 38 nm, which is similar to other caliciviruses. When running the purified TV through SDS-PAGE, only a single protein band of approximately 60 kDa was revealed (Farkas et al. 2008). These results suggest that this protein might be the capsid protein VP1.

Figure 10. Transmission electron microscope pictures of Tulane virus (adapted from Farkas et al. 2008)
1.3.4 Genome structure of Tulane virus

The complete genome sequence of wild type TV isolated from rhesus macaque stool samples and also tissue culture-adapted, plaque-purified TVs has been compared; the two sequences were identical including the primer binding sites and junction regions. The full-length genome of tissue culture-adapted TV is 6714 nt without the poly (A) tail, which is the shortest of all known calicivirus genomes. It has a ribonucleoside composition of 27.46% A, 24.58% C, 22.07% G, and 25.87% U residues. The TV genome starts with a 14-nt-long 5’ untranslated region (UTR) of GGGTGAAGCGAAGCTATG. Like other caliciviruses, the genome of TV contains three ORFs. ORF1 starts from nt 15 to 4358, ORF2 from nt 4380 to 5984, and ORF3 from nt 5984 to 6640 (Figure 11). The genome ends with a 74-nt-long 3’ UTR, followed by a poly(A) tail of 26 to 62 residues at the terminus. ORF1 encodes the nonstructural proteins, while ORF2 and ORF3 encode the capsid protein VP1 and minor capsid protein VP2. Sequencing results demonstrated that TV and human norovirus have comparable nt lengths encoding the structural and nonstructural regions, with the exception of 5’ end. The N-terminal protein encoded by TV has a much smaller molecular mass compared to human norovirus, which explains the difference in the genome sizes between the two viruses (Farkas et al. 2008; Wei et al. 2008).
ORF1 and ORF2 of TV are separated by 21 nt, and they are both in frame +2 relative to the first nucleotide, which is different from the case for noroviruses. The ORF3 of TV is in frame +3 and overlaps with the last nucleotide of the ORF2 termination codon, which is similar to noroviruses. The predicted initiation codons of all three ORFs are in a strong consensus context for translation initiation of eukaryotic mRNA (Kozak 1991). The 5’ UTR of TV did not exhibit significant homologies with the 5’ UTR of putative subgenomic RNA, which is different from noroviruses. However, the 5’ UTR aligned well to a region 21 nt downstream from the ORF2 initiation codon (Figure 12) (Farkas et al. 2008).
Figure 12. Alignment of the 5’ end of genomic and subgenomic RNAs of TV.

Initiation codons of the nonstructural polyprotein and VP1 are boxed (adapted from Farkas et al. 2008).

Differences between stool sample isolated-TV and cell culture-adapted TV have been reported. After about 25 passages in the LLC-MK2 cell line, notable mutations have been observed, mainly in protein-encoding regions including the P domain of capsid protein VP1 and the C terminus of minor structural protein VP2. Such results indicate either the presence of numerous quasi-species of the viruses in vivo, or adaptive mutations essential for replication and adaption to cell culture (Wei et al. 2008).

1.3.5 Proteins of Tulane virus

1.3.5.1 Nonstructural proteins

The nonstructural protein encoded by ORF1 of TV has the typical organization of caliciviruses and has the highest overall amino acid identity (22 to 24%) with noroviruses compared with other members of the Caliciviridae. ORF1 of TV contains all the calicivirus conserved amino acid motifs including protease, VPg, NTPase, and polymerase (Farkas et al. 2008). A putative polyprotein cleavage map of the TV nonstructural protein was proposed based on sequence alignments with other caliciviruses cleavage maps and amino acid residues at cleavage sites. The TV nonstructural protein is
cleaved into six functional proteins including the N-terminus protein (26.4 kDa), the NTPase (38 kDa), P16 (15.7 kDa), VPg (10.8 kDa), the protease (18 kDa), and the polymerase (52.9 kDa). The N-terminus protein, P16, and VPg are relatively smaller (21 to 41% difference) compared to noroviruses, which explains the difference in sizes between the TV and noroviruses polyproteins (Figure 13). The similarity between TV and human norovirus could be utilized for understanding the basic virology of the uncultivable human norovirus, while the differences such as the smaller sizes of the N-terminus protein, P16, and VPg could indicate possible an explanation for TV’s capability to replicate in cell lines *in vitro.*
Figure 13. Predicted nonstructural polyprotein cleavage map of TV compared with published GI (Southampton), GII (MD145), and GV (MNV-1) norovirus cleavage maps (adapted from Farkas et al. 2008)

A recent study on the protease of TV validated four of the five cleavage sites of this predicted cleavage map using recombinant polyprotein fragments by in trans cleavage and N-terminal sequencing. Though the cleavage site between the N-terminus protein and the NTPase could not be confirmed by N-terminal sequencing, the Q/S scissile bond at residue 233-234 remains the primary candidate site for cleavage, as it is the only potential cleavage site within 113 adjacent amino acids. It has also been reported that though TV and norovirus proteases share less than 27% amino acid sequence identity, the catalytic
triad composed of a nucleophilic cysteine thiol at position 139, a histidine at position 30, and a glutamic acid at position 54 is well conserved, and conventional cysteine protease inhibitors were found to reduce the activity of the TV protease. Moreover, the study demonstrated partially reciprocal substrate cleavage between TV and norovirus proteases. These findings not only help us to better understand the molecular biology and replication cycle of TV, but demonstrate the potential for developing antivirals targeting the protease of human norovirus (Wei, Meller, and Jiang 2013).

In addition to the protease, the NTPase of TV is also important for replication. A study using an RNA interference (RNAi) technique demonstrated that replication of viral RNA, translation of the capsid protein, and assembly of the viral particles was dramatically hampered when treated with 120 pmol of siRNA targeting NTPase for 4 h prior to cell inoculation with TV. Though the function of NTPase is still unclear for TV, such results indicate another potential antiviral strategy against TV and human norovirus (Fan et al. 2013).

1.3.5.2 Structural proteins

The TV capsid protein VP1 and minor structural protein VP2 have also been characterized. VP1 of TV is 534 amino acids long with the molecular mass of approximately 57.9kDa. It shows highest (20 to 25%) overall amino acid identity with noroviruses (Farkas, et al., 2008). The TV VP1 contains the conserved PPG (PPN) motif and FXXLXPP hinge between the S and P domains, which characteristic of most
caliciviruses. The P domain of VP1 of caliciviruses has been associated with multiple functions including host receptor recognition, immune regulation, and involvement in viral replication (Tan et al. 2003; Tan and Jiang 2005). There have been reports of the presence of soluble P domains in norovirus infected patients, suggesting the possible function of the P domain in viral replication and pathogenesis (Greenberg, Valdesuso et al. 1981; Graham et al. 1994). It was reported that the S and P domains of TV VP1 were found in cells infected with TV but not in the culture medium supernatant, which indicated these protein fragments were involved in the viral replication but were not part of the mature virion (Fan et al. 2013). However, this was the first successful attempt to visualize the S domain protein and the molecular mass detected was bigger than expected, it remains to be confirmed whether the P and S domain proteins were synthesized during TV viral replication or were natural degradation products of VP1 without any function (Fan et al. 2013).

TV VP2 is 218 amino acids long and is calculated to be about 22.8kDa in molecular weight. It also exhibits the highest (22 to 26%) amino acid identity with noroviruses (Farkas et al. 2008). Interestingly, SDS-PAGE analysis of purified TV revealed a single protein of about 60kDa, indicating that VP1 may be the only component of the TV capsid (Farkas et al. 2008). The function of VP2 remains unclear, though various studies have demonstrated that it is essential for viral replication. It has been reported that both ORF2 and ORF3 are essential for the production of infectious TV (Wei et al. 2008). Moreover, siRNAs targeting the VP1 and VP2 genes could effectively
inhibit the replication of TV (Fan et al. 2013). These results indicate that both VP1 and VP2 are required for the assembly of infectious TV particles. Considering their sequence similarity, TV VP2 may function similarly to VP2 of human norovirus, which is involved in stabilization of VP1, involvement in RNA packaging, and regulation of protein synthesis (Bertolotti-Ciarlet et al. 2003; Glass et al. 2000).

1.3.6 Replication cycle of Tulane virus

Since the discovery of TV, the reverse genetic system, protein expression, replication, receptor binding, and recovery of TV have all been extensively studied. Some known characteristics of the TV replication cycle include: naked capped full-length RNA is infectious in the LLC-MK2 cell line; a subgenomic RNA is present during replication but is not required for viral infection; ORF1 encodes a polyprotein that is further processed by the viral protease into six nonstructural proteins that are involved in the replication of viral genome; ORF2 and ORF3 are both essential for the generation of virion; P and S domain proteins of TV were present in infected cells but not in mature virions; different HBGA antigens affect TV binding and infectivity (Fan et al. 2013; Wei et al. 2008; Wei, Meller, and Jiang 2013; Farkas, Cross et al. 2010; Farkas et al. 2008). However, the details of the viral life cycle of TV are still not fully understood. Since TV can be grown in cell culture, the details of the replication scheme can be elucidated by the direct findings of future studies. Understanding the replication strategy of TV, in combination with the studies of MNV-1 replication, will give insight into the potential
replication strategy of human norovirus (Sosnovtsev et al. 2006; Wobus, Thackray, and Virgin 2006).

The proposed replication cycle of TV includes 11 steps (Figure 14):

Step 1: Attachment. The viral capsid binds to HBGAs present on the surface of the host cell.

Step 2: Entry and uncoating. The viral particle enters the host cell through endocytosis. The genomic RNA is released within the endocytic vesicles, and then upon degradation of the vesicle is released into the cell.

Step 3: Translation of ORF1. The Vpg linked to the 5’ end of the RNA genome is recognized by the host cell ribosomes and serves as the translational start site; ORF1 is translated into the nonstructural polyprotein precursor.

Step 4: Processing of the nonstructural proteins. The polyprotein precursor is cleaved by the viral protease and the nonstructural proteins and their precursors are released.

Step 5: Assembly of the replication complex. The nonstructural proteins and precursors form the enzymatic complex that is responsible for the genome replication and synthesis of antigenomic RNA.

Step 6: Synthesis of antigenomic RNA. The RdRp synthesizes a full length antisense copy of the full length TV genome.

Step 7: Replication of genomic RNA using the antigenomic RNA as template. The RDRP uses the antigenome RNA has a template to produce multiple copies of full length genomic RNA.
Step 8: Synthesis of subgenomic RNA using antigenomic RNA as template. The RdRp using the antigenome RNA from Step 6 as a template, transcribes subgenomic RNA containing ORF2 and ORF3. This subgenomic RNA encodes VP1 and VP2 and is used to yield high amounts of the structural proteins.

Step 9: Translation of subgenomic RNA into structural proteins. A termination-reinitiation mechanism observed in other caliciviruses might be recruited to regulate the ratio of VP1/VP2 (Luttermann and Meyers 2007; Napthine et al. 2009).

Step 10: Virion assembly. The newly synthesized genomic RNA is packed into capsid made of VP1 and VP2 structural protein.

Step 11: Modification and release of the mature virion from the host cell.

Figure 14. Proposed replication cycle of TV (adapted from Rohayem et al. 2010)
1.3.7 Receptors of Tulane virus

1.3.7.1 Histo-blood group antigens (HBGAs) are the cellular receptors for TV

Virus replication cycle starts with attachment, which requires the virus to bind to its cellular receptor on the surface of the host cells. It has been identified that, similar to human norovirus, the histo-blood group antigens (HBGAs) are the functional receptors for TV (Farkas, Cross et al. 2010; Huang et al. 2003; Hutson et al. 2002; Tan et al. 2003).

HBGAs are carbohydrate complexes that are present on the surface of erythrocytes as well as mucosal epithelium of the intestinal, genitourinary, and respiratory tracts (Ravn and Dabelsteen 2000). HBGAs also exist in the form of free oligosaccharides in several types of body secretions including saliva, blood, milk, and the mucosal secretions of the intestines (Tan and Jiang 2005). There are three families of HBGAs: Lewis, ABO, and secretor. Respectively, each is specifically recognized by different norovirus strains. It was reported that though humans and non-human primates have four ABO blood group phenotypes, only humans and the anthropoid apes express the ABH antigens on red blood cells. Thus the ABO HBGA type of other non-human primates is determined by analyzing saliva samples or antibodies in the serum (Apol et al. 2000). Since the HBGA synthesis system is controlled by multiple gene families that contain silent alleles, the HBGA phenotypes of the human population are polymorphic and complicated (Marionneau et al. 2001). In rhesus macaques, group B has been reported to be the major ABO blood type (Linden et al. 2008; Moor-Jankowski and Socha 1978).
polymorphism of HBGAs adds to the complexity in the understanding of the virus-receptor interaction.

1.3.7.2 TV-HBGA interaction

Recently HBGAs have been identified as the cellular receptors for TV (Farkas, Cross et al. 2010). Both saliva binding assays and plaque reduction assays were used to confirm the interaction between TV and HBGAs. Using the saliva binding assay, it was found that prototype TV bound to six of eight type A and seven of eight type B saliva samples, while no viral binding was observed for the eight type O saliva samples. Additionally, it was found that TV did not bind to Lewis-type antigens using the saliva binding assay. To further confirm the attachment of TV to type A and B antigens, synthetic BSA-conjugated type A and B trisaccharides with sugar moieties corresponding to type A and B saliva samples were used. It was found that TV also attached to these synthetic type A and B moieties. These results were further confirmed by plaque reduction assays. Pre-incubation of prototype TV with type A or type B saliva samples induced significant plaque reduction (7 to 79% for type A and 50 to 90% for type B), whereas type O samples and the synthetic PAA-conjugated oligosaccharides had no plaque reduction effect regardless of Lewis type. Interestingly, the BSA-conjugated type A and B trisaccharides increased the plaque formation by 2- to 12-fold in a dose dependent manner.
These results indicate that TV utilizes the HBGAs as its cellular receptor, similar to the human noroviruses. However, there were some inconsistencies observed in the findings. The discrepancies in the results may be explained by the co-receptor/receptor theory (Farkas, Cross et al. 2010). It is possible that the HBGA molecule is a co-receptor for TV and the virus needs to interact with a smaller unknown molecule on the cell surface to trigger conformational changes or other reactions that facilitate viral entry. This hypothesis is supported by previous studies showing that the expression of HBGAs alone on the cell surface did not increase the efficiency of norovirus infection (Guix et al. 2007; White et al. 1996).

The *in vitro* study results were found to coincide with the results obtained from the study of TV receptor binding *in vivo*. The *in vivo* study, which focused on the HGBA phenotype of rhesus macaques and the relative susceptibility to TV infection, found that out of 57 TV-infected rhesus macaques, 96% (55) were blood type B and 4% (2) were type O. These results indicated that both blood type B and type O rhesus macaques were susceptible to TV infection. The susceptibility of blood type A rhesus macaques to TV infections remains unknown due to the fact that this phenotype was rare in the study population (Farkas, Cross et al. 2010). The HBGA phenotypes of the rhesus macaque population in this study were predominately blood type B, with 97% of the animals expressing the B antigen. A more polymorphic population should be used to further investigate the susceptibility of different ABO blood types to TV infection in the rhesus macaque model.
1.3.7.3 Comparison between TV- and human norovirus-HBGA interaction

Many research efforts have focused on the study of the mechanism of the interaction between noroviruses and the HBGA receptors. It has been demonstrated that the major viral capsid protein VP1, specifically the P2 domain of the VP1 protein, is responsible for the specificity of receptor binding (Tan et al. 2003; Tan and Jiang 2005; Tan et al. 2008). Using the VLPs of the prototype Norwalk virus as a ligand, it was shown that Norwalk virus binds to secretors but not non-secretors HBGAs (Marionneau et al. 2002). In addition, Norwalk virus VLPs were also found to bind to differentiated Caco2 cells which readily express the H antigen on their outer membrane (Marionneau et al. 2002). Moreover, it has also been demonstrated that norovirus VLPs can recognize the A, but not B antigens (Huang et al. 2003). Recently, a new model for the characterization of noroviruses based on the multi-selection for HBGA binding was proposed (Figure 15). In this model, human noroviruses were divided into three groups (the H, A/B, and Lewis binding groups) based on the major residues of the three saccharides in HBGA. This model emphasizes the critical roles of the major binding residues that are also the determinants of the three major human HBGA families (the ABO, secretor and Lewis families). Thus the proposed model is likely to represent all possible repertoires of HGBAs that interact with noroviruses. Moreover, the novel method of classification provides ability to explain additional variations of norovirus-HBGA interaction (Tan and Jiang 2011).
Figure 15. Newly proposed multi-selection model of norovirus-HBGA interaction

(Tan and Jiang 2011)

(A) HBGA product with the five saccharides indicated in different shapes and colors. (B) Classification of GII and GI NoVs into three binding groups (A/B, H and Lewis binding groups) and their targets at the A/B, H and Lewis epitopes, respectively.
Based on these findings, it can be concluded that TV and human norovirus have preferential affinity for different types of these antigens (Table 2) although both of them utilize HBGAs as their cellular receptors.

Table 2. Comparison between the HBGAs receptor interaction of TV and human norovirus

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Viral receptor</th>
<th>Antigens binding specificity</th>
<th>High susceptibility group</th>
<th>Low susceptibility group</th>
<th>Binding model</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV</td>
<td>HBGAs</td>
<td>Only bind to A/B antigen group, no association has been reported for other groups</td>
<td>Type B</td>
<td>Type A and type O</td>
<td>Co-receptor/receptor model</td>
</tr>
<tr>
<td>Human norovirus</td>
<td>HBGAs</td>
<td>Strains binding to A/B, H, and Lewis antigen groups have all been reported</td>
<td>Type O</td>
<td>Type B</td>
<td>Multi-selection model</td>
</tr>
</tbody>
</table>

1.3.8 Pathogenesis of Tulane virus

1.3.8.1 TV infection in juvenile rhesus macaques

The primary host of TV is assumed to be the rhesus macaque, as the virus was initially isolated from stool samples obtained from these primates exhibiting symptoms of gastroenteritis. Since the discovery of TV, studies have focused on the prevalence of TV in rhesus macaque population. A study showed that 11% (57 out of 500) stool samples
from the rhesus macaque in Tulane National Primate Research Center (TNPRC) were positive for recovirus isolates, of which TV (GI.1) is the prototype. Some of the recovirus-positive animals had a clinical history of watery diarrhea within a 1-month period prior to sample collection, and TV virus-neutralizing antibodies were detected in serum samples from a cohort of the infected animals exhibiting diarrheal symptoms (Farkas, Cross et al. 2010).

Another study focusing on the detection of anti-norovirus, anti-sapovirus, and anti-TV antibodies in rhesus macaques of TNPRC showed a high prevalence of TV neutralizing antibody in the population compared to antibodies against the other viruses (Farkas, Dufour et al. 2010). While only 10% of the animals shed TV in their stool samples, 382 (69%) of the 556 serum samples collected from TNPRC rhesus macaques had sufficient anti-TV antibodies to neutralize TV at minimum dilution of 1:20. These results suggest that TV is a matrix disease in the macaques colony studied.

Recently, experimental inoculation of TV into juvenile rhesus macaques confirmed that TV infection could cause enteric disease (Sestak et al. 2012). Three seronegative juvenile (less than 3 year-old) rhesus macaques were inoculated intragastrically with cell culture-adapted TV. Two of the three animals developed symptoms of diarrhea and fever within two days after virus inoculation, whereas the other animal remained asymptomatic throughout the observational period. No symptoms of vomiting or viremia were found in any of the three animals.
All three rhesus macaques shed the virus for 8 days, and one animal shed TV for 10 days. On either PID 2 (day 2 post-inoculation) or PID 3 the highest levels of viral shedding in the stool was observed, and a peak shedding level of $10^5$ RNA copies per gram of stool was detected by real time RT-PCR. On average a juvenile rhesus macaque produces 60 to 100 grams of stool per day, hence the level of virus shed in stool exceeded the amount in the initial inoculum ($10^6$), which indicates TV replication was occurring in the host.

In addition, all three animals developed TV neutralizing antibody responses by PID 7. The TV neutralizing antibody titer peaked between PID 7 and 9, and persisted at this titer up to PID 38 followed by a steady decline to pre-inoculation levels by PID 80. None of these rhesus macaques were positive for antibody responses against recovirus strain FT285 from a different genotype, indicating no cross-reactivity between TV (GI.1) and FT285 (GI.2) specific antibodies.

Biopsies of the duodenum of TV infected rhesus macaques on PID 3 showed histologic changes including villous blunting and lymphocytic infiltration in the lamina propria, but no significant damage was observed in the intestinal epithelium. Confocal microscopy examination of the intestinal brush border and the epithelial layer revealed no significant damage to the enterocytes, indicating the possibility that the virus may translocate into the lamina propria without replicating in the enterocytes. Consistent with this notion, numerous cells in the lamina propria were positive for TV antigen but not in
the epithelium of the duodenum, and many TV antigens exhibited perinuclear fluorescence while TV-specific antibodies colocalized with calnexin in some cells (Sestak et al. 2012).

These results demonstrated that cell culture-adapted TV could cause clinical disease, viral shedding, immune responses, and histologic lesions in rhesus macaques. The visualization of TV antigens in tissue biopsies suggested putative viral replication in the lamina propria of duodenum. This TV-rhesus macaque model could be used to study the replication, pathogenesis, and immunity of other enteric caliciviruses including human norovirus.

1.3.8.2 Potential zoonotic transmission of TV

In addition to the detection of TV neutralizing antibody in a majority of the rhesus macaques colony, it was also found that 88% of the 100 human serum samples taken from primate caretakers in TNPRC were positive for TV virus-neutralizing antibodies. Though the majority of the samples only neutralized TV at dilutions less than 1:16, two serum samples still demonstrated neutralizing effect at dilution of 1:256. This finding suggested potential zoonotic transmission of TV from rhesus macaques to humans (Farkas, Cross et al. 2010).

Low levels of antibody cross-reactivity between TV and human norovirus has also been reported (Farkas, Dufour et al. 2010). Low but consistent cross-reaction between
TV specific antibodies and different norovirus genotypes was detected by ELISA using hyperimmune sera. However, the human serum samples collected during a human norovirus gastroenteritis outbreak investigation failed to neutralize TV even at 1:10 dilution, indicating that antibodies against human norovirus may not cross react with TV.

The fact that TV and human norovirus reciprocal antibody binding was detected further suggested that TV and norovirus are closely related. The high prevalence of TV neutralizing antibody in primate caretakers indicated frequent exposure to TV and possibility of zoonotic transmission. However, the transmission of TV from non-human primates to human or vice versa still needs more investigation.

1.3.8.3 Comparison of TV and norovirus pathogenesis

1.3.8.3.1 Human norovirus

The disease caused by human norovirus infection is acute gastroenteritis, which is characterized by a rapid onset and typical symptoms of vomiting and watery diarrhea. Other symptoms include nausea, abdominal cramping, fever, chills, headache, and dehydration. Vomiting has been associated more frequently with GII.4 strains compared to other genotypes (Friesema et al. 2009). The incubation period for this virus is usually 1 to 3 days and the illness normally persists for 2 to 3 days in healthy individuals (Koopmans 2008; Koopmans and Duizer 2004). However it has been reported that symptoms can persist beyond 4 days in children under 12 years of age and elderly persons over 80 years of age (Rockx et al. 2002; Lopman et al. 2004). Norovirus
infection typically leads to a self-limited disease in healthy individuals. However, more severe complications include volume depletion and electrolyte disturbances. Elderly and persons with chronic diseases are more likely to suffer these complications and even death (Mattner et al. 2006).

Recent human volunteer studies have added to our knowledge on the duration and magnitude of viral shedding during norovirus infections. The magnitude and duration of virus shedding after volunteers infected with Norwalk virus was recently investigated (Atmar et al. 2008). Eleven out of 16 persons developed gastroenteritis and the symptoms lasted for 1 to 2 days. Viral shedding was detected by RT-PCR at 18 h post inoculation and lasted for 13 to 56 days, with a peak RNA titer of $9.5 \times 10^{10}$ genomic copies per gram of feces as determined by quantitative RT-PCR (Atmar et al. 2008). The median viral shedding peak load reported in this study (about $10^{11}$ genomic copies per gram of feces) was much higher than the $10^7$ to $10^8$ median viral loads reported in prior studies (Chan et al. 2006; Ozawa et al. 2007).

Progress has also been made in the study of pathologic changes caused by norovirus infection. In 1973, a human volunteer study was conducted to investigate human infection with Norwalk virus (Agus et al. 1973). Intestinal biopsy specimens were obtained from normal volunteers before, during, and after administration of the Norwalk virus. Histologic changes including villi blunting, microvilli shortening, dilation of endoplasmic reticulum, and increased intracellular multivesiculate bodies were observed
in the small intestine of acutely ill volunteers. Biopsy specimens obtained 2 weeks after illness from the same individuals showed normal histologic patterns. These results indicated that the acute gastroenteritis induced by Norwalk virus caused definite but reversible pathophysiologic lesions of the small intestine in humans (Agus et al. 1973). These results were later confirmed by another study showing that similar reversible lesions were observed in the peroral jejunal biopsies of volunteer subjects acutely ill with viral gastroenteritis induced by Hawaii agent. Increased serum antibody levels were found in three of the four ill volunteer subjects (Dolin et al. 1975). No histologic changes were observed in the gastric fundus or in the antrum or colonic mucosa. However, no information on lesions in other portions of the intestines of these volunteers is available.

In most individuals exposed to norovirus, generation of histologic lesions was correlated with the symptoms of diarrhea. However, in one study typical mild mucosal lesions were observed in biopsies from volunteers who did not show any clinical symptoms after challenge with the Hawaii agent (Schreiber, Blacklow, and Trier 1974). A recent study showed that human norovirus infection lead to structural and functional changes in the duodenum. Observations included epithelial barrier dysfunction, reduction of sealing tight junctional proteins, increases in epithelial apoptosis, and stimulated active anion secretion (Troeger et al. 2009). To date, the role of the small intestine in norovirus infection remains unknown because the cellular virus replication sites are not identified, and more distal involvement of the small intestine has not been studied (Glass, Parashar, and Estes 2009).
1.3.8.3.2 Murine norovirus

The first norovirus that infects mice, the prototype MNV-1, was first isolated from severely immunocompromised mice (Karst et al. 2003). It was reported that MNV-1 induced systemic and lethal infection in mice lacking the recombination-activating gene 2 (RAG2) and signal transducer and activator of transcription 1 (STAT1) (RAG2/STAT1−/− mice). Later studies demonstrated that both wild type and immunocompromised mice could be infected by peroral (p.o.) and intranasal (i.n.) inoculation (Hsu et al. 2005; Karst et al. 2003).

MNV-1 infection could cause severe disease in STAT1−/− mice by all inoculation routes, such as intraperitoneal, footpad, and all methods mentioned above. Viral RNA was detected in all the examined organs (including lung, liver, spleen, proximal intestine, and brain) and blood and feces of MNV-1 infected STAT1−/− mice on PIDs 1, 3, and 7. The histopathological analysis of these mice showed signs of pneumonia and loss of splenic architecture on PID 3, and severe liver inflammation by PID 7. Signs of encephalitis were also observed in the brain of RAG2/STAT1−/− mice after direct intracerebral inoculation (Karst et al. 2003). However, to date, the exact cause of death of MNV-1 infected mice remains unknown.

MNV-1 can also infect wild type mice including adult 129 and juvenile CD1 mice, though no clinical symptoms have been observed. Despite lack of symptoms in these wild type mice, viral RNA was detected in the liver, spleen, and proximal intestine at PID 1
after p.o. inoculation. Viral RNA level was not detectable on PID 3, but was detectable again in feces on PID 7, and in the mesenteric lymph node, spleen, jejunum, and feces at weeks 5 and 8 post inoculation with three new MNV isolates (Wobus, Thackray, and Virgin 2006; Hsu et al. 2005). In addition to viral RNA, seroconversion was also detected. However, neither the adult 129 mice nor juvenile CD1 mice exhibited any clinical symptoms after inoculation with MNV-1. Absence of clinical symptoms could be due to the fact that the systemic infection caused by MNV-1 is controlled by innate immunity. Also, mice have a structurally different digestive system that lacks emetic reflex, so the symptom of vomiting is impossible in the mice model. In addition, studies have only examined a few MNV strains and several mouse strains. Inclusion of more MNV strains as well as other mouse strains might help to better understand the pathogenesis of MNV.

1.3.8.3.3 Comparison of TV, MNV, and human norovirus pathogenesis

The pathogenesis of TV, human norovirus, and MNV is compared as shown in Table 3.
Table 3. Comparison of the pathogenesis of three caliciviruses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TV</th>
<th>Human NoV</th>
<th>MNV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genus</td>
<td>Recovirus</td>
<td>Norovirus</td>
<td>Norovirus</td>
</tr>
<tr>
<td>Growth in tissue culture</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Susceptible host</td>
<td>Rhesus macaque</td>
<td>Humans</td>
<td>Mice</td>
</tr>
<tr>
<td>Type of infection</td>
<td>Enteric</td>
<td>Enteric</td>
<td>Systemic</td>
</tr>
<tr>
<td>Symptoms of infection</td>
<td>Mild diarrhea and fever</td>
<td>Acute gastroenteritis including watery diarrhea, projectile vomiting, abdominal cramp, sometime fever</td>
<td>No symptom in wild type mice; systemic lethal infection in immunocompromised mice</td>
</tr>
<tr>
<td>Duration of symptoms</td>
<td>1 day to a week</td>
<td>Typically 1 to 3 days; longer in immunocompromised population</td>
<td>Up to a week or till death in immunocompromised mice</td>
</tr>
<tr>
<td>Viral detection in organs</td>
<td>N/A</td>
<td>Small intestine only</td>
<td>Liver, spleen, lung, brains, lymph node, proximal intestine</td>
</tr>
<tr>
<td>Viral shedding in feces</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Duration of fecal shedding</td>
<td>Up to 10 days</td>
<td>Up to 20 to 40 days, longer in immunocompromised population</td>
<td>Up to 8 weeks in immunocompromised mice</td>
</tr>
<tr>
<td>Presence of viremia</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Presence of histological changes</td>
<td>Villous blunting, lymphocytic infiltration in lamina propria of duodenum</td>
<td>Villous blunting and microvillous shortening of jejunum</td>
<td>Signs of pneumonia, loss of splenic architecture, liver inflammation, and encephalitis</td>
</tr>
<tr>
<td>Cost of model</td>
<td>Expensive</td>
<td>Expensive</td>
<td>Cheap</td>
</tr>
</tbody>
</table>
Since it is the only norovirus that can replicate in tissue culture, MNV has been widely used as a surrogate for human norovirus study. However, since MNV utilizes different cellular receptor and causes systemic infection, it may not be an ideal model for the study of pathogenesis of human norovirus. TV has been proposed as a better surrogate for pathogenesis study because it shares the same cellular receptors as human norovirus, causes enteric infection and gastroenteritis, and bears more similarity to human norovirus infection in terms of the viral shedding patterns and histologic changes.

1.3.9 Immunology and host susceptibility of Tulane virus and human norovirus

1.3.9.1 Immune response caused by TV infection

To date the study of the immunology and host susceptibility of TV is very limited. Experimental inoculation of juvenile rhesus macaques demonstrated that TV induced host immune response starting from PID 7. The response featured the development of TV specific neutralizing antibodies. By PID 7 to PID 9, the antibody titer reached peak (1:160 to 1:640) in all three TV infected animals, and the neutralizing antibody persisted at high levels till weeks 4 or 5 post inoculation. After week 5, the antibody titers started to decline, however low levels (1:40 to 1:80) of neutralizing antibody still existed by the end of the study (PID 80) (Sestak et al. 2012). No neutralizing antibody response was detected against a GI.2 recovirus FT285 in hyperimmune serum samples from TV-infected animals, indicating the antibody induced was strain specific. The rapid production of TV specific antibody on PID 7 indicated an early memory response, which
is consistent with the low levels of TV neutralizing antibodies prior to inoculation. The decline of antibody titer at week 5 was probably due to absence of re-exposure that usually takes place in the colony animals (Cannon et al. 2009; Tan and Jiang 2010). If the inoculation could include re-exposure and re-infection that mimics what really happens in the animal colony, the immune response might be stronger and last for a longer time. In general these findings are consistent with what have been reported for human norovirus in human volunteers (Wyatt et al. 1974). Previous studies have suggested that innate immunity including IFN- or STAT1-dependent immune responses, might be responsible for the rapid control of infection cause by MNV, while adaptive immunity mediated by B and/or T cells might be responsible for viral clearance (Wobus, Thackray, and Virgin 2006). This might be the same case for TV. Development of efficient assays targeting on humoral, mucosal, or cellular immune response will help to further reveal the details of immunity triggered by TV.

High prevalence of TV neutralizing antibodies in the rhesus macaque population has also been reported. Sixty-nine percent (69%) of the serum samples collected from rhesus macaques in TNPRC were able to neutralize TV at dilution of 1:20 or higher (up to 1:1280). Recent data showed that approximately 80% rhesus macaques housed at three separate US colonies were sero-positive for TV (Sestak et al. 2012). Such high prevalence indicated TV infections were common in colony macaques. Interestingly, there seemed to be an association between age and antibody presence. While TV-specific neutralizing antibody was detected in 43% of animals less than 1 year old, the percentage
increased to 94% in animals more than 3 years old, indicating that the prevalence of TV neutralizing antibody increased with age (Farkas, Dufour et al. 2010). This distinct pattern is different from the observations for human norovirus and sapovirus antibody prevalence in the same rhesus macaque population (Table 4). The reason why TV antibody production is age-associated remains unclear. Possible explanation could be difference between the mechanisms of immune response to different viruses, as well as the difference of viral infection and clearance. It is also possible that animals of older age have been exposed to TV, which leads to higher chance of infection and re-infection(s) that stimulate the immune response.

Table 4. Prevalence of TV antibodies in rhesus macaques grouped by age (adapted from Farkas et al., 2010)

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>TV (GI.1 recovirus)</th>
<th>Norwalk (GI.1 norovirus)</th>
<th>MOH (GII.5 norovirus)</th>
<th>Mex14917 (G1.3 sapovirus)</th>
<th>Mex340 (GII.2 sapovirus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>56/131 (43)</td>
<td>37/55 (67)</td>
<td>33/55 (60)</td>
<td>25/55 (45)</td>
<td>37/55 (67)</td>
</tr>
<tr>
<td>1-2</td>
<td>137/222 (62)</td>
<td>23/47 (49)</td>
<td>23/47 (49)</td>
<td>31/47 (66)</td>
<td>24/47 (51)</td>
</tr>
<tr>
<td>2-3</td>
<td>105/114 (92)</td>
<td>8/28 (29)</td>
<td>8/28 (29)</td>
<td>14/28 (50)</td>
<td>15/28 (53)</td>
</tr>
<tr>
<td>&gt;3</td>
<td>84/89 (94)</td>
<td>46/58 (79)</td>
<td>33/58 (57)</td>
<td>24/58 (52)</td>
<td>30/58 (52)</td>
</tr>
</tbody>
</table>

It should be pointed out that TV neutralizing antibodies were also detected in humans. Out of the 100 human serum samples taken from primate caretakers in TNPRC,
88% were positive for TV neutralizing antibodies. Though the majority of the samples neutralized TV at low dilution less than 1:16, two serum samples still demonstrated neutralizing effect at dilution of 1:256. It remains unknown if TV could cause infection and symptoms in humans. This finding suggested potential infection of TV in humans, as well as the potential zoonotic nature of TV (Farkas, Cross et al. 2010).

1.3.9.2 Immune responses caused by human norovirus in healthy people.

The immunology of human norovirus is still poorly understood. Early human volunteer studies demonstrated that challenge with Norwalk virus conferred short-term but not long term immunity to re-infection with the same virus. Dolin et al. reported that the Norwalk virus appeared to be relatively host specific for man and conferred at least short-term immunity, however widespread natural immunity to this agent might be incomplete or absent because of the high frequency of disease induced in unselected volunteers (Dolin et al. 1972). The immunity of viral gastroenteritis induced by Norwalk virus was further examined by another study (Parrino et al. 1977). Twelve volunteers were challenged and re-challenged with Norwalk agent and symptoms, jejunal biopsies and serum antibody were evaluated. In the first challenge, six volunteers developed gastroenteritis; when re-challenged 27 to 42 months later, the six who were ill in the first challenge again had gastroenteritis with jejunal lesions. The other six volunteers who were infected in the first challenge did not demonstrate illness or jejunal lesions in the second challenge. Four volunteers were subject to a third challenge 8 weeks after the second one, and three of them did not develop gastroenteritis. Moreover there was no
clear correlation between serum antibody level and protection against Norwalk virus infection. These findings indicate both short term and long term immunity could be induced by viral gastroenteritis, and factors other than serum antibody are important in immunity to Norwalk gastroenteritis (Parrino et al. 1977). Later, cross-challenge studies using the serotypically distinct Norwalk virus (GI), Hawaii virus (GII), and Montgomery County agents (GI) demonstrated that Norwalk virus and Hawaii virus failed to confer immunity to subsequent disease caused by the other, while Norwalk-induced disease seemed to confer immunity to Montgomery County agent challenge. Such results suggested there is lack of heterotypic immunity of different strains of human norovirus (Wyatt et al. 1974). A recent study showed that Snow Mountain virus (GII norovirus) challenge induced production of serum IgG, IgA, and T-cell response that could cross-react with Hawaii virus (GII) but not GI Norwalk virus (Lindesmith et al. 2005).

The correlation between pre-existing antibodies and norovirus infection susceptibility has been controversial. Researchers have found that some individuals with a high level of antibodies against norovirus were even more susceptible to norovirus challenge than those with lower level or no antibodies (Parrino et al. 1977; Greenberg, Wyatt et al. 1981). Later, it was found that HBGAs, the receptor for human norovirus, play a critical role in susceptibility of norovirus infection. Virus-receptor interaction is highly specific. Though cross-reactive antigenic epitopes have been observed among different norovirus strains, these epitopes might not necessarily induce cross-neutralizing antibodies (Kitamoto et al. 2002; Hardy et al. 1996). Such results suggest receptor
specificity plays an important role in host susceptibility, although acquired immunity is also involved in resistance to norovirus infection. The fact that individuals with high level of antibodies were still susceptible to norovirus infection indicates that these individuals have matched HBGA receptors with the challenge virus, and the strains that they also have previously been exposed to have same antigenic epitopes with the challenge virus but belong to different neutralization types. Therefore, the higher levels of antibody may suggest exposure to certain strains in the past but does not necessarily indicate protective immunity (Tan and Jiang 2005).

1.3.9.3 Immune responses caused by human norovirus in immunocompromised population

It has been reported that symptoms and severity of MNV infection were different in wild type and immunocompromised mice. For human norovirus, more severe disease and prolonged duration of viral shedding were also documented in immunocompromised humans. Intestinal transplant pediatric patients that were diagnosed with human norovirus infection developed secretory or osmotic diarrhea (Kaufman et al. 2003; Kaufman et al. 2005; Morotti et al. 2004). Due to immunosuppressive therapy, these patients had prolonged diarrhea ranging from 17 to 326 days, as well as prolonged viral shedding up to 80 days. The clinical symptoms and human norovirus RNA detection remitted after reduction of the immunosuppressive therapy. Lopman and colleagues demonstrated that patients in health care institutes including hospitals and nursing homes had a median duration of norovirus gastroenteritis for 3 days, which is significantly higher longer than
that for all other groups (Lopman et al. 2004). It is suggested that infection in hospitalized persons may be more severe than that in other groups. Another clinical study conducted in patients with human norovirus infection showed that higher viral concentration and older age were independently associated with prolonged diarrhea that last over 4 days (Lee et al. 2007). Severe protracted diarrhea and prolonged viral shedding in hospitalized, elderly, and immunocompromised patients indicates that active viral replication was enhanced and viral clearance was delayed (Goller et al. 2004; Lee et al. 2007; Mattner et al. 2006). The detailed differences upon norovirus infection between immunocompetent and immunocompromised hosts are summarized in Table 5.

**Table 5. Characteristics of norovirus gastroenteritis in immunocompetent versus immunocompromised humans (adapted from Bok and Green 2012)**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Immunocompetent hosts</th>
<th>Immunocompromised hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence</td>
<td>Leading cause of gastroenteritis worldwide</td>
<td>Not established; estimated at about 17 to 18%</td>
</tr>
<tr>
<td>Seasonality</td>
<td>Peak in winter months</td>
<td>Year-round</td>
</tr>
<tr>
<td>Clinical features</td>
<td>Acute onset, duration of 24 to 48 h</td>
<td>Acute onset, indefinite duration</td>
</tr>
<tr>
<td>Viral shedding</td>
<td>20 to 40 days</td>
<td>Weeks to years</td>
</tr>
<tr>
<td>Amount of virus</td>
<td>$10^8$ to $10^9$ genome copies per gram of stool</td>
<td>$10^5$ to $10^8$ genome copies per gram of stool, depending on level of immunosuppressive therapy</td>
</tr>
<tr>
<td>Evolution of virus in host</td>
<td>Small number of stable variants</td>
<td>Markedly diverse variants</td>
</tr>
<tr>
<td>Tissue tropism</td>
<td>Small intestine</td>
<td>Small intestine</td>
</tr>
<tr>
<td>Complication</td>
<td>Dehydration</td>
<td>Dehydration, malnutrition, dysfunction of intestinal barrier</td>
</tr>
</tbody>
</table>

57
1.3.10 Animal models for enteric caliciviruses

Animal models are important for the study of viral pathogenesis, immunology, and vaccine. Considering the enteric nature of TV and the fact that it utilizes HBGAs as cellular receptors, non-human primates and gnotobiotic animals might be good candidates for the development of TV animal models.

1.3.10.1 Non-human primate models for human norovirus

Great efforts have been made to develop animal models for enteric viruses including human noroviruses, hepatitis A and E viruses (Dienstag et al. 1975; Purcell and Emerson 2001; Bok et al. 2011). Several non-human primate species including rhesus macaque, pigtail macaque, and chimpanzee have been challenged with human noroviruses, and the studies have achieved various levels of success.

As early as 1978, the first experimental infection of chimpanzees with Norwalk virus was reported (Wyatt et al. 1978). The chimpanzees were inoculated with fecal filtrate of human origin containing the Norwalk virus of epidemic viral gastroenteritis by stomach tube. None of the chimpanzees developed clinical symptoms including diarrhea or vomiting, however, viral antigens in feces were detected from five of nine animals. In addition, a significant increase in Norwalk virus-specific antibody was found in serum. Subsequent infection was also conducted by feeding four additional chimpanzees with fecal filtrate prepared from one of the animals infected with human norovirus. The results
showed that viral antigen was detected in two of four chimpanzees and all four chimpanzees developed antibody response, demonstrating that viral replication had occurred in chimpanzees despite absence of clinical illness (Wyatt et al. 1978).

Later a sequential passage study of Toronto virus through a newborn pigtail macaque (*Macaca nemestrina*) was conducted (Subekti et al. 2002). The inoculum, a fecal filtrate from a patient during epidemic of viral gastroenteritis containing Toronto virus, was administrated via nasogastric tube to three newborn pigtail macaques. Symptoms characterized by diarrhea, vomiting, and dehydration occurred in all three animals, and viral RNA was detected in stool samples of all inoculated monkeys using RT-PCR and oligonucleotide probe analysis. The sequential infection of two additional newborn macaques using a fecal filtrate prepared from the three previously infected animals demonstrated the presence of viral RNA in stool samples, suggesting that viral replication occurred with association of clinical illness. These results indicate pigtail macaques are susceptible to norovirus infection, and sequential passaging of norovirus through an immunoincompetent newborn pigtail macaque model may allow for the adaptation of human norovirus to primate host (Subekti et al. 2002).

In 2005, an experimental Norwalk virus infection in non-human primates including common marmosets, cotton top tamarins, cynomolgus, and rhesus macaques was reported (Rockx et al. 2005). Rhesus macaques were the only species susceptible to oral Norwalk virus infection, with long time viral shedding and Norwalk virus specific IgM
and IgG detected in one animal. In contrast, based on short-term viral shedding, only a low level of replication may have occurred in common marmosets and cotton top tamarins; viral replication was not detected in cynomolgus macaques. No clinical symptoms or antibody responses were observed in these species. These results suggested that rhesus macaques may be a promising animal model to study the immune response and pathogenesis after norovirus infection (Rockx et al. 2005).

Recently, a chimpanzee model study has been reported using six seronegative chimpanzees were inoculated intravenously with Norwalk virus (Bok et al. 2011). None of the chimpanzees developed clinical illness of gastroenteritis, but the onset and duration of virus shedding in stool and serum antibody response were similar to those observed in humans. Viral RNA was detected in intestinal and liver biopsies concurrent with the detection of viral shedding in stool. In addition, Norwalk virus antigen expression was observed in cells of the small intestinal lamina propria. When rechallenged with human norovirus 4 to 24 months later, two previously infected chimpanzees were resistant to reinfection. Moreover, chimpanzees intramuscularly vaccinated with VLPs derived from GI norovirus were protected from infection when challenged 2 and 18 months after vaccination (Bok et al. 2011). This study demonstrated that VLPs vaccine could induce protective homologous immunity, as well as established chimpanzee as a viable animal model for the study of human norovirus replication and immunity.

1.3.10.2 Non-human primate models for Tulane virus

60
To date the only animal model reported for TV is rhesus macaque, its natural host. Experimental inoculation demonstrated TV caused mild symptoms, viral shedding, immune response, and histologic changes in the rhesus macaques (Sestak et al. 2012). Other nonhuman primates such as chimpanzee and pigtail macaque may be good candidate species for TV although they have not been tested. However, the access to non-human primates is extremely limited, and there are increasingly strict regulations about the use of non-human primates for research. In fact, NIH has now prohibited the use of chimpanzee in experiments. These factors might make it more difficult to study TV.

1.3.10.3 Gnotobiotic animal models

The use of gnotobiotic pigs as an animal model for the study of human norovirus as well as TV has recently been investigated (Duan et al. unpublished observation). Gnotobiotic pigs present a number of important advantages over other animal models for investigating the pathogenesis and immune responses of enteric viruses. First, pigs closely resemble humans in their gastrointestinal anatomy, physiology, and in the development of mucosal immunity (Kim 1975; Yuan et al. 1996). Second, gnotobiotic animals are born devoid of maternal antibodies but are immunocompetent, allowing assessment of primary immune responses (Kim 1975; Mehrazar and Kim 1988). Third, the gnotobiotic status assures that exposure to extraneous enteric pathogens is eliminated as a confounding variable. Lastly, it has been reported that the newborn piglets are susceptible until at least 6 weeks of age to infection and disease with several human enteric viruses including human norovirus and rotavirus (Yuan et al. 1996; Cheetham et
al. 2006). All these features make gnotobiotic pigs a good model to study the pathogenesis and immunity of enteric viruses. In fact, both gnotobiotic pigs and calves have been used for pathogenesis and vaccination studies of human norovirus.

The gnotobiotic pig model was first developed in 2006 for the study of pathogenesis and viral replication of human norovirus (Cheetham et al. 2006). Forty-eight out of fifty-five (85%) animals developed mild diarrhea after oral inoculation with GII.4 human norovirus or pig-passaged intestinal contents. Pigs were positive for viral shedding in rectal swab fluids and intestinal contents by RT-PCR and antigen ELISA from post-inoculation days (PID) 1 to 4. Seroconversion was detected by antibody ELISA in 13 of 22 inoculated pigs after PID 21. Immunofluorescent microscopy revealed sporadic infection of duodenal and jejunal enterocytes with a few stained cells in the ileum in 18 out of 31 human norovirus inoculated pigs. Transmission electron microscopy of intestines from inoculated pigs showed disrupted enterocytes with cytoplasmic membrane vesicles containing calicivirus-like particles of 25 to 40 nm in diameter. However, only one out of seven pigs showed mild histopathologic lesions in the proximal small intestine. Taken together these results suggested that human norovirus replicated in gnotobiotic pigs (Cheetham et al. 2006). A subsequent study demonstrated that the binding pattern of GI/GII human norovirus VLPs to tissues might be associated with A/H HBGAs. By comparing the A/H phenotypes of 65 human norovirus-inoculated gnotobiotic pigs from the previous study, it was found that significantly more A+ and H+
pigs shed virus or had seroconversion than non-A+ and non-H+ pigs (Cheetham et al. 2007).

Study has also found that human norovirus infection induced low levels of antibodies and low numbers of antibody-secreting cells (ASC), both systemically and in the gut mucosa, and 65% seroconversion in gnotobiotic pigs orally-inoculated with GII.4 human norovirus. Moreover, it seemed that there is correlation between clinical illness and seroconversion, as pigs with higher diarrhea scores were more likely to seroconvert and developed higher intestinal IgA and IgG antibody titers (Souza, Cheetham et al. 2007). Later, a human norovirus GII.4 HS66 VLP-based vaccine adjuvanted with immunostimulating complexes (ISCOM) or mutant E. coli LT toxin (mLT) was tested using this model. The gnotobiotic pigs were orally or intranasally vaccinated, and challenged with same strain at day 28 post vaccination. It was found that both vaccines induced high rates of sero- (100%) and copr antibody-conversion (75–100%). Though only 57% of unvaccinated controls shed virus, both vaccines induced increased protection (75-100%) against viral shedding and diarrhea compared to controls. In addition, the VLP+mLT vaccine induced Th1/Th2 serum cytokines and cytokine secreting cells, whereas the VLP+ISCOM vaccine induced Th2 biased responses with significantly increased IgM, IgA and IgG antibody-secreting cells in intestine (Souza, Costantini et al. 2007).
Gnotobiotic calves have also been assessed as an alternative animal model to study the pathogenesis and immune responses to the human norovirus (Souza et al. 2008). After inoculation with human norovirus HS66 strain, all five gnotobiotic calves developed diarrhea and shed virus, with one calf had viremia. Intestinal lesions was observed in the duodenum and jejunum of one of the two examined gnotobiotic calves, with lesions similar to but less severe than caused by bovine enteric caliciviruses. Viral capsid antigen was also detected in the jejunum of the proximal small intestine of one of two calves tested by immunohistochemistry. Of all the inoculated calves, 67% sero- and 100% coproantibody- converted with IgA and/or IgG antibodies specific to human norovirus HS66 strain, though at low titers. Elevated level of antibodies and cytokines were observed in serum, feces, intestine, and intestinal contents of the human norovirus inoculated calves (Souza et al. 2008). These results indicate that gnotobiotic calves also support the replication of human norovirus.

Although these animal experiments gave us very exciting results, the robustness of these models remains to be further tested. In addition, there are limitations for this model. The gnotobiotic animals are expensive and require special facilities and expertise, and there are not many institutions that have such facilities or expertise to maintain these animals.

1.4. Tulane virus as an ideal surrogate for human norovirus
Currently, the study of human norovirus heavily relies on cultivable calicivirus surrogates due to the fact that it cannot replicate in tissue culture. It has proposed that TV is a good surrogate for human norovirus because it is cultivable, and has similar size, morphology, buoyant density, and genomic organization to those of human norovirus. Moreover, TV utilizes HBGAs as cellular receptors and causes enteric disease in its host, making it an improved surrogate for the study of norovirus-host interaction compared to other surrogates (Table 6).

### Table 6. Comparison of human norovirus and several animal calicivirus research surrogates

<table>
<thead>
<tr>
<th>Virus</th>
<th>Genome</th>
<th>Viral Particle (nm)</th>
<th>Cellular Receptor</th>
<th>Growth in tissue culture</th>
<th>Reverse genetic system</th>
<th>Host</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuNoV</td>
<td>7.7 kb</td>
<td>28-35</td>
<td>HBGAs</td>
<td>No</td>
<td>No</td>
<td>Human</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td>MNV</td>
<td>7.3 kb</td>
<td>28-35</td>
<td>sialic Acid</td>
<td>Yes</td>
<td>Yes</td>
<td>Mice</td>
<td>Systemic Disease</td>
</tr>
<tr>
<td>FCV</td>
<td>7.6 kb</td>
<td>28-35</td>
<td>sialic Acid</td>
<td>Yes</td>
<td>Yes</td>
<td>Felines</td>
<td>Respiratory Disease</td>
</tr>
<tr>
<td>TV</td>
<td>6.7 kb</td>
<td>27-40</td>
<td>HBGAs</td>
<td>Yes</td>
<td>Yes</td>
<td>Rhesus macaques</td>
<td>Gastroenteritis</td>
</tr>
</tbody>
</table>
CHAPTER 2
PATHOGENESIS OF TULANE VIRUS IN GNOTOBIOTIC PIGS

2.1. Abstract

Enteric calicivirus infections such as human norovirus (NoV) are the leading cause of acute nonbacterial gastroenteritis worldwide. The pathogenesis of these viruses is poorly understood primarily because many of them cannot be propagated in vitro. Tulane virus (TV), a newly discovered enteric primate calicivirus recovered from feces of rhesus macaques, is cultivable and recognizes human blood group antigens (HBGAs) similar to human NoV. TV is now recognized as an improved surrogate for the study human NoV. In this study, we developed a gnotobiotic pig model to examine the pathogenesis of TV. Twenty-eight newborn gnotobiotic piglets were orally inoculated with 2x10^8 PFU of TV and euthanized at 3, 4, 5, 6, and 10 post-inoculation days (PID). Twelve of the twenty-eight inoculated piglets developed mild diarrhea. Infectious TV was detected in stools on PID 1, reached a peak titer on PID 1-3; and viral RNAs remained detectable through PID 10. Viral RNA was detected in intestinal contents and intestine tissue segments of duodenum, jejunum, and ileum. Piglets developed TV-neutralizing serum antibodies by PID 6. Immunofluorescent staining showed that TV antigens were present in villous tip (epithelial) cells and cells within the lamina propria of the duodenum and jejunum but not
ileum or transverse colon. Histologic examination demonstrated mild villous atrophy, increased numbers of mononuclear inflammatory cells and occasional neutrophils and macrophage/monocytes undergoing necrosis/apoptosis in duodenal and proximal jejunal tissues. These results demonstrate that TV caused mild diarrhea, viral shedding, and intestinal pathologic lesions in gnotobiotic pigs similar to those described for TV in primates and human NoV in humans. Taken together, these results demonstrate that TV infection of gnotobiotic pigs can be used as a novel model to study the replication and pathogenesis of enteric caliciviruses in vivo.

2.2. Introduction

The Caliciviridae is a group of closely related viral genera (Norovirus, Sapovirus, Lagovirus, Vesivirus, Bacovirus, and Recovirus) that infect many different animal species including humans (Farkas et al. 2008; Green et al. 2000). Most of these agents are enteric pathogens whose replication and chief clinical manifestations are gastroenteritis and potentially life-threatening diarrhea. Examples of these viruses include human norovirus (NoV), porcine NoV, bovine BoV, human sapovirus, and porcine sapovirus. Human NoV is the major food-and water-borne virus that accounts for more than 95% of nonbacterial acute gastroenteritis and over 50% of the outbreaks of food-related illnesses in the USA (Li et al. 2012).

Despite the fact that human NoV infections have been known for over 40 years, to date, all attempts at the in vitro cultivation of the human NoVs have been unsuccessful
(Duizer et al. 2004; Straub et al. 2007; Chan, Wong, and Leung 2007). This peculiar feature of this viral group has greatly hindered development of efficacious vaccines and other anti-viral therapies for human NoV-associated gastroenteritis. A few Caliciviridae species have been adapted to tissue culture propagation. Both feline calicivirus (FCV) and murine norovirus (NoV) replicate in vitro, and the general physical characteristics and virus-cell interactions characteristic of the Caliciviridae have been established largely with these two agents. Murine NoV would, at first glance, represent an ideal surrogate research model because it belongs to the genera Norovirus and can be grown in cell culture. However, murine NoV, unlike human NoV is a systemic, not mucosal, surface-restricted viral infection and the host species (chiefly inbred murine strains) experience neither a significant enteric infection phase nor clinically apparent diarrhea. Thus, murine NoV has a different in vivo pathogenesis profile than do the various human NoV genogroups and genotypes (Cannon et al. 2006; Karst et al. 2003; Wobus, Thackray, and Virgin 2006). Moreover, murine NoV does not utilize histo-blood group antigen (HBGA) receptors for cell attachment and entry as does human NoV. In spite of these drawbacks, significant progress in our understanding of the replication, gene expression, and pathogenesis of Caliciviridae infections has been made with the murine NoV model.

In spite of the dramatic genomic diversity among and within the human NoVs, progress in reproducing gastroenteritis and fecal shedding of infectious virions has been achieved by infection of human volunteers (Agus et al. 1973; Dolin et al. 1972; Gallimore et al. 2004; Graham et al. 1994; Kaufman et al. 2003; Kaufman et al. 2005;
Morotti et al. 2004; Chan, Ho, and Sung 2011; Parrino et al. 1977; Tacket et al. 2003), various primate species (Bok et al. 2011; Sestak et al. 2012; Rockx et al. 2005; Subekti et al. 2002; Wyatt et al. 1978) and more recently non-primate species such as gnotobiotic calves and swine (Cheetham et al. 2006; Souza et al. 2008; Souza, Cheetham et al. 2007). The latter (swine), share similar-to-identical HBGA receptors with human/primate HBGAs. In general, these animal models replicate the essential features of human NoV infection in humans in that gastroenteritis is induced, infectious virus and viral RNAs are shed in feces, and sero-conversion after infection is achieved. Attempts to develop subunit vaccines using virus-like particles (VLPs), generated by expression of viral capsid in cell culture with these animal models have also shown limited success (Atmar et al. 2011; LoBue et al. 2006; LoBue et al. 2009; Souza, Costantini et al. 2007; Tan et al. 2011; Ma and Li 2011).

In 2008, a new enteric calicivirus identified as Tulane virus (TV) or monkey calicivirus was isolated in stools of rhesus macaques at the Tulane National Primate Research Center (Farkas et al. 2008). This agent, recently classified as a Recovirus genus within the Caliciviridae family, has biological, physical, and genetic characteristics similar to other members of the Caliciviridae including buoyant density, particle size, morphology, and genome organization. Notably, TV recognizes the type A and B HBGAs similar to human NoV. However, unlike human NoVs, TV replicates to high titers in primate-origin LLC-MK2 cell line(s). Moreover, in vitro passage does not attenuate TV as a recent study in TV-seronegative juvenile rhesus macaques
demonstrated that cell culture-derived TV induced gastroenteritis, and concomitant histologic lesions of viral infection in the upper small intestine, viral shedding and seroconversion (Sestak et al. 2012). Thus genetic/antigenic similarities to human NoV and its robust propagation in cell culture make TV an improved surrogate for human NoV in vivo studies.

Gnotobiotic pigs are germ-free and immunocompetent pigs at birth, which lack maternal antibodies and exposure to microbial antigens (Krakowka and Eaton 1997). Gnotobiotic pigs are excellent models for studying enteric human pathogens because pigs and humans share many similarities in gastrointestinal structure, physiology, and immunology. In addition, the presence of HBGAs in swine makes them particularly suitable for the study of human NoV-mediated disease (Saif et al. 1996; Tan and Jiang 2010). Currently, the gnotobiotic pig has been used as an animal model to study the pathogenesis and immunogenicity of human enteric viruses, such as rotavirus (Yuan et al. 1996) and human NoV (Cheetham et al. 2006). It was found that gnotobiotics, orally inoculated with various wild-type human NoV fecal isolates, develop mild diarrhea and histologic evidence of enteritis, viral shedding and seroconversion (Cheetham et al. 2006; Souza et al. 2008; Souza, Cheetham et al. 2007), providing compelling evidence that human NoV was able to replicate in gnotobiotic pigs (Cheetham et al. 2006). In addition, gnotobiotic pigs have been used as animal models to evaluate the efficacy of human NoV vaccine candidates. Specifically, a virus-like particle (VLP)-based human NoV vaccine
candidate induced norovirus-specific immune responses and protected pigs from viral shedding, diarrhea, and intestinal histologic lesions (Souza, Cheetham et al. 2007).

Currently, the pathogenesis and immunology of enteric caliciviruses is poorly understood. Given the fact that human NoV is not cultivable, understanding these questions require us to establish a proper surrogate virus in a suitable animal model. The objectives of this study are to determine the pathogenesis of TV in gnotobiotic pigs, and to determine if the TV- gnotobiotic pig is a good model for human NoV infection in vivo.

2.3. Materials and methods

2.3.1. Cell culture and virus inoculum preparation

Tulane virus was generously provided by Dr. Xi Jiang, Cincinnati Children’s Hospital Medical Center, and then propagated in cell culture at The Ohio State University. Briefly, the rhesus macaque kidney cell line LLC-MK2 (ATCC, Manassas, VA) were cultured in T150 cell culture flasks (Corning, Tewksbury, MA) to 90% confluence in OPTI-MEM medium (Invitrogen, Carlsbad, CA) supplemented with 2% fetal bovine serum (FBS). After removal of cell culture medium, LLC-MK2 cells were infected with TV at MOI of 0.1, and the flasks were gently agitated every 15 min during incubation at 37°C, 5% carbon dioxide (CO₂). At 1 h post-inoculation, 15.0 ml of OPTI-MEM with 2.0% FBS was added to the cultures, and the flasks were incubated at 37°C, 5% CO₂. TV was harvested from monolayers when extensive cytopathic effects (CPE) were observed, generally between 40-48 h after infection. For this, 15.0 ml of supernatant from each
flask was collected into sterile conical tubes and stored on ice. The infected cells and residual medium left in the flask were subjected to three freeze-thaw cycles (-80°C, room temperature) to release cell-associated virus. The media from multiple flasks were combined, and residual cell debris was removed by centrifugation (3000×g, 30 min) at 4°C. Infectious TV titer was quantitated by plaque assays as previously described (Wei et al. 2008). In this way, a master stock of TV was made and stored at -80°C. Aliquots (10 ml containing 2×10⁸ PFU/ml) were used to orally infect piglets; the same stock inoculum was used in all in vivo studies.

2.3.2. Delivery of gnotobiotic piglets

All animal protocols used in this study have been approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University (IACUC-OSU). Three litters of gnotobiotic piglets were derived from date-mated pregnant sows (n=3) by Caesarian section essentially by procedures described previously (Krakowka and Eaton 1997). Neonatal piglets were transferred into sterile pentub isolation units containing six partitions, an exterior heat source was applied, and the piglets were fed a liquid Parmalat® milk replacement diet per os three times daily (100 to 150ml per feeding). Uninfected controls were maintained in separate isolation units. The average size of litters ranged from 10-16 piglets.
2.3.3. Animal challenge studies

Thirty-three 2-day-old gnotobiotic piglets from three litters were divided into mock-inoculation control (n=5) and TV-infected group (n=28). A 1.0 ml of pre-inoculation blood sample for serum was collected from each piglet by jugular venipuncture. Piglets in the TV-inoculated group were orally inoculated with 10 ml (2x10^8 total plaque-forming units or PFU) of TV; separately-housed controls were inoculated with 10 ml of OPTI-MEM containing 2.0%FBS. After inoculation, the piglets were observed and evaluated daily for weight and body temperature changes, and anorexia. Daily rectal mucosal/fecal swabs were collected from each piglet and diarrhea/fecal consistency score was assigned to each using a subjective scale wherein 0 = normal, 1 = creamy, 2 = pasty and 3 = watery. Pigs with fecal consistency scores of 2 or 3 were scored as diarrhea-positive. Piglets were terminated on PIDs 3 (n=4), 4 (n=9), 5 (n=5), 6 (n=5), and 10 (n=5).

Prior to termination, a blood sample for serum was collected from heavily sedated piglets and each piglet received Euthol® solution intravenously to effect. Intestinal contents from the duodenum, proximal jejunum, ileum, transverse colon and descending colon were collected from each pig. For this, the intestine segment was clipped at both ends and 0.5 ml of OPTI-MEM was injected by a sterile syringe into the intestinal lumen, gently massaged and then 0.5 ml liquid was withdrawn with the same syringe. Adjacent intestinal tissue segments (duodenum, jejunum, ileum, and colon) were collected from each pig for viral detection and histologic examination.
2.3.4. Enumeration of infectious TV by plaque assay

Plaque assays were performed on tissue culture TV, all fecal samples, intestinal contents and intestinal tissue segment homogenates. LLC-MK2 cells were seeded onto 6-well plates with 2ml OPTI-MEM containing 2% FBS and incubated for 12 h, 37°C, 5% CO₂ to 90% confluence. Samples containing suspect virus were diluted 1:10 with OPTI-MEM, homogenized, vortexed for 1 min, and clarified by centrifugation at 5000×g for 10 min. Tissue segments were diluted 1:10 with OPTI-MEM containing 2% (v/v) FBS, homogenized by Douche homogenizer, then clarified by centrifugation at 8000×g for 5 min. Ten-fold serial dilutions of supernatant were made and 400 µl of each was added to each well in duplicate after the removal of cell culture medium. For intestinal contents, samples were vortexed, dispersed by vigorous pipetting, and then used for serial dilutions and subsequent infection. The plates were gently shaken to evenly spread the inoculum every 10 to 15 min. At 1 h post infection, 2.5 ml of overlay consisting of 5% FBS, 1.4% NaHCO₃, 2.5% HEPES, 1% Glutamine, and 0.25% agarose in MEM was added to each well, and plates were incubated at 37°C, 5% CO₂ for 48 hr before fixing with 10% (v/v) formaldehyde in PBS and staining with 0.05% (w/v) crystal violet in 10% (v/v) ethanol.

2.3.5. Quantification of viral genomic RNA by RT-qPCR

The sample processing was the same for fecal samples and intestinal contents as described in plaque assay, while tissue samples were ground in liquid nitrogen, dispersed through 20 gauge needle/syringe for at least 20 times. The serum samples were used
directly for RNA extraction. The total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) as directed by the manufacturer. Two-step reverse transcription (RT) was conducted using primer (5’-TTGCAGGAGGGTTTCAAGATG-3’) targeting on the VP1 region of TV using Superscript III transcriptase kit (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. The RT products were then used to perform real-time PCR using primers and probes specifically targeting the VP1 gene of TV (forward: 5’-TTGCAGGAGGGTTTCAAGATG-3’, reverse: 5’-CACGGTTTCATTGTCCCCATA-3’, probe: 5’-TGATGCACACATGTGGGA-3’, Applied Biosystems, Foster City, CA) on a StepOne Real Time PCR system (Applied Biosystems). A standard plasmid for TV was constructed by inserting the sequence of entire ORF2 (encoding viral protein VP1) into pGEM T-easy vector (Promega, Madison, WI). The plasmid of known concentration was ten-fold serial diluted to generate standard curve for real-time PCR. The amplification cycles were: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, 1 min at 60°C. The threshold for detection of fluorescence above background was set within the exponential phase of the amplification curve. For each assay, 10-fold dilutions of standard plasmid were generated, and negative controls samples and ddH2O were included in each assay.

2.3.6. Virus neutralization assay

Serum samples collected from each pig before sacrifice were tested for the presence of TV-specific virus-neutralizing (VN) antibodies as previously described (Farkas, Dufour et al. 2010). Briefly, serum samples were titrated in five replicate wells of 96-well
plates (Corning, Lowell, MA) to neutralize 100 TCID$_{50}$ of TV. Briefly, 100 TCID$_{50}$ of TV was mixed with serum in OPTI-MEM to achieve 2-fold serial dilution starting from 1:4. The virus/serum mixture was incubated at 37°C for 1h. The 96-well plate was seeded with $10^4$ of LLC-MK2 cells/well one day prior to assay, and 100µl of virus/serum mixture was added to each well. Plates were observed 96 h post infection, by which time all cells in virus control exhibited CPE characterized by cell degeneration and detachment. TV-specific VN antibody titer was determined as the highest dilution which the cell monolayer was at least 50% intact.

2.3.7. Immunofluorescence microscopy

The method used was indirect immunofluorescence on whole intestinal tissue mounts adopted from a previous study (Cheetham et al. 2006). Briefly, fresh intestinal tissues were sectioned into small pieces and then fixed with 4.0% (v/v) paraformaldehyde-0.2% (v/v) glutaraldehyde in 0.1 M potassium phosphate buffer (PPB), pH 7.4, for 2 h, 22°C, washed 4 times with PPB, and quenched with PPB containing 50mM glycine at 4°C overnight. Tissue sections were then permeabilized with 0.1% Triton X-100 in PBS for 1 h at 22°C washed twice with PBS and blocked with PBS containing 2.0% (w/v) bovine serum albumin (BSA) with 5.0% (v/v) normal goat serum at 4°C overnight. Then tissues were incubated in 1:500 diluted rabbit anti-TV antiserum (a generous gift from Dr. Xi Jiang) for 12 h at 4°C in incubation buffer consisting of 10 mM PPB containing 150 mM NaCl, 10 mM sodium azide, and 0.2% (w/v) BSA. After 6 washes with PBS, tissues were incubated in a 1:1,200 dilution of goat anti-rabbit IgG
labeled with AlexaFluor488 (Invitrogen, A11034), in incubation buffer 12 h, 4C. After 3 washes with PBS, samples were counterstained with the nuclear stain SYTOX (Invitrogen, S11368), and actin stain AlexaFluor633-labeled phalloidin (Invitrogen, A222884). The samples were then trimmed into small 3×2 mm pieces and mounted onto slides, and were examined using an Olympus Spectral confocal microscope system at The Ohio State University.

2.3.8. **Histological examination**

In addition to intestine segments from each pig, portions of kidney, spleen and liver were also collected at necropsy. Tissues were fixed in 10% (v/v) phosphate-buffered formalin for 24-36 h, dehydrated in a graded ethanol series, embedded in paraffin, cut in 5-micrometer sections, and collected on glass slides. The sections were de-paraffinized, rehydrated and then stained with hematoxylin and eosin. Slides were examined by conventional light microscopy.

2.3.9. **Statistical analysis**

Statistical analysis of one-way ANOVA was performed by using Minitab statistical analysis software (Minitab, Inc., State College, PA), a P-value of 0.05 was considered statistically significant.

2.4. **Results**

2.4.1. **TV infection caused mild diarrhea in gnotobiotic pigs**
Mild (score = 1) to moderate (score = 2) diarrhea was observed in 12 of 28 TV-inoculated piglets (Table 7). Diarrhea commenced on PID 3 as was most prominent on PIDs 4 and 5. In some piglets the symptom persisted to PID 10. All five controls remained diarrhea-negative at any time. No differences in weight, body temperature, and anorexia were observed between TV-inoculated and control piglets.

Table 7. Diarrhea, fecal virus shedding and viral RNA detection in TV-infected gnotobiotic pigs

<table>
<thead>
<tr>
<th></th>
<th>PID 3</th>
<th>PID 4</th>
<th>PID 5</th>
<th>PID6</th>
<th>PID10</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrhea&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4/9</td>
<td>3/5</td>
<td>2/5</td>
<td>3/5</td>
<td>12/28</td>
</tr>
<tr>
<td>Fecal viral shedding&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2/4</td>
<td>5/9</td>
<td>3/5</td>
<td>3/5</td>
<td>4/5</td>
<td>17/28</td>
</tr>
<tr>
<td>Fecal viral RNA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3/4</td>
<td>7/9</td>
<td>5/5</td>
<td>4/5</td>
<td>5/5</td>
<td>24/28</td>
</tr>
</tbody>
</table>

<sup>a</sup> Diarrhea/fecal consistency score was assigned to each pig using a subjective scale where 0 = normal, 1 = creamy, 2 = pasty and 3 = watery. Pigs with fecal consistency scores of 2 or 3 were scored as diarrhea-positive.

<sup>b</sup> Number infected (numerator) divided by number in group (denominator)

<sup>c</sup> Infectious viral particles detected by plaque assay

<sup>d</sup> Viral RNAs detected by RT-qPCR
2.4.2. Fecal shedding of infectious TV was observed in inoculated pigs

The results of fecal viral shedding by PID number are summarized in Table 8 and Figure 16. Fecal infectious virus excretion was detected on PIDs 1 through 5. Seventeen out of 28 (60.7%) TV-inoculated pigs shed infectious virus in feces up to 5 days after challenge. The average duration of viral shedding was two days (range 1-5 days). The detected mean infectious viral titer in feces declined from about $10^5$ PFU/g fecal matter (PID 1) to $10^3$ PFU/g fecal matter (PID 5), and was undetectable by plaque assay after PID 5. No infectious virus was detected in mock infected controls.

![Figure 16. Quantification of infectious TV in fecal samples by plaque assay.](image)

Fecal samples were collected from all piglets in each group daily. Samples were diluted in MEM, homogenized, and clarified by centrifugation. The presence of infectious virus
particles was detected by plaque assay. Data are expressed as the average viral titer ± 1 standard deviation.

Table 8. Duration of diarrhea, fecal virus shedding and viral RNA detection in TV-infected gnotobiotic pigs

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of total pigs</th>
<th>Pigs with diarrhea (%) a,b</th>
<th>Mean onset of diarrhea (PID)c</th>
<th>Fecal viral shedding (%) b</th>
<th>Mean onset of virus shedding (PID)d</th>
<th>Mean virus shedding duration (days)e</th>
<th>Fecal viral RNA detection (%) b</th>
<th>Mean viral RNA shedding duration (days)f</th>
</tr>
</thead>
<tbody>
<tr>
<td>PID 3</td>
<td>4</td>
<td>0/4 (0)</td>
<td>0</td>
<td>2/4 (50)</td>
<td>1</td>
<td>1</td>
<td>3/4 (75)</td>
<td>3</td>
</tr>
<tr>
<td>PID 4</td>
<td>9</td>
<td>4/9 (44)</td>
<td>4</td>
<td>5/9 (55)</td>
<td>1</td>
<td>1.67</td>
<td>7/9 (78)</td>
<td>4</td>
</tr>
<tr>
<td>PID 5</td>
<td>5</td>
<td>3/5 (60)</td>
<td>4</td>
<td>3/5 (60)</td>
<td>1</td>
<td>1.5</td>
<td>5/5 (100)</td>
<td>5</td>
</tr>
<tr>
<td>PID 6</td>
<td>5</td>
<td>2/5 (40)</td>
<td>4</td>
<td>3/5 (60)</td>
<td>1</td>
<td>1</td>
<td>4/5 (80)</td>
<td>6</td>
</tr>
<tr>
<td>PID 10</td>
<td>5</td>
<td>3/5 (60)</td>
<td>5</td>
<td>4/5 (80)</td>
<td>1</td>
<td>3</td>
<td>5/5 (100)</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>12/28 (43)</td>
<td>4/25</td>
<td>17/28 (61)</td>
<td>1</td>
<td>1.73</td>
<td>24/28 (86)</td>
<td>5.6</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>0/5 (0)</td>
<td>0</td>
<td>0/5 (0)</td>
<td>0</td>
<td>0</td>
<td>0/5 (0)</td>
<td>0</td>
</tr>
</tbody>
</table>

a Diarrhea/fecal consistency score was assigned to each pig using a subjective scale where 0 = normal, 1 = creamy, 2 = pasty and 3 = watery. Pigs with fecal consistency scores of 2 or 3 were scored as diarrhea-positive.

b Indicates number of positive samples in total samples

c The average of day on which diarrhea was first observed. Average based on all TV-positive inoculated pigs in this group

d The average of day on which infectious virus was first detected in fecal samples.

e The average time during which infectious virus was detected in fecal samples.

f The average time during which viral RNA was detected in fecal samples
2.4.3. TV RNA in feces

We next determined whether viral RNA was present in feces using RT-qPCR. As shown in Figure 17A, TV-RNA was detected in feces in 24 out of 28 TV-infected pigs from PID 1 through 10; the average TV-RNA level during this interval was $10^5$ genomic RNA copies/g of fecal matter. Interestingly, even after infectious virus could no longer be detected by plaque assays (PID 5), levels of TV-RNAs comparable to those seen on PIDs 1-4 were documented ($P > 0.05$). Since RT-qPCR assay cannot discriminate infectious and noninfectious virus particles, we suspected that high levels of RNA copies detected may be from unencapsidated viral TV-RNA(s) and whole or fragmented free viral TV-RNAs in fecal samples. To address this possibility, fecal extract samples of 11 TV-infected piglets from the same litter from PID 1 to PID 3 were treated with DNase-free RNase for 1 h followed by RNA extraction and RT-qPCR. Within these 11 piglets, 6, 8, and 4 of them had infectious virus shedding at PID 1, 2, and 3 respectively (Figure 17B). However, 9, 10, and 11 pigs were TV RNA positive at PIDs 1, 2, and 3 respectively (Figure 17C). This demonstrated that some pigs were negative for infectious virus but were positive for viral RNA. As shown in Figure 17C, there was no significant difference in RNA copies in RNase-treated and untreated samples ($P > 0.05$), suggesting that there is little naked TV-RNA presented in fecal samples. No TV-RNAs were detected in control piglet feces at any time interval tested.
Figure 17. Quantification of TV RNA in fecal samples by RT-qPCR.
(A) RNA copies in fecal samples. Fecal samples were collected from all piglets in each group daily. Samples were diluted in MEM, homogenized, and clarified by centrifugation. The total RNA was extracted from fecal samples using an RNeasy Mini Kit. The viral genomic RNA was quantified by real-time RT-PCR by targeting viral VP1 gene. (B) Infectious virus in fecal samples. Fecal samples from 11 piglets collected PID1 to PID3 were processed and then quantified by plaque assay. (C) The effect of RNase treatment on TV RNA copies in fecal samples. Fecal samples from 11 piglets collected PID1 to PID3 were processed and then treated with DNase-free RNase for 1 h, followed by RNA extraction and real-time PCR. Data are expressed as the average RNA copies ± 1 standard deviation.

2.4.4. Infectious TV in intestinal contents and tissues

For intestinal contents (Figure 18), only samples taken on PID 4 and 5 contained infectious TV. In these positive samples, an average viral titer of only $10^2$ PFU/g of intestinal content was detected; intestinal contents from all three piglets collected on PID 5 were positive for infectious TV. All samples taken from pigs sacrificed on PIDs 3, 6 and 10 as well as all colon samples were negative for infectious TV. Regarding the intestinal tissue itself, only one sample (ileum of one TV-inoculated pig sacrificed on PID 3) tested positive by plaque assay; all others were negative. No infectious virus was detected in mock infected controls.
Figure 18. Quantification of infectious TV in the intestinal contents by plaque assay.

Intestinal contents were collected from different sections of small intestine when piglets were sacrificed on different PIDs. Approximately 2cm of intestine segment was clipped at both ends, 0.5 ml of OPTI-MEM was injected by a sterile syringe into the intestinal lumen, gently massaged and then all the liquid was withdrawn with the same syringe. Samples were homogenized, and infectious virus particles were detected by plaque assay. Data are expressed as the average virus titer ± 1 standard deviation.
2.4.5. TV viral RNA was detected in intestinal contents and tissues

The real-time RT-PCR results for TV-RNAs in intestinal contents and tissues are shown in Figure 19 and 20, respectively. Most small intestinal lumen samples (18/28 for duodenum, 19/28 for jejunum, and 27/28 for ileum) tested positive for TV-RNAs (Figure 19). Intestinal contents collected from the ileum had significant higher (P<0.05) viral RNA levels \(10^4\) TV RNA copies/g of sample) than those detected in the duodenal and jejunal contents (an average of less than \(10^3\) TV-RNA copies/g sample). RT-qPCR identified TV-RNAs in some (12/28) ileum tissues and a portion (13/28) of the jejunal tissues, and only a few of the (5/28) duodenal tissues (Figure 20). TV RNA levels in duodenal and jejunal samples averaged \(10^4\) TV-RNA copies/g of tissue, whereas ileum tissues were higher (an average of \(10^5\) TV RNA copies/g tissue). For both intestinal tissues and intestinal contents, there was no significant difference (P>0.05) between TV-RNA levels in piglets sacrificed on different PIDs. No TV-RNAs were detected in mock infected controls.
Figure 19. Quantification of TV RNA in intestinal contents by real time RT-PCR.

Total RNA was extracted from intestinal contents from Figure 18. The TV genomic RNA copies were quantified by real-time RT-PCR. Data are expressed as the average RNA copies ± 1 standard deviation.
Figure 20. Quantification of TV RNA in intestinal tissues by real time RT-PCR.

Total RNA was extracted from intestinal tissues collected from TV-inoculated piglets on different PIDS. The TV genomic RNA copies were quantified by real-time RT-PCR. Data are expressed as the average RNA copies ± 1 standard deviation.

2.4.6. Virus-neutralizing antibody in serum

TV virus-neutralizing antibodies were detected in serum samples of piglets sacrificed on PID 6 and later (Figure 21). Three of five serum samples from piglets sacrificed on PID 6 showed TV-specific neutralizing assay with the average titer of $2^{4.7}$ GMT. For piglets sacrificed on PID 10, sera of all five piglets could protect cells from
TV at highest dilution of 1:128. The average titer at PID 10 was $2^{7.6}$ GMT. These data demonstrated that production of virus-neutralizing antibody started as early as PID 6 and the level of antibody increased with longer time post inoculation.

![Figure 21. Detection of neutralizing antibodies from TV-infected pigs.](image)

Serum samples collected from piglets before sacrifice were mixed with 100 TCID$_{50}$ of TV to achieve 2-fold serial dilution starting from 1:4, and the mixture was incubated at 37 °C for 1h. The inactivated virus serum mixture was then titrated in five replicate wells on 96-well plated seeded with LLC-MK2 cells and observed at 96h PI. TV neutralizing antibody titer was determined as the concentration which can protect 50% of the cell monolayer. Data are expressed as the average antibody titer ± 1 standard deviation.
2.4.7. Gross pathologic findings

No significant gross pathologic change was found in pig intestines and other organs throughout PID 4-10. However, in some TV-infected pigs, excess fluid and gas was found in small intestine (particularly in jejunum section), and “paste-like” matter was found in the cecum (Figure 22).
Figure 22. Gross pathological changes in intestine.

The gross pathological changes of intestine in gnotobiotic piglets inoculated with TV and mock inoculated control. (A) and (C) The jejunum of TV-inoculated pig at PID6 contains excess fluid and gas. (B) The excess gas and paste-like consistency of the feces in the cecum of TV-inoculated pig was observed at PID6. (D) Normal consistency of intestinal content of small intestine and cecum in mock-inoculated control at PID 10.
2.4.8. **Histologic findings**

Histologic examination of intestinal sections demonstrated mild villous atrophy (shortening and blunting of villi), increased numbers of mononuclear inflammatory cells and occasional neutrophils and macrophage/monocytes undergoing necrosis/apoptosis were present in duodenal and proximal jejunal tissues of TV-infected but not control gnotobiotic piglets (Figure 23 and 24). In some TV-infected pigs, “gland abscesses” were found in the glandular lumen of duodenum. There was loss (necrosis) of villous tip epithelia with condensation of the lamina propria in that area. Adjacent intestinal epithelial cells have migrated to cover the necrosis/defect. Occasional squamoid changes in villous tip lining epithelia, presumably secondary to prior epithelial cell necrosis/apoptosis and loss were also seen (Figure 23).
Figure 23. Histologic changes in duodenal tissues.

(A) and (D), Mock-inoculated control piglet at PID 3. (B) TV-inoculated piglet at PID 4. Changes of villi in the proximal duodenum was observed, which results in the shortening and blunting of duodenal villi, termed villous atrophy. (C) TV-inoculated piglet at PID 6 showed villous blunting and villous atrophy. (E) TV-inoculated piglet on PID 6. Necrosis
of villous tip epithelium with condensation of the lamina propria beneath the area of epithelial loss was observed. Also, adjacent intestinal epithelial cells have assumed a flattened of squamoid appearance as a response to this injury. (F) TV-inoculated piglet at PID 4 with gland abscess in the glandular lumen. (G) TV-inoculated piglets on PID 10. The circled area on top was suspected to be intestinal epithelial cells that have but not yet fully developed from cells that covered a focal area of epithelial loss from infection. (H) TV-inoculated piglets on PID 6. Necrosis was observed in the villous tip epithelia. Adjacent intestinal epithelial cells are beginning to migrate laterally to cover the defect. (I) TV-inoculated piglets on PID 4. Villous atrophy, increased numbers of inflammatory cells in the lamina propria with single cell necrosis were observed in blunted villous tips.
Figure 24. Histologic changes in jejunal tissues.

(A) and (D), Mock-inoculated control piglet at PID 3. (B) TV-inoculated piglet at PID 4 showed villous atrophy with shortening and blunting of villi. (C) TV-inoculated piglet at PID 4 showed necrosis of inflammatory cells in the lamina propria. (E) TV-inoculated piglet at PID 4 showed necrosis of inflammatory cells in the lamina propria. (F) TV-inoculated piglet at PID 4 with mononuclear inflammatory cells exiting in the vasculature of the villous tip.

As shown in Table 9, histologic changes were found in most duodenum and jejenum sections, but not in ileum and colon samples. Ten pigs demonstrated histological
changes in both duodenum and jejunum, whereas five other pigs only showed histological changes in the duodenum. In all, mild, multi-focally distributed lesions were present in intestinal segments from 15 of 23 TV inoculated piglets from PID 4 or later.

Table 9. Summary of histologic changes in intestinal tissue

<table>
<thead>
<tr>
<th>PID</th>
<th>Duodenum*</th>
<th>Jejunum*</th>
<th>Ileum*</th>
<th>Colon*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7/9</td>
<td>5/9</td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3/5</td>
<td>2/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3/5</td>
<td>2/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2/5</td>
<td>1/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td><strong>Total</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td><strong>15/23</strong></td>
<td><strong>10/23</strong></td>
<td><strong>0/23</strong></td>
<td><strong>0/23</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup> Indicates number of samples with histologic changes in total of intestinal samples

<sup>a</sup> Minimal histological changes including lesions, villous tip edema, mononuclear infiltrate, and villous atrophy were observed on PIDs 4 and 10

<sup>b</sup> Mild histological changes including mixed mononuclear cell infiltrates in villous tips, mild inflammatory edema of the villous tip lamina propria and necrosis of villous tips were observed in PIDs 5 and 6.

<sup>c</sup> The 10 pigs that had histological changes in the jejunum also had histological changes in duodenum. Five pigs demonstrated changes in the duodenum but not jejunum.
2.4.9. Detection of TV antigen-positive cells in the intestine by IFA

Confocal fluorescent microscopy was applied to visually confirm that TV infects cells of the small intestine. The whole-mount tissue IFA was performed on fresh tissues from pigs at PIDs 4, 5, 6, and 10 using TV polyclonal antibody. As summarized in Table 4, most duodenum and jejunum tissues from TV-infected pigs were IFA positive. However, IFA signal was not detected in the ileum or transverse colon. As shown in Figure 25, positively stained cells (green) were detected as discrete aggregates of signal in villous tips and immediately adjacent sides of individual villi of the duodenum and jejunum (Figure 25). As confirmed by sectional observation, TV antigen was detected at different sites within infected cells, either as peri-nuclear aggregates or diffusely distributed in the cytoplasm. Co-localization in cell nuclei was not observed. While some of the cells were epithelial-origin enterocytes, the majority of the TV antigen-positive cells were found in the lamina propria. These cells remain to be identified. No cells were stained positive in any tissue segments from controls.
Figure 25. Detection of TV antigen in intestinal tissues by indirect immunofluorescent assay (IFA)
Fresh intestinal tissues were sectioned into small pieces, fixed, quenched, and permeabilized. The tissues were incubated with TV-specific polyclonal antibody, followed by incubation with goat anti-rabbit IgG Alexa488. Nuclei and actin were counterstained with SYTOX orange and phallotoxin Alexa633. The stained tissues were mounted onto slides and examined using Olympus FV1000 Confocal Microscopy Image system.

<table>
<thead>
<tr>
<th>PID</th>
<th>Duodenum*</th>
<th>Jejunum*</th>
<th>Ileum*</th>
<th>Colon*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>5/6</td>
<td>5/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>5</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
<td>0/6</td>
</tr>
<tr>
<td>6</td>
<td>2/3</td>
<td>2/3</td>
<td>0/3</td>
<td>ND*</td>
</tr>
<tr>
<td>10</td>
<td>1/3</td>
<td>2/3</td>
<td>ND*</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* Indicates number of IFA-positive samples in total of intestinal samples.

a ND: not done.

2.5. Discussion

Enteric caliciviruses are the leading causative agents for nonbacterial gastroenteritis. However, research on the most important enteric calicivirus, human NoV, has been
severely hampered due to the fact that it cannot be grown in cell culture and lacks a suitable small animal model. Therefore, there is an urgent need to develop an in vivo model using a proper surrogate virus that is genetically and clinically similar to human NoV. In this study, we determined the pathogenesis of TV, a primate calicivirus, in gnotobiotic piglets. We found that TV caused diarrhea, viral shedding, and intestinal lesions in gnotobiotic pigs similar to those described for TV in primates and human NoV in humans (Sestak et al. 2012; Agus et al. 1973; Atmar et al. 2008; Dolin et al. 1975; Schreiber, Blacklow, and Trier 1974).

Increasing evidence suggests that TV is an improved surrogate for human NoV. TV closely resembles human NoV in aspects of viral structure (nonenveloped icosohedral morphology), size (27-30 nm), genome organization (6.7-7.7 kb), environmental stability (resistance to acid and heat), clinical symptoms (enteric infection), and receptor binding (HBGAs). To date, TV is the only member in the genus Recovirus within family Caliciviridae. Serologic surveys of captive primates indicate high incidence of subclinical TV infection in primates (Farkas, Dufour et al. 2010). More recently, a recovirus, the most closely related calicivirus to human NoV, was found to cause symptoms identical to HuNoV infection in humans (Sestak et al. 2012). Tissue culture-adapted TV was inoculated into three seronegative juvenile rhesus macaques to assess the pathogenecity of TV in the species of origin (Sestak et al. 2012). Two of three macaques developed diarrhea and pyrexia by PID 2 and all three shed virus in feces for more than a week. All TV-infected rhesus macaques seroconverted by PID 7. Endoscopic duodenal
biopsies were performed on PID 3 and lymphocytic infiltration of lamina propria and villous blunting were seen but changes attributable to virus infection of intestinal epithelia were not present; TV antigen-positive cells were detected in the lamina propria but not in epithelial. Our experiments sought to replicate these results using gnotobiotic swine instead of captive primates.

Gnotobiotic swine is an ideal model to study the pathogenesis and immunology of enteric human viruses. Previously, we (Ma et al, 2012, unpublished data) and others (Cheetham et al. 2006) have shown that gnotobiotic piglets are susceptible to oral infection with several different strains of nonculturable human NoVs (Cheetham et al. 2006; Souza, Cheetham et al. 2007; Souza, Costantini et al. 2007). Inoculated piglets develop a mild and self-limiting infection after being orally challenged with these agents. Though less is known about the expression and variations of HBGA-like antigens in swine tissues, what is clear is that swine share many HBGA antigenic epitopes with human-origin HBGAs and it is likely that this unusual feature of pigs permits infection by the norovirus species. The primary value of TV versus other human NoVs is its cultivability and hence the opportunity to obtain almost unlimited quantities of infectious virus for detailed in vivo and in vitro studies.

In this study, mild diarrhea was observed in most TV-inoculated pigs, while the control pigs from the same litters remains asymptomatic throughout the observation interval. Diarrhea did not occur in all infected piglets, was not life threatening nor was it
accompanied by pyrexia. Regarding the latter, we have observed similar lack of febrile responses of gnotobiotes in both experimental bacterial infections in pig (Eaton et al. 1991), various viral infections including porcine circovirus 2 (PCV2) (Krakowka et al. 2001) and porcine rotavirus infection (Krakowka, unpublished data, 2008). Viable infectious (plaque-forming) virus was detected in feces on PIDs 1 (17 of 28), 2 (17 of 28), 3 (10 of 28), 4 (4 of 24), and 5 (5 of 15). Peak viral titer (10^5 PFU/g) occurred on PID 1. Average viral titer on PIDs 2 and 3 were slightly greater than 10^3 PFU/g feces. Intestinal contents collected from PIDs 4 and 5 contained low levels of infectious virus. Given the high stability of this group of viral agents it is probable that some portion of infectious virus recovered 24 h after challenge was derived from the oral inoculum as “pass through” virus. Although post-infection fecal viral titers never exceeded that of the oral inoculum (2x10^8 PFU), the total volume of fecal output over the first 4-5 days after infection indicates that recovered virus represents newly synthesized virus within infected piglets. Confocal microscopy performed on tissue sections identified TV antigen-positive cells, chiefly in the lamina propria of the duodenum and proximal jejunum. This provided strong evidence that TV gene expression occurred in pig intestines. Importantly, TV caused significant histologic lesions in duodenal and proximal jejunal tissues including mild villous atrophy, increased numbers of mononuclear inflammatory cells and occasional neutrophils and macrophage/monocytes undergoing necrosis/apoptosis. Finally, TV-infected piglets developed TV-specific neutralizing antibodies. Taken together, these data support the hypothesis that gnotobiotic swine support TV replication in the proximal small intestine.
The RT-qPCR assay for viral RNAs is routinely used for human NoV diagnostic purposes, in epidemiologic investigations and to monitor virus shedding in the feces of both human NoV-infected humans and in various surrogate animal models of NoV infection. In this study, we found that high levels of viral RNA were detected by RT-qPCR at every PID interval from 1 through 10 despite the fact that infectious particles were undetectable by plaque assay after PID 5. Moreover, viral RNAs were routinely detected in the ileum, even though infectious virus was not recovered from this site nor were virus-infected cells detected by IFA in sections of ileum. It thus appears that the assay can detect viral RNAs (or viral RNA fragments) in samples that are apparently devoid of infectious virus. One explanation for this apparent lack of concordance between assays is the presence of inhibitors in sample homogenates that may reduce the efficacy of the viral plaque assay. Another possibility is that some virus particles were neutralized by TV-specific IgA in GI tract which cannot be detected by plaque assay. Alternatively, it may be that RT-qPCR overestimates the actual amount of infectious virus in samples as it represents the amplified sum of both encapsidated viral RNAs and noninfectious RNA fragments present in the samples. However, RNase treatments suggest that there was little naked TV-RNA presented in feces. Inhibitors in samples can be overcome by sample dilution but this manipulation may yield false negative results. Another variable is sampling bias or artifact. Histologic evaluation of the sampled intestine repeatedly emphasized the multifocal nature of the inflammatory changes attributable to virus infection. Thus, a single sample, even of small and anatomically
defined ileum and duodenal sections of the intestine, may not be representative of the entire segment of bowel. If this is the case, then increasing the number of samples per site and/or increasing the number of piglets per PID interval would improve the chances of detecting infectious virus in various tissues. This approach should be further evaluated. Regards the most complete assessment of viral infection is best accomplished by using all three methods of viral detection.

Based upon these data, the pathogenesis of TV infection in gnotobiotic pigs can be constructed. After oral inoculation, infectious virus attaches and replicates in the cells of the duodenum and proximal jejunum. While the specific cell types which support viral replication in pigs has not yet been identified, it is clear that viral RNA replication and protein translation occurs duodenum and jejunum and assembled viral particles are released into the intestinal lumen resulting in detection of infectious virus and viral RNAs in the intestinal contents and feces. Histologic changes in the proximal small intestine develop and mild diarrhea ensues. The infectious period arbitrarily identified by recovered infectious virus lasts 2-3 days and thereafter is limited by unidentified innate immune mechanisms and later by the adaptive immune response. The duration of effective antiviral immunity could not be determined with the experimental design used nor could the possibility of intermittent shedding of infectious virus be precisely determined.
In summary, the great advantage of the described TV-gnotobiotic pig model of human NoV infections is that TV can be readily replicated \textit{in vitro}. This system is easily manipulated (repeated infection and co-infection with human NoV for example) and the gnotobiotic conditions used assure ease of replication of the data. With pigs, the HBGA type(s) can be individually determined and correlated to disease susceptibility. Further, serial \textit{in vitro} passage of virus in porcine cell lines may increase or alter expression of disease. Finally, novel immune therapies such as vaccinations with VLPs or treatment with alpha interferon can be adequately tested for anti-replicative or anti-diarrheal effects in this model.
CHAPTER 3
ADAPTION OF TULANE VIRUS TO PIG MACROPHAGE CELLS AND PASSAGE OF TULANE VIRUS IN GNOTOBIOTIC PIGLETS

3.1. Abstract

The immunopathogenesis of enteric caliciviruses is poorly understood. In humans, naturally occurring human NoV infection fails to induce effective long-term adaptive immunity for protection against repeated infections. Previous studies using murine norovirus (MNV) model system found that MNV has tropism for macrophages and dendritic cells which leads to impairment of the host innate and adaptive immune responses in mice. In this study, we attempted to adapt Tulane virus (TV) to pig macrophage cell line (3D/4), and to pass TV in vivo using the gnotobiotic pig as a model. It was found that TV replicates efficiently in pig macrophages although a slight decrease in TV titer was observed from passages 1 to 5. To determine whether macrophage cell-adapted TV had altered pathogenesis in vivo, $10^7$ PFU was inoculated into newborn gnotobiotic piglets. It was found that macrophage cell-adapted TV caused low levels of virus and RNA shedding in feces but did not cause any histologic changes or TV-specific antigen expression in the intestine. Fecal extracts (containing $10^4$ PFU of TV) from TV-infected piglets were used for oral inoculation of naive piglets. It was found that piglets...
shed $10^{2.5}$ PFU/g of infectious TV in feces for at least 4 days without detectable histologic changes and TV antigens in the intestine, suggesting that the virus shed in feces in the second passage exceeded the level of the inoculation dose. Taken together, we successfully demonstrated that TV, the only cultivable primate calicivirus, has tropism for macrophages. In addition, *in vivo* passage of TV may lead to the adaption of TV to piglets.

### 3.2. Introduction

Human norovirus (NoV), an enteric calicivirus, is responsible for more than 95% of nonbacterial acute gastroenteritis. The immunopathogenesis of human NoV is poorly understood due to the fact that it lacks an appropriate cell culture and animal model. Interestingly, naturally occurring human NoV infection fails to induce both early antiviral innate immune responses and effective long-term adaptive immunity for protection against repeated infections. To date, our understanding of the biology and pathogenesis of caliciviruses is largely shaped by the discovery of murine norovirus (MNV). *In vitro*, it was found that MNV has a tropism for cells of hematopoietic lineage, specifically, macrophages and dendritic cells. *In vivo*, it was found that MNV causes systemic infection in immunocompromised mice lacking the recombination-activating gene 2 (RAG2) and signal transducer and activator of transcription 1 (STAT-1) (RAG2/STAT1−/−). In addition, *in vivo* studies demonstrated that both innate and adaptive immune responses were critical in control of MNV infection and viral clearance from the intestine and other tissues (Wobus et al. 2004; Wobus, Thackray, and Virgin...
These observations in MNV, coupled with the fact that there is no long-term immunity induced by naturally occurring human NoV infection, have led to the hypothesis that human NoV and other enteric caliciviruses may also impair the function of macrophages and dendritic cells. The impairment of these cells can result in a less effective innate anti-viral immunity as well as the induction of an aberrant adaptive immune response. However, there is a lack of direct evidence to support this hypothesis.

Although MNV serves as a useful model to understand the pathogenesis of caliciviruses, whether the results obtained from MNV studies can be directly applied to human NoV remains highly debated. MNV differs from human NoV in clinical manifestations (systemic infection vs diarrhea/vomiting) and host receptors (sialic acid vs histo-blood group antigens). Tulane virus (TV), also known as primate calicivirus or rhesus enteric calicivirus, was discovered in the stool samples of rhesus macaques in 2008 (Farkas et al. 2008). This virus was recently classified into a new genus, named Recovirus, within the Caliciviridae family. TV shares many physical and biological characteristics such as morphology, virion structure, and genome organization, with human norovirus (NoV). In addition, TV utilizes histo-blood group antigens (HBGAs) as cellular receptors for viral attachment, as human NoV does. However, unlike human NoVs, TV replicates to high titers in primate-origin LLC-MK2 cell lines. A recent study demonstrated that cell culture-derived TV induced gastroenteritis, and concomitant histologic lesions of viral infection in the upper small intestine, viral shedding, and
seroconversion in juvenile rhesus macaques (Sestak et al. 2012). Thus, TV is potentially an improved surrogate to study the pathogenesis and immunology of human NoV.

In Chapter 2, we have demonstrated that tissue culture-adapted TV induced mild diarrhea, accompanied by viral and RNA shedding in feces, seroconversion and antibody production, and histologic lesions and viral antigen expression in small intestine. These results indicated that TV can cause enteric infection in gnotobiotic piglets similar to those described for TV in primates and human NoV in humans.

The objectives of Chapter 3 are to: (i) determine whether TV can adapt to macrophages as MNV does; (ii) determine whether macrophage cell-adapted TV can alter the virulence of TV in pigs; and (iii) determine whether TV can be repeated passed in vivo in gnotobiotic pigs.

3.3. Material and methods

3.3.1. Cell cultured virus stock preparation

The Tulane virus was generously provided by Dr. Xi Jiang, Cincinnati Children’s Hospital Medical Center. TV was propagated in LLC-MK2 cells at The Ohio State University. The original TV master stock was prepared as previously described in Chapter 2.

3.3.2. Adaption of TV in pig macrophage cells
Another TV stock, TV P5, was prepared by serial passing TV five times in pig macrophage cells (3D/4 cell line, kindly provided by Susan Ringler in Dr. Krakowka’s group). Briefly, the 3D/4 cells were cultured in T150 cell culture flasks (Corning, Tewksbury, MA) to 90% confluence in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10.0% v/v fetal bovine serum (FBS). LLC-MK2 cultured TV was used for the first infection using the MOI of 1 and same procedures as TV culture in LLC-MK2 cells. At 48 hr post infection, the virus, designated TV P1, first passage in 3D/4 cells, was harvested using the same procedure as previously described. TV P2 was then prepared using TV P1 as inoculum, TV P3 using TV P2, etc., until the fifth passage TV P5 was obtained. All virus infection and harvest procedures were performed as previously described, and the viral titer was enumerated using plaque assay. Aliquots of TV P5 (10 ml containing roughly 10⁷ PFU/ml) were used to orally infect piglets; the same virus stock inoculum was used for all in vivo studies.

3.3.3. Inoculum preparation using animal samples

For in vivo serial passage of TV, stool samples that were positive for TV were processed and used as inoculum. Briefly, fecal samples collected on each day post inoculation was diluted with one volume of OPTI-MEM, homogenized, and centrifuged at 3,000g for 30 minutes. The supernatant was filtered using 0.8um and then 0.22um filters. The filtrate was then titrated by plaque assay and RT-qPCR. Due to the small volume of sample, some of the TV-positive samples were combined and thoroughly mixed to make the final inoculum. Aliquots were made according to viral titer/viral RNA
level, and aliquots from the same inoculum stock were used for the piglets in the same group.

### 3.3.4. Animal challenge studies

All animal protocols used in this study have been approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University (ILACUC-OSU). Gnotobiotic pigs were Caesarian-derived from date mated pregnant sows (n=2) and maintained as gnotobiotes on a liquid Parmalat\textsuperscript{R} milk replacement diet as previously described (Krakowka and Eaton 1997). Eleven 2-day-old gnotobiotic piglets from two litters were divided into control (n=1), and TV P5 (n=4), TV P0 (n=3), and TV P1 (n=3). Piglets in the TV P5 group were sacrificed on day 3 post inoculation (PID 3) whereas piglets in the latter two groups and the control were sacrificed on PID 4. A 1.0 ml pre-inoculation blood sample for serum testing was collected from each piglet by jugular venipuncture. Piglets in the infected groups were orally inoculated with 10.0 ml of cell cultured virus or small intestinal or fecal filtrate; separately-housed controls were inoculated with 10.0 ml of OPTI-MEM with 2.0% FBS, which is the medium used for virus culture. The TV P5 group was inoculated with $10^7$ PFU of \textit{in vitro} cultured TV at passage 5; the TV P0 group was inoculated with $2 \times 10^8$ PFU of original TV stock; and the TV P1 group was inoculated with the fecal filtrates from the TV P0 group. After inoculation, the piglets were observed and evaluated daily for weight and body temperature changes, and anorexia. Daily rectal mucosa/fecal swabs were collected from each piglet and a diarrhea/fecal consistency score was assigned to each using a subjective...
scale wherein 0 = normal, 1 = creamy, 2 = pasty and 3 = watery. Pigs with fecal consistency scores of 2 or 3 were scored as diarrhea-positive.

Prior to termination, a blood sample for serum testing was collected from piglets sedated with Euthol® solution administered intravenously. Intestinal contents from the duodenum, proximal jejunum, ileum, and transverse colon were collected from each pig. To collect the intestinal contents, the intestine segment was clipped at both ends and 0.5 ml of OPTI-MEM was injected by sterile syringe into the intestinal lumen, gently massaged, and then 0.5 ml liquid was withdrawn using the same syringe. Adjacent intestinal tissue segments (duodenum, jejunum, ileum, and colon) were collected from each pig for viral detection by plaque assay, RT-qPCR, immunofluorescence by confocal microscopy and for histologic examination.

3.3.5. Enumeration of infectious TV by plaque assay

Plaque assays were performed on tissue culture TV, all fecal samples, intestinal contents, and intestinal tissue segment homogenates as previously described. Briefly, LLC-MK2 cells were seeded onto 6-well plates with 2ml OPTI-MEM/2%FBS and incubated for 12 hrs, at 37°C and 5% CO₂ to 90% confluence. Samples suspect of containing virus were diluted 1:10 with OPTI-MEM/2%FBS, homogenized, vortexed for 1 min, and clarified by centrifugation at 5000rpm for 10 min. Tissue segments were diluted 1:10 with OPTI-MEM/2%FBS and homogenized by Douche homogenizer, then clarified by centrifugation at 8000 rpm for 5 min. Ten-fold serial dilutions of supernatant
were made and 400 ul of each was added to each well in duplicate after the removal of cell culture medium. For intestinal contents, samples were vortexed, dispersed by vigorous pipeting, and then used for serial dilutions and subsequent infection. The plates were gently shaken to evenly spread the inoculum every 10 to 15 min. At 1 hr PI, 2.5ml overlay consisting of 5% FBS, 1.4% NaHCO₃, 2.5% HEPES, 1% Glutamine, and 0.25% agarose in minimum Eagle medium (MEM) was added to each well, and plates were incubated at 37°C, 5% CO₂ for 48 hr before fixing with 10% (v/v) formaldehyde in PBS and staining with 0.05% (w/v) crystal violet in 10% (v/v) ethanol.

3.3.6. Quantification of viral genomic RNA by RT-qPCR

The sample processing was the same for fecal samples and intestinal contents as described in plaque assay, whereas tissue samples were ground in liquid nitrogen, dispersed through 20 gauge needle/syringe for at least 20 times, and centrifuged at full speed for 10 min. The serum samples were used directly for RNA extraction. The total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA) as directed by the manufacturer. Two-step reverse transcription (RT) was conducted using primer (5’-TTGCAGGAGGGTTTCAAGATG-3’) targeting on the VP1 region of TV using Superscript III transcriptase kit (Invitrogen, Carlsbad, CA) following manufacture’s protocol. The RT products were then used to perform real-time PCR using primers and probe specifically targeting the VP1 gene of TV (fw: 5’-TTGCAGGAGGGTTTCAAGATG-3’, rv: 5’-CACGGTTTCATTGTCCCCATA-3’, probe: 5’-TGATGACACACATGTGGGA-3’, ABI, Foster City, CA) on a StepOne Real
Time PCR system (Applied Biosystems, Foster City, CA). A standard plasmid for Tulane virus was constructed by inserting the sequence of entire ORF2 (encoding viral protein VP1) into pGEM T-easy vector (Promega, Madison, WI). The plasmid of known concentration was ten-fold serial diluted to generate standard curve for real-time PCR. The real time PCR amplification conditions were: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, 1 min at 60°C. The threshold for detection of fluorescence above background was set within the exponential phase of the amplification curve. For each assay, 10-fold dilutions of standard plasmid were generated, and negative controls samples and ddH2O were included in each assay.

3.3.7. Immunofluorescence microscopy

The method used was indirect immunofluorescence on whole intestinal tissue mounts adopted from a previous study (Cheetham et al. 2006). Briefly, fresh intestinal tissues collected on sacrifice day were sectioned into small pieces and then fixed with 4.0% (v/v) paraformaldehyde-0.2% (v/v) glutaraldehyde in 0.1 M potassium phosphate buffer (PPB), pH 7.4, for 2 h, 22°C, washed 4 times with PPB, and quenched with PPB containing 50 mM glycine at 4°C overnight. Tissue sections were then permeabilized with 0.1% Triton X-100 in PBS for 1 h at 22°C washed twice with PBS and blocked with PBS containing 2.0% (w/v) bovine serum albumin (BSA) with 5.0% (v/v) normal goat serum (NGS) at 4°C overnight. Next, tissues were incubated in 1:500 diluted rabbit anti-TV antiserum (a generous gift from Dr. Xi Jiang) for 12 h at 4°C in incubation buffer consisting of 10 mM PPB containing 150 mM NaCl, 10 mM sodium azide, and 0.2%
(w/v) BSA. After 6 washes with PBS, tissues were incubated in a 1:1200 dilution of goat anti-rabbit IgG labeled with AlexaFluor488 (Invitrogen, A11034), in incubation buffer 12 h, 4°C. After 3 washes with PBS, samples were counterstained with the nuclear stain SYTOX (Invitrogen, S11368), and actin stain AlexaFluor633-labeled phalloidin (Invitrogen, A222884). The samples were then trimmed into small 3×2 mm pieces and mounted onto slides, and were examined using an Olympus Spectral confocal microscope system (FV1000 confocal system, Olympus).

3.3.8. Histological examination

Segments of duodenum, proximal jejunum, ileum, and transverse colon were collected for histological examination. Tissue segments were fixed in 10% (v/v) phosphate-buffered formalin for 24-36 h, dehydrated in a graded ethanol series, embedded in paraffin, cut in 5-micrometer sections, and transferred to glass slides. The sections were de-paraffinized, rehydrated, and then stained with hematoxylin and eosin. Slides were examined by conventional light microscopy.

3.3.9. Statistical analysis

Statistical analysis of one-way ANOVA was performed by using Minitab statistical analysis software (Minitab, Inc., State College, PA), a P-value of 0.05 was considered statistically significant.

3.4. Results
3.4.1. TV replicates efficiently in pig macrophage cells

Natural human NoV infection fails to induce effective long-term immunity to protect against future exposure by unknown mechanism the remains unknown. One possibility is that human NoV infection, similar to MNV, impairs the functions of macrophages and dendritic cells which in turn stops the induction of robust innate and adaptive immunities. To explore this hypothesis, we first determined whether TV, the only enteric cultivable primate calicivirus, also has a tropism of macrophage cells. We chose to use pig macrophage cells because gnotobiotic piglets support the replication of TV in vivo as demonstrated in Chapter 2. Initial stock (designated P0) of TV that was grown in LLC-MK2 cells was used to infect 3D/4 pig macrophage cells at an MOI of 1. After extensive cytopathic effect (CPE) was observed, cell culture supernatant (designated as P1) was harvested and used for the next passage. Using this procedure, TV was passed 5 times in 3D/4 pig macrophage cells. The viral titer of each passage was determined by plaque assay. As shown in Table 11, TV replicated efficiently in pig macrophage cells. However, a slight decrease in viral titer was observed from P0 to P5. Viral titer decreased from $2 \times 10^7$ PFU/ml (P0, original TV) to $10^6$ PFU/ml (P5, the fifth passage in 3D/4 cells), with each passage showing a decrease of approximately 0.2-0.3 log in titer (Table 11).
Table 11. Viral titer of \textit{in vitro} passaged TV in 3D/4 cells

<table>
<thead>
<tr>
<th>Passage</th>
<th>Viral titer (log_{10} PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0 (original TV)</td>
<td>7.30</td>
</tr>
<tr>
<td>P1</td>
<td>7.01</td>
</tr>
<tr>
<td>P2</td>
<td>6.89</td>
</tr>
<tr>
<td>P3</td>
<td>6.65</td>
</tr>
<tr>
<td>P4</td>
<td>6.12</td>
</tr>
<tr>
<td>P5</td>
<td>6.03</td>
</tr>
</tbody>
</table>

3.4.2. \textbf{Virulence of TV grown in macrophage cells}

3.4.2.1. \textbf{Rationale for the experiment design}

Although TV has a tropism for pig macrophage cells \textit{in vitro}, it is not known whether macrophage cells are the target for TV infection \textit{in vivo}. We chose to use macrophage cell-adapted TV for inoculation of piglets for two reasons. First, if TV indeed infects the macrophage cells in the intestine, it may lead to the impairment of the innate and adaptive immunities, which in turn results in enhanced viral virulence. Second, passage of TV may result in the attenuation of the virus, which will likely reduce the pathogenesis. Therefore, in this experiment, 4 piglets were inoculated with the fifth passage of macrophage cell-adapted TV (designated TV P5). After virus inoculation fecal samples were collected. At PID 3, piglets were euthanized and the terminal samples were collected for analysis.
3.4.2.2. Pathogenesis of TV grown in pig macrophage cells

3.4.2.2.1. Absence of clinical signs or viremia in pigs inoculated with macrophage cell adapted TV

None of piglets inoculated with TV P5 exhibited clinical signs. No significant changes were observed in body temperature, body weight, or anorexia during the three day study period. Viremia was not detected in any blood samples of inoculated piglets.

3.4.2.2.2. Viral shedding in feces

The results of fecal viral shedding for piglets inoculated with TV P5 are summarized in Figure 26(A). Infectious TV was detected in the fecal samples from all four piglets at PID 1, but only one piglet was positive for infectious TV on PID2. No piglet shed infectious virus on PID3. The average infectious viral titer at PIDs 1 and 2 was approximately $10^3$ PFU/g feces.
Figure 26. Detection of (A) infectious virus and (B) viral RNA in fecal samples of TV P5 inoculated piglet

Fecal samples were collected from all piglets in each group daily. Samples were diluted in MEM, homogenized, and clarified by centrifugation. (A) The presence of infectious virus particles was detected by plaque assay. (B) The total RNA was extracted from fecal samples using an RNeasy Mini Kit. The viral genomic RNA was quantified by real-time RT-PCR by targeting viral VP1 gene. Data are expressed as the average ± 1 standard deviation.
3.4.2.2.3. **Viral RNA in feces.**

The viral RNA in feces was determined by RT-qPCR. As shown in Figure 26(B), TV RNAs were detected in all four TV P5 piglets from PID1 to PID3. The viral RNA level remained at approximately $10^{4-5}$ RNA copies per gram feces during the three days. No significant difference in viral RNA levels was observed from PID 1 to PID 3 ($P>0.05$).

3.4.2.2.4. **Infectious TV in intestinal contents.**

As shown in Figure 27(A), low levels of infectious virus were detected in the small intestinal content filtrate of pigs inoculated with TV P5. The viral titer was approximately $10^{1.1}$ PFU/ml small intestinal content, with one piglet containing the highest titer of $10^{1.4}$ PFU/ml small intestinal content.
Figure 27. Detection of (A) infectious virus and (B) viral RNA in intestinal contents of TV P5 inoculated piglets

Intestinal contents were collected from different sections of small intestine when piglets were sacrificed on PID 3. Approximately 2cm of intestine segment was clipped at both ends, 0.5 ml of OPTI-MEM was injected by a sterile syringe into the intestinal lumen, gently massaged and then all the liquid was withdrawn with the same syringe. (A) The presence of infectious virus particles was detected by plaque assay. (B) The total RNA was extracted from fecal samples using an RNeasy Mini Kit. The viral genomic RNA was quantified by real-time RT-PCR by targeting viral VP1 gene. Data are expressed as the average ± 1 standard deviation.
3.4.2.2.5. Viral RNA in intestinal contents.

The RT-qPCR results for TV RNA in the intestinal contents are shown in Figure 27(B). All small intestinal content samples from piglets inoculated with TV P5 were found to be positive of TV RNAs. No significant difference in viral RNA levels ($10^3$ to $10^4$ TV RNA copies/ml sample) was found for samples collected from different intestinal contents.

3.4.2.2.6. Absence of histologic changes or TV antigens in intestine tissues

Piglets inoculated with TV P5 were sacrificed on PID 3, intestinal sections were examined for histologic changes and the presence of TV antigen as described in Chapter 2. As shown in Table 12, no histologic changes or TV antigens were found in duodenum, jejunum, ileum, and transverse colon of any of the infected piglets.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>No. of piglets</th>
<th>Diarrhea (%)</th>
<th>Fecal virus shedding a (%)</th>
<th>Detection of viral RNA in ICs (%)</th>
<th>TV antigen detection</th>
<th>Histologic changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV P5c</td>
<td>4</td>
<td>0/4 (0)</td>
<td>4/4 (100)</td>
<td>4/4 (100)</td>
<td>- b</td>
<td>-</td>
</tr>
</tbody>
</table>

a Detected by plaque assay and RT-qPCR.

b -, negative.

c TV that has been passed five times in 3D/4 pig macrophage cells. Dose: $10^7$ PFU.
3.4.3. *In vivo* passage of TV grown in LLC-MK2 cells

3.4.3.1. Rationale for the experiment design

Next, we decided to examine whether TV grown in LLC-MK2 cells can be adapted to piglets. The stock TV grown in LLC-MK2 cells (designated TV P0) with titer of $2 \times 10^7$ PFU/ml was inoculated into three newborn piglets. The feces from the TV P0 inoculated piglets were collected and filtered. The fecal filtrate (designated TV P1, the first passage from *in vivo*) was used to inoculate another three piglets.

3.4.3.2. Pathogenesis of TV grown in LLC-MK2

The original TV P0 stock grown in LLC-MK2 cells were inoculated into 4 piglets. At PID 4, piglets were euthanized, and infectious TV, TV RNA, intestinal histologic changes, and TV antigens were determined using identical protocols described in Chapter 2.

Figure 28 shows the levels of infectious TV and TV RNA in feces. Low levels of infectious virus ($10^2$ to $10^3$ PFU/g feces) were detected in the fecal filtrates of TV P0 inoculated piglets on PID 1 through PID 3 [Figure 28(A)]. RT-qPCR results showed that all three fecal filtrates from PID 1 to PID 3 contained about $10^4$ to $10^5$ RNA copies/g feces [Figure 3(B)].
Figure 28. Detection of (A) infectious virus and (B) viral RNA in fecal samples of TV P0 inoculated piglets

Fecal samples were collected from all piglets in each group daily. Samples were diluted in MEM, homogenized, and clarified by centrifugation. (A) The presence of infectious virus particles was detected by plaque assay. (B) The total RNA was extracted from fecal samples using an RNeasy Mini Kit. The viral genomic RNA was quantified by real-time RT-PCR by targeting viral VP1 gene. Data are expressed as the average ± 1 standard deviation.

Figure 29 shows the titers of infectious TV and TV RNA in the intestinal contents. Infectious TV was detected in the contents of the duodenum and jejunum from two
piglets inoculated with TV P0, but not in ileum and transverse colon [Figure 29(A)]. RNA was detectable in duodenum (1/3), jejunum (1/3), ileum (3/3), and transverse colon (1/3) [Figure 29(B)].

![Graph showing viral titer in various intestinal sections](image)

![Graph showing viral RNA in various intestinal sections](image)

**Figure 29. Detection of (A) infectious virus and (B) viral RNA in intestinal contents of TV P0 inoculated piglets**

Intestinal contents were collected from different sections of small intestine when piglets were sacrificed on PID 4. Approximately 2cm of intestine segment was clipped at both ends, 0.5 ml of OPTI-MEM was injected by a sterile syringe into the intestinal lumen, gently massaged and then all the liquid was withdrawn with the same syringe. (A) The
presence of infectious virus particles was detected by plaque assay. (B) The total RNA was extracted from fecal samples using an RNeasy Mini Kit. The viral genomic RNA was quantified by real-time RT-PCR by targeting viral VP1 gene. Data are expressed as the average ± 1 standard deviation.

Figure 30 shows the histologic changes in intestines observed in piglets inoculated with TV P0. Similar to the observations in Chapter 2, TV caused mild histologic changes in duodenum, jejunum, but not ileum, or transverse colon.

Figure 30. Histologic changes in tissue segments of piglets inoculated with TV P0 or TV P1

TV P0-inoculated piglets at PID 4 showed mild villous atrophy with shortening and blunting of villi. Necrosis of inflammatory cells in the lamina propria was also observed
in the TVP0-inoculated piglets. No histologic change was observed in TV P1-inoculated piglets or mock inoculation control.

Figure 31 shows the presence of TV antigen in the intestines of piglets inoculated with TV P0. Similar to the observations in Chapter 2, TV antigens were detected in duodenum and jejunum, but not in ileum or colon.

![Image showing antigen detection in tissue segments of piglets inoculated with TV P0 or TV P1 by indirect immunofluorescent assay (IFA)]

Fresh intestinal tissues were sectioned into small pieces, fixed, quenched, and permeabilized. The tissues were incubated with TV-specific polyclonal antibody,
followed by incubation with goat anti-rabbit IgG Alexa488. Nuclei and actin were counterstained with SYTOX orange and phallotoxin Alexa633. The stained tissues were mounted onto slides and examined using Olympus FV1000 Confocal Microscopy Image system.

3.4.3.3. In vivo passage of TV in pigs

The fecal samples that were positive for infectious TV were combined to make a pool of TV P1 inoculum. Each piglet received an aliquot of 5 ml fecal filtrate containing a total of $10^4$ PFU of TV.

3.4.3.3.1. Fecal shedding of infectious TV

The results of fecal viral shedding of pigs inoculated with TV P1 are summarized in Figure 32(A). Fecal infectious virus excretion was detected on PID 1 through 4. One pig shed infectious virus in feces from a maximum of 4 days after challenge, although the other two shed for only one day. The average infectious viral titer in feces remained approximately $10^{2.5}$ PFU/g fecal matter from PID 1 to 4 with no significant change ($P>0.05$). Considering the shedding time and the amount of feces (20-50g) produced by each piglet per day, the amount of virus released in feces exceeded that in the inoculum. Therefore, this data suggests that TV may replicate in the second passage in piglets. No infectious virus was detected in mock infected controls.
Figure 32. Detection of (A) infectious virus and (B) viral RNA in fecal samples of TV P1 inoculated piglets

Fecal samples were collected from all piglets in each group daily. Samples were diluted in MEM, homogenized, and clarified by centrifugation. (A) The presence of infectious virus particles was detected by plaque assay. (B) The total RNA was extracted from fecal samples using an RNeasy Mini Kit. The viral genomic RNA was quantified by real-time RT-PCR by targeting viral VP1 gene. Data are expressed as the average ± 1 standard deviation.
3.4.3.3.2. TV-RNA was detected in feces

The TV-RNA in feces was quantified by RT-qPCR. As shown in Figure 32(B), TV-RNA was detected in feces in two of the three TV-infected pigs one PID 1 and PID 2, all three on PID 3, and one of three on PID4. The average TV-RNA level was about $10^3$ genomic RNA copies/g of fecal matter. No significant difference (P>0.05) was found between TV-RNA levels on different PIDs. No TV-RNAs were detected in control piglet feces at any time interval tested.

3.4.3.3.3. Infectious TV was detected in intestinal contents

Infectious TV was detected in the intestinal content samples collected on PID 4. Two of three pigs were positive for infectious TV in the duodenal and jejunal contents, only one pig had infectious virus in the ileum content, and all transverse colon contents were negative for infectious TV [Figure 33 (A)]. In the positive samples, an average viral titer of only $10^{1.5}$ PFU/g of intestinal content was detected. No infectious virus was detected in mock infected controls.
Intestinal contents were collected from different sections of small intestine as previously described when piglets were sacrificed on PID 4. (A) The presence of infectious virus particles was detected by plaque assay. (B) The total RNA was extracted from fecal samples using an RNeasy Mini Kit. The viral genomic RNA was quantified by real-time RT-PCR by targeting viral VP1 gene. Data are expressed as the average ± 1 standard deviation.
3.4.3.3.4. **TV-RNA was detected in intestinal contents**

The RT-qPCR results for TV-RNAs in intestinal contents are shown in Figure 33 (B). Most small intestinal content samples (1/3 for duodenal contents, 2/3 for jejunum contents, and 2/3 for ileum content) and all transverse colon contents tested were positive for TV-RNAs. All sample tested had approximately $10^{2.7}$ TV RNA copies per ml of intestinal content with no significant difference ($P>0.05$) between TV-RNA levels in different intestinal contents. No TV-RNAs were detected in mock infected controls.

3.4.3.3.5. **Histology and TV antigen**

No significant histological changes (Figure 30) or TV antigen (Figure 31) was found in duodenum, jejunum, ileum, or transverse colon from any of the piglets inoculated with TV P1 (Table 13).

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>No. of piglets</th>
<th>Diarrhea (%)</th>
<th>Fecal virus shedding a (%)</th>
<th>Detection of virus in ICs a (%)</th>
<th>TV antigen detection</th>
<th>Histological changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV P0 c</td>
<td>3</td>
<td>1/3 (33)</td>
<td>3/3 (100)</td>
<td>3/3 (100)</td>
<td>+ b</td>
<td>+</td>
</tr>
<tr>
<td>TV P1 d</td>
<td>3</td>
<td>0/3 (0)</td>
<td>3/3 (100)</td>
<td>3/3 (100)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a Detected by plaque assay and RT-qPCR

b +, positive; -, negative

c Original TV cultured in LLC-MK2 cells. Dose: $2 \times 10^8$ PFU.

d The fecal filtrate of pigs inoculated with TV P0. Dose: $10^4$ PFU.
3.5. Discussion

In this study, we adapted TV to pig macrophage cells, and passed TV repeatedly in vivo in gnotobiotic pigs. We found that TV caused typical cytopathic effects and replicated efficiently in pig macrophage cells, suggesting that TV has a tropism for macrophage cells. We found that pig macrophage cell-adapted TV did not cause any clinical symptoms or intestinal pathological changes in piglets. In addition, we found that feces infiltrate from TV-infected pigs can cause viral shedding but not histologic changes or TV antigen expression in the second passage in gnotobiotic piglets. These findings fill in a major knowledge gap in our understanding of the cell tropism of TV and the adaption of TV to a non-primate host, the piglets.

To date, the immunopathogenesis of enteric caliciviruses is poorly understood primarily because many of them cannot be grown in cell culture and lack a small animal model. Studies using MNV-mice model found that MNV-1 has a tropism for macrophage and dendritic cells that leads to the impairment of the ability to induce innate and adaptive immunities against viral infection. However, whether these findings can be applied to human NoV and other enteric caliciviruses is not known because MNV causes a systemic of infection (characterized by the presence of virus in blood, liver, spleen and lungs) but not gastroenteritis and enteric infection in mice. TV is a good model to study the immunopathogenesis of human NoV because it causes gastroenteritis and enteric infection and utilizes the same cellular receptors as human NoV does.
Our results showed that TV replicates efficiently in pig macrophage cell cultured \textit{in vitro}. This lead to our hypothesis that TV may also target macrophages \textit{in vivo}. To test this hypothesis, we inoculated $10^7$ PFU of macrophage cell adapted TV into newborn gnotobiotic piglets. However, little virus shedding in feces and no significant intestinal pathological changes or TV antigens were detected. Several possible explanations for this result exist. First, $10^7$ PFU of TV may not be sufficient to cause viral shedding and changes in piglets. As demonstrated in Chapter 2, $2 \times 10^8$ PFU of TV (grown in LLC-MK2 cells) caused significant gastroenteritis and intestinal pathological lesions. We used $10^7$ PFU of macrophage cell-adapted TV in this experiment because the viral titer achieved in the macrophage cells was relatively low ($10^{5-6}$ PFU/ml). In contrast, TV can grow to a much higher titer ($10^{7-8}$PFU/ml) in LLC-MK2 cells. Clearly, future experiments are required to compare the pathogenesis of TV grown in LLC-MK2 cells and macrophage cells using the same inoculation doses. Second, passage of TV 5 times in pig macrophage cells may cause mutations in the viral genome, which can result in the attenuation of the virus. Therefore, future experiments are needed to sequence the full-length genome of TV grown in pig macrophage cells. Finally, it may be necessary to increase the number of passages of TV in macrophage cells. It will be interesting to compare the virulence of TV after prolonged passages (for example P10, P20, and P30).

The natural host of TV is the rhesus macaque. In this study, we adapted TV to gnotobiotic pigs. The feces collected from TV-infected piglets were used as inoculum to
infect naive piglets. The piglets received a total of $10^4$ PFU of the \textit{in vivo} passed TV and on average shed $10^{2.5}$ PFU infectious virus per gram of feces for four days. Considering the fact that a piglet produces about 20 to 50 g of feces per day on average, the virus shed in feces has exceeded the level of the inoculation dose. This result indicates that TV replicates in the intestines in the second passage in piglets. However, no histologic lesions or TV antigens were detected in the intestine of piglets after infection using the pig adapted TV. This result conflicts with the finding that a significant amount of infectious TV was shed during the second passage in the piglets. There are two possibilities to explain this data. First, it is possible that TV was attenuated after one passage in piglets. Currently, we are sequencing the viral genome isolated in the feces from the first passage in piglets. Second, $10^4$ PFU of TV may not be sufficient to cause detectable intestinal histologic lesions or TV antigen expression. Thus, further experiments should aim to increase the virus inoculation level for the second passage in the piglet. To achieve this aim, the key is to generate a large pool of feces containing high titers of TV from the first passage in pigs.

In summary, we found, for the first time, that TV replicated efficiently in pig macrophage cells. In addition, \textit{in vivo} passage of TV yielded promising preliminary results. The piglets receiving fecal filtrates (containing $10^4$ PFU of TV) shed $10^{2.5}$ PFU/g feces for 4 days. These findings will be useful for the study of the immunopathogenesis of TV and other enteric calicivirus in the future.
CHAPTER 4 CONCLUSION

Firstly, this study demonstrates that LLC-MK2 cell origin TV caused mild gastroenteritis, viral shedding, and intestinal histological lesions in gnotobiotic piglets. To our knowledge, this is the first demonstration that a non-human primate calicivirus can cause gastroenteritis in piglets, with similar manifestations to human NoV in humans. The gnotobiotic pig model has the potential for broad application in the study of human and animal caliciviruses. The pig gut shares high similarity with humans and primates which make it an ideal model to study enteric pathogens. This research indicates that the gnotobiotic pig supports TV infection, and this model can be used for future pathogenesis and vaccine studies.

Secondly, this study successfully adapted TV to pig macrophage cell line. TV causes typical cytopathic effect (CPE) and replicates efficiently in pig macrophage cells, suggesting that TV has a tropism for macrophages. This discovery will be useful for the study of the immunopathogenesis of TV in vivo. Another calicivirus, MNV, has also been shown to have tropism for macrophage cells. It is believed that the infection of macrophage and dendritic cells by these viruses leads to suppression of the immune responses and causes the absence of long-term immunity following infection. Long-term
immunity is rarely observed following infection with caliciviruses, and the fact that TV infects macrophage cells may help to explain this phenomenon.

Thirdly, this study demonstrates that macrophage cell adapted TV does not cause gastroenteritis or significant intestinal pathological changes in gnotobiotic piglets. The adaptation of TV to the macrophage cells may have induced mutations which limited the virus infectivity in vivo. Future studies should target sequencing the entire RNA genomes of both the porcine macrophage cell line and rhesus macaque kidney cell line adapted viruses for comparison.

Finally, this study demonstrates that feces collected from TV-infected piglets can cause viral shedding in piglets but lack significant intestinal pathological changes. Specifically, the fecal infiltrates (containing $10^4$ PFU of TV) from TV-infected piglets can cause $10^{2.5}$ PFU/g of viral shedding in feces for at least 4 days, suggesting that in vivo passage of TV may lead to the adaption of TV to piglets.

In summary, this research has demonstrated that the gnotobiotic pig can serve as a model for the study of TV infection in vivo. Additionally, it was found that TV has tropism for macrophage cells. Understanding the pathogenesis and cell tropism of TV can be used as a model for other caliciviruses that cannot be grown in vitro, such as human NoV.
LIST OF REFERENCES


140


