Development of molecular techniques for the detection and pathogenesis study of swine corona- and corona-like virus

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Development of molecular techniques for the detection and pathogenesis study of swine corona-and corona-like virus

by

Theerapol Sirinarumitr

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Major Professors: John P. Kluge and Prem S. Paul

Iowa State University

Ames, Iowa

1998
This is to certify that the Doctoral dissertation of

Theerapol Sirinarumitr

has met the thesis requirements of Iowa State University

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

For the Major Program

Signature was redacted for privacy.

For the Graduate College
This Dissertation is dedicated to my parents, my wife, my brother, and all of my teachers
# TABLE OF CONTENTS

## ABSTRACT

## CHAPTER 1. GENERAL INTRODUCTION
- Dissertation Organization
- Introduction

## CHAPTER 2. LITERATURE REVIEW
- The History of Coronaviruses
- Coronaviruses
- The Transmissible Gastroenteritis Virus
- The Porcine Respiratory Coronavirus
- Methods for the Detection of TGEV and PRCV
- The History of Arteriviruses
- Arteriviruses
- The Porcine Reproductive and Respiratory Syndrome Virus
- Methods for the Detection of PRRSV

## CHAPTER 3. *IN SITU* HYBRIDIZATION TECHNIQUE FOR THE DETECTION OF SWINE ENTERIC AND RESPIRATORY CORONAVIRUSES, TRANSMISSIBLE GASTROENTERITIS VIRUS (TGEV) AND PORCINE RESPIRATORY CORONAVIRUS (PRCV), IN FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUES
- Abstract
- Introduction
- Materials and Methods
- Results
- Discussion
ABSTRACT

In situ hybridization (ISH) technique was first developed to detect and differentiate transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) in cell culture and tissue sections using \(^{35}\text{S}\)-labeled RNA probes. RNA probe generated from plasmid PSP.FP\(_2\) detected both TGEV and PRCV, whereas PSP.FP\(_1\) probe detected only TGEV. The TGEV RNA was detected mainly within the enterocytes at the tips of villi and within a few crypt epithelial cells. The PRCV RNA was detected mainly in the bronchiolar epithelial cells and in lesser amount in type I and type II pneumocytes, alveolar macrophages and bronchial epithelial cells. Since the ISH technique using a radiolabeled probe is time consuming and not user friendly, the nonisotopic ISH technique using fluorescein-labeled RNA probe was developed. A rapid ISH technique using radiolabeled and fluorescein-labeled probes was able to decrease hybridization time from 20 hours to 2 hours without compromising the intensity of the signal and tissue morphology. By the rapid nonisotopic ISH technique, the entire procedure could be performed within about 7-8 hours.

The mechanism of TGEV-induced cell death is not known. We demonstrated TGEV induced apoptosis in swine testes cell cultures by gel electrophoresis, electron microscopy, and terminal deoxytransferase digoxigenin-dUTP nick end labeling (TUNEL) technique. By electron microscopy, we showed that infected-ST cells from TGEV-inoculated wells were undergoing cell lysis, however, uninfected-ST cells were undergoing apoptosis. Double labeling technique also demonstrated that TGEV positive cells were negative for apoptosis and apoptotic cells were negative for TGEV RNA. Our results indicated that TGEV induced apoptosis in uninfected bystander cells, thus amplifying the cytopathic effect of TGEV.

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically emerging virus in swine. We demonstrated that PRRSV induced apoptosis both in vitro and in vivo and apoptotic cells were uninfected bystander cells. In the lungs of PRRSV-infected
pigs, the apoptotic cells were predominantly alveolar macrophages, lymphocytes, pulmonary intravascular macrophages, and type I and type II pneumocytes. In the lymph nodes of PRRSV-infected pigs, the apoptotic cells were predominantly lymphocytes and macrophages. A large number of macrophages and lymphocytes undergoing apoptosis might be the reason that PRRSV-infected pigs are susceptible to secondary infection.
CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation contains four separate manuscripts. Each individual manuscript is complete in itself and includes an Abstract, Introduction, Materials and Methods, Results, Discussion, and Reference section. The Ph.D. candidate, Theerapol Sirinarumitr, is the senior author and principal investigator for each manuscript. Part of the literature review, methods for the detection of TGEV and PRCV, has been published in the *Advances in Experimental Medicine and Biology* Volume 42: *Mechanisms in the Pathogenesis of Enteric Diseases*. The first manuscript describes the development of *in situ* hybridization technique for the detection and differentiation of transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV) in formalin-fixed paraffin-embedded tissues using radiolabeled ribonucleic acid (RNA) probes and has been published in the *Journal of Virological Methods*. The second manuscript describes the rapid *in situ* hybridization technique for the detection of TGEV in tissues using radiolabeled and fluorescein-labeled riboprobes and has been published in the *Molecular and Cellular probes*. The third manuscript describes the usage of techniques to study TGEV-induced cell death in cell culture and has been submitted to *Archives of Virology*. The fourth manuscript describes how porcine reproductive and respiratory syndrome virus (PRRSV)-induced cell death in cell culture and in swine tissues and has been submitted to the *Journal of General Virology*. In addition, a current review of the literature relevant to this dissertation as well as a General Conclusions are included. The General Conclusions section summarizes our study in view of what have been studied by others, and discusses ideas and areas that are worthy of continued research. Literature citations contained within the General Introduction, Literature
Introduction

Transmissible gastroenteritis (TGE) is an economically important disease because it is highly contagious and characterized by vomiting, severe diarrhea, and high mortality in piglets during the first few weeks of life. TGEV has been extensively studied, however, the mechanism of TGEV-induced cell death is still unclear. In recent years, another coronavirus has been identified in swine and has been designated as porcine respiratory coronavirus (PRCV). The most commonly used method for TGEV detection in diagnostic cases is immunofluorescence method on frozen sections of intestines. This technique has many problems as sections or villus enterocytes often wash off the slides during processing. In addition, the emergence of PRCV, closely related antigenically to TGEV, creates a diagnostic problem. Another virus similar to TGEV in genomic organization is Porcine reproductive and respiratory syndrome virus (PRRSV). PRRSV has been shown to cause reproductive failure in pregnant sows characterized by stillbirths, mummified fetus, and weak born piglets (Wensvoort et al., 1991; Terpstra et al., 1991; Plana Duran et al., 1992).

My colleagues and I have been interested in developing an improved diagnostic technique for the detection and differentiation of TGEV and PRCV, to improve such a technique so that it is appropriate for the diagnostic laboratory and more user friendly, and to understand the mechanism of TGEV-and PRRSV-induced cell death. In the first study, my colleagues and I report on an in situ hybridization technique using radiolabeled RNA probe for the detection and differentiation of TGEV and PRCV in the swine testes cell culture infected with TGEV and PRCV. In this study, we also optimized the in situ hybridization conditions for the detection and differentiation of TGEV and PRCV in tissues.
from experimentally and naturally infected pigs. However, we realize that in situ hybridization technique using radiolabeled RNA probe is a time-consuming technique and is not user friendly. Therefore, in the second study, a rapid in situ hybridization technique was developed using radiolabeled and fluorescein-labeled RNA probes. The rapid in situ hybridization technique decreases hybridization time from overnight to 2 hours for both radiolabeled and nonradiolabeled RNA probes. The entire process could be performed within about 7-8 hours using the combination between rapid in situ hybridization and nonradiolabeled RNA probes. Thus, the in situ hybridization technique is more suitable and more user friendly for diagnostic purpose. In the third study, my colleagues and I determined the mechanism of TGEV-induced cell death in the swine testes cell culture. In this study, TGEV was shown to kill infected cells by cell lysis and unexpectedly the uninfected bystander cells by apoptosis. According to the results, TGEV-induced apoptosis in bystander cells might be one of the mechanisms for amplifying the cytopathic effect of TGEV. The result of this study might lead to a new strategy for treatment of virus infection. In the fourth study, my colleagues and I determined the mechanism of PRRSV-induced cell death. We showed PRRSV induced apoptosis in cell cultures as well as lungs and lymph nodes of PRRSV-infected pigs. The apoptotic cells were mostly bystander cells both in vitro and in vivo. The apoptotic cells were mostly alveolar macrophages in the lungs and mostly lymphocytes and macrophages in the lymph nodes. This may in part explain why PRRSV-infected pigs frequently have secondary infections with both bacteria and viruses and might be the reason that PRRSV-infected pigs have a dramatic reduction in the numbers of the alveolar macrophages and in the numbers of circulating lymphocytes and monocytes.
CHAPTER 2. LITERATURE REVIEW

The History of Coronavirus

Coronaviruses are large, enveloped, plus-strand RNA viruses that belong to the family of Coronaviridae and cause diseases in both humans and animals (Holmes and Lai, 1996). Coronaviruses have a distinctive morphology because the viral envelopes have petal-shaped glycoproteins projecting from the envelopes of virions which result in a crownlike appearance (McIntosh, 1996). Avian infectious bronchitis (IBV) was the first disease described that was caused by coronavirus in 1931 (Schalk and Hawn, 1931). Later, TGE in swine was described in 1946 (Doyle and Hutching, 1946) and mouse hepatitis virus (MHV) was recognized in 1949 (Cheever et al., 1949). In 1965, Tyrell and Bynoe recovered an ether-labile virus, strain B814, from a boy with a cold. Hamre and Proknow (1966) also recovered five virus strains and the prototype strain 229E was shown to be similar to that of IBV and B814. McIntosh et al. (1967) have isolated other strains of human coronaviruses and these viruses were antigenically and morphologically related to MHV. For several years, the characteristic morphology was the only criterion for classifying coronaviruses. Recently, property of structural proteins, genomic RNA and mRNAs, nucleic acid homology and antigenic cross-reactivity have been used to classify coronaviruses.

Coronaviruses are divided into three serological groups, and generally infect only one species or several closely related species (Holmes, 1996). Table 1 shows the species infected by coronaviruses.
<table>
<thead>
<tr>
<th>Antigenic group</th>
<th>Abbreviation</th>
<th>Name</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>HCV-229E</td>
<td>Human respiratory coronavirus</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>TGEV</td>
<td>Transmissible gastro-enteritis virus</td>
<td>Pig</td>
</tr>
<tr>
<td></td>
<td>PRCV</td>
<td>Porcine respiratory coronavirus</td>
<td>Pig</td>
</tr>
<tr>
<td></td>
<td>CCV</td>
<td>Canine coronavirus</td>
<td>Dog</td>
</tr>
<tr>
<td></td>
<td>FECV</td>
<td>Feline enteric coronavirus</td>
<td>Cat</td>
</tr>
<tr>
<td></td>
<td>FIPV</td>
<td>Feline infectious peritonitis virus</td>
<td>Cat</td>
</tr>
<tr>
<td></td>
<td>RbCV</td>
<td>Rabbit coronavirus</td>
<td>Rabbit</td>
</tr>
<tr>
<td>II</td>
<td>HCV-OC43</td>
<td>Human respiratory coronavirus</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>MHV</td>
<td>Mouse hepatitis virus</td>
<td>Mouse</td>
</tr>
<tr>
<td></td>
<td>SDAV</td>
<td>Sialodacryoadenitis virus</td>
<td>Rat</td>
</tr>
<tr>
<td></td>
<td>HEV</td>
<td>Hemagglutinating encephalomyelitis virus</td>
<td>Pig</td>
</tr>
<tr>
<td></td>
<td>BCV</td>
<td>Bovine coronavirus</td>
<td>Cow</td>
</tr>
<tr>
<td></td>
<td>RbECV</td>
<td>Rabbit enteric coronavirus</td>
<td>Rabbit</td>
</tr>
<tr>
<td></td>
<td>TCV</td>
<td>Turkey coronavirus</td>
<td>Turkey</td>
</tr>
<tr>
<td>III</td>
<td>IBV</td>
<td>Infectious bronchitis virus</td>
<td>Chicken</td>
</tr>
</tbody>
</table>

*Modified from Holmes and Lai (1996)*
Coronaviruses

Coronavirus particles are generally round, yet moderately pleomorphic, with a diameter ranging from 100 to 150 nanometers (nm) (McIntosh, 1996). The coronavirus has an envelope with a single layer of widely spaced, club-shaped projections. These projections are about 20 nm in length (McIntosh, 1996). Coronavirus particles have a molecular mass of 6 to $9 \times 10^6$ and a buoyant density of 1.18 to 1.21 g/ml in sucrose and 1.14 to 1.23 g/ml in CsCl (Bohl, 1989; Brian et al., 1980; Garwes and Pocock, 1975).

Coronaviruses appear to use a specific receptor to infect specific tissues because inhibition of phagocytic or endosomal pathways did not affect coronavirus infection of susceptible cells (Kooi, et al., 1992; Payne et al., 1990). The MHV receptor for entering murine enterocytes and hepatic cells was found to be mmCGM1 (murine carcinoembryonic antigen gene family member 1) which belongs to the carcinoembryonic antigen family of proteins (Williams et al., 1991). Recently, mmCGM2 was found to be an alternative receptor for MHV (Yokomori and Lai, 1992). The aminopeptidase N was also found to be a receptor for HCV-229E (Yeager et al., 1992) and TGEV (Delmas et al., 1992), and this enzyme is ubiquitous in respiratory tissue and the intestinal tract.

The genomic RNA of coronaviruses is 27 to 32 kb in size (Spaan et al., 1988; Lai, 1990), which is the largest of all RNA virus genomes. The virus genome is a single-stranded, positive-sense RNA which is capped at the 5' end and is polyadenylated at the 3' end (Spaan et al., 1988). Coronavirus replication occurs exclusively in the cytoplasm of infected cells and can occur normally in enucleated cells (Wilhelmsen et al., 1981). After penetration into the cells, a virus-specific RNA-dependent RNA polymerase is synthesized (Holmes and Lai, 1996). In all coronaviruses, gene 1 contains two overlapping open reading frames (ORFs 1a and 1b) which can be translated by a ribosomal frame-shifting mechanism. The deduced amino acid contains several putative domains. These domains include
"membrane anchor, cysteine rich, and protease domains in ORF 1a" (Lee et al., 1991; Olsen, 1993) and "polymerase, helicase, and zinc-finger domains and protease cleavages sites in ORF 1b" (Denison et al., 1991; Lee et al., 1991; Olsen, 1993). The plus-strand genomic RNA is transcribed to both full-length negative-strand RNA (Spaan et al., 1988) and subgenomic negative-strand RNAs (Lai, 1990) using virus-specific RNA-dependent RNA polymerase. The negative-strand RNAs then serve as templates for the transcription of genomic RNA and subgenomic RNAs (Spaan et al., 1988). A characteristic feature of coronavirus subgenomic mRNAs is the presence of the common sequence at 5' end which is called the leader sequence. This sequence is about 60 to 70 nucleotides long and is believed to play an important role in the regulation and transcription of coronavirus genomic RNA and subgenomic mRNAs. This leader sequence is found only at a short intergenic sequence that contains a 7-to 18-nucleotide sequence homologous with a sequence near the 3' end of the leader sequence (Shieh et al., 1989). This homologous region containing seven nucleotides (UCUAAC) which is conserved among most coronaviruses and is believed to be the binding point of leader RNA (Lai, 1990).

At least 5 to 7 subgenomic mRNAs are produced in the infected cells depending on virus species (Holmes and Lai, 1996). The subgenomic mRNAs form a nested set with a common 3' end; thus, each mRNA contains all of the sequence in the next smaller mRNA plus an additional gene at the 5' end. All subgenomic mRNAs, except the smallest one, are structurally polycistronic but they are functionally monocistronic. However, some of the mRNAs of MHV, BCV, and IBV have recently been found to be bicistronic and tricistronic in nature (Boursnell et al., 1985; Budzilowicz and Weiss, 1987; Liebowitz et al., 1985; Liu et al., 1991; Senanayake et al., 1992).

All coronaviruses have at least three major structural proteins which are the spike glycoprotein (S), the nucleocapsid protein (N), and the integral membrane protein (M)
(Spaan et al., 1988). The S glycoprotein is the structural protein of the large, petal-shaped spikes (Holmes and Lai, 1996). The S glycoprotein has a size of 180-200 kd and has many important biological functions: attachment to the specific cell receptors, fusion of viral envelope with cell membrane, induction of cell fusion, binding at Fc receptor of immunoglobulin, induction of neutralization antibodies and cell mediated immunity (Holmes and Lai, 1996). The deduced amino acid sequences of S gene reveal several potential N-linked glycosylation sites and 4 structural domains: a short carboxyterminal cytoplasmic domain, a transmembrane domain, and two large external domains (S1 and S2) (Holmes and Lai, 1966). The cytoplasmic domain is a cysteine-rich domain and may play a role in assembly of spikes or interaction with other viral proteins. The transmembrane domain is attached to the S2 domain. The region between S1 and S2 domains of group II and III coronaviruses has a trypsin-cleavage motif which is comprised of a cluster of basic amino acids. However, the S glycoprotein of group I coronaviruses does not have protease-sensitive sequence. Each spike is composed of three S glycoproteins held together with noncovalent bonds (Holmes and Lai, 1996).

The N protein has molecular mass of 50-60 kd and is translated in the cytoplasm by free polysomes (Holmes and Lai, 1996). The N protein is phosphorylated protein and binds to virus RNA. The N protein appears to play an important role in virus transcription as the level of N protein may act as indicator to switch between mRNA synthesis and genomic RNA synthesis (Lai, 1990). The other biological functions of N protein are formation of nucleocapsid and induction of cell mediated immunity (Holmes and Lai, 1996).

The M protein has a molecular mass of 25 to 30 kd and is glycosylated transmembrane protein (Holmes and Lai, 1996). The M protein has several domains: short extracytoplasmic domain at the amino-terminus, and three transmembrane domains, and large cytoplasmic domain at the carboxy-terminus. Glycosylation occurs at the N-terminal
domain of M protein and is O-linked for IBV and TGEV. However, it is N-linked glycosylation for MHV. The M protein is synthesized on membrane-bound polysomes in the rough endoplasmic reticulum (RER). The mature M protein accumulates in the golgi apparatus and is not transported to the plasma membrane. The M protein has several biological functions: determination of budding site, formation of envelope, interaction with viral nucleocapsid, and induction of IFN-α (Holmes and Lai, 1996).

The hemagglutinin-esterase (HE) protein is found only in group II coronaviruses (Holmes and Lai, 1996). The HE protein has a molecular mass of 65 to 75 kd and is cotranslationally glycosylated by the addition of N-linked glycans in the RER. The HE glycoprotein is found to be disulfide-linked dimer on the virions and has molecular mass of 130-140 kd. The HE protein, like the HE protein of influenza virus, binds to 9-O-acetylated neuraminic acid on cell membrane. The HE protein has several biological functions: hemagglutination, hemabsorption, and cleavage of 9-O-acetyl neuraminic acid (Holmes and Lai, 1996).

The binding of the N protein to viral RNA to form helical nucleocapsids may require a specific sequence and is believed to happen with the full-length genomic RNA (Holmes and Lai, 1996). However, recently the encapsidation of subgenomic mRNAs has been demonstrated. It is not clear that the encapsidation of subgenomic mRNAs is a specific event. Packaging of genomic RNA requires a specific sequence of 61 nucleotides at the 3’ end of gene 1b (Holmes and Lai, 1996). Virions are formed by budding of nucleocapsids to intracellular membranes that contain M protein. These intracellular membranes are called budding compartment and located between the RER and the Golgi. The S glycoprotein is not required for formation or release of virions (Holmes and Lai, 1996). Later on budding of virions can also be observed in the RER and at the outer leaflet of the nuclear envelope. The large and small spikes formed by the S and HE glycoprotein, respectively, are
incorporated into virions during budding. Infectious virus is released normally from the intact cells.

**Transmissible Gastroenteritis Virus**

Transmissible gastroenteritis (TGE) has been recognized as a virus-induced gastroenteritis disease since 1946 when the virus was isolated for the first time (Doyle and Hutchings, 1946). Transmissible gastroenteritis continues to be an economically important disease because it is highly contagious and causes vomiting, severe diarrhea and high mortality in piglets during the first few weeks of life. The etiological agent of TGE is referred as transmissible gastroenteritis virus (TGEV).

TGEV belongs to the genus coronavirus of the family Coronaviridae and is a pleomorphic enveloped virus, with an overall diameter of 60-160 nm (Okaniwa et al., 1968; Phillip et al., 1971; Granzow et al. 1981). There is a single layer of club-shaped surface projections of 12-25 nm in length.

The nucleic acid of TGEV is a single-stranded, positive-sense polyadenylated RNA genome which has a size of >23 kb (Siddell et al., 1983; Laude et al. 1990) and can be infectious (Brian et al., 1980). The full-length negative-sense RNA is first synthesized from the positive-sense RNA genome after infection and is used as the template for synthesis of the 3'-terminal nested set of 7-8 subgenomic mRNAs, depending on the strain of TGEV (Britton et al., 1989; Spaan et al., 1988; Wesley et al., 1990a). The subgenomic mRNAs, with the exception of the smallest one, are polycistronic in nature, and only the unique 5' open reading frame is translated (Sanchez et al., 1992; Spaan et al., 1988). The subgenomic mRNA 2 (~8.2 kb) encodes S protein or peplomer (Wesley et al., 1991b). The S protein (molecular mass of 195-220 kD), which is glycosylated (Garwes et al., 1976), is important for cell attachment, membrane fusion and induction of neutralizing antibodies to...
TGEV (Garwes et al., 1978/79). Laude et al. (1995) examined pathogenicity for newborn piglets of a series of neutralization escape mutants encoding mutation in S gene. Several amino acid changes were correlated with a dramatic loss of enterovirulence, thus indicating that crucial virulence determinants are associated with this domain of the S gene. Ballesteros et al. (1997) showed that two nucleotide changes (214 and 655) may be in principle responsible for the loss of enteric tropism, but only the change in nucleotide 655 was found in the respiratory isolates. It is most likely that a single change at nucleotide 655 may lead to a substitution in amino acid 219 of the S protein and is responsible for the loss of enteric tropism. In order to infect enterocytes, two different domains of the S protein, mapping between amino acids 522 and 744 and around amino acid 219, respectively, are involved. The first domain binds to porcine aminopeptidase N, the cellular receptor for TGEV. The other domain may serve as the binding site for a co-receptor essential for the enteric tropism of TGEV. In the case of the virulent Miller strain of TGEV, the two nonstructural proteins, 3a and 3-1, are encoded by the 3.8 kb subgenomic mRNA 3 and by the 3.5 kb subgenomic mRNA 3-1, respectively (Wesley et al., 1989). In the case of the virulent FS/72 British strain and the Purdue strain of TGEV, the 3a and 3-1 regions are encoded by a single 3.8 kb subgenomic mRNA 3 (Britton et al., 1989; Kapke et al., 1988). The genes 3a and 3-1 have been hypothesized to play an important role in the virulence, tissue tropism, plaque morphology, persistence in swine leukocytes and pathogenesis of TGEV (Wesley et al., 1990 a), because the small plaque (SP) mutant of TGEV has an S gene similar to wild type TGEV, but there is a large deletion (462 nucleotides) in 3a and 3-1 regions (Wesley et al., 1990a). The SP mutant of TGEV was not pathogenic for three-day-old piglets and was found to replicate in unidentified cells in the lamina propria instead of the villus enterocytes of the small intestine like other virulent TGEV (Wesley et al., 1990a; Woods et al., 1981; Woods et al., 1978). The subgenomic mRNA 4 encodes for a small
The subgenomic mRNA 5 (2.5 kb) encodes for the integral-membrane protein (M), a glycoprotein with a molecular mass about 29-31 kD, which has three membrane-spanning domains and is firmly embedded in the viral envelope. The M glycoprotein is presumed to be responsible for mediating complement-dependent neutralization and interferon induction because the hydrophilic N terminus protrudes out from the virion and has a single accessible glycosylation site. The subgenomic mRNA 6 (1.7 kb) encodes for the nucleocapsid (N) protein (molecular mass about 45 to 50 kD) which is a basic nonglycosylated phosphoprotein. The N protein is associated with viral RNA and serves as a nucleocapsid (Britton et al., 1988a). The subgenomic mRNA 7 (0.5 kb) is the smallest subgenomic mRNA and has been shown to encode for a possible DNA binding protein in TGEV-infected cells (Garwes et al., 1989; Britton et al., 1990).

TGEV is very stable in the frozen condition but somewhat labile at room temperature or above. There was a 10 fold drop in titer when the virus was stored at -18° C for 18 months (Haelterman and Hutchings, 1965) but there was 10 fold reduction in titer every 24 hours when virus was stored at 37° C (Young et al., 1955). TGEV is highly sensitive to light since a $10^5$ pig-infectious dose of TGEV in fecal material was inactivated within 6 hours after direct exposure to sunlight (Halteman, 1963). Most TGEV strains are moderately trypsin resistant and relatively stable during incubation at pH 3.0 for 30 minutes (Harada et al., 1968; Laude et al., 1981). These properties may contribute to the ability of the virus to survive in the environment of the stomach and of the small intestines. TGEV is inactivated by several disinfectants including sodium hypochlorite, sodium hydroxide, formaldehyde solution, iodine, phenolic, and quaternary ammonium compounds (Nakao et al., 1978; Brown, 1981).
TGE is a disease which has an appearance of an epizootic, enzootic or intermittent enzootic. The epizootic form of TGEV occurs when the virus is introduced into a herd where most if not all of the pigs are susceptible (Bohl, 1989). The disease rapidly spreads among pigs of all ages. In suckling pigs, the disease is characterized by severe diarrhea, dehydration, vomiting and high mortality, especially in pigs under 2 to 3 weeks of age. The mortality in piglets under two weeks of age may reach 100%, but, in nursery pigs, those of three to eight weeks of age, the mortality is usually less than 10 to 20% (Hill, 1989; Saif and Bohl, 1986). The older pigs develop inappetence, vomiting, and diarrhea that last from two to four days (Hill, 1989; Saif and Bohl, 1986). Lactating sows commonly become sick and develop agalactia which contributes to the severity of the disease in the suckling pigs. The disease usually occurs in the winter months because of low temperatures and less sunlight, thus allowing and enhancing TGEV survival in the environment. If there is no influx of susceptible pigs, the disease usually terminates in a few weeks.

The enzootic form of TGE refers to the persistence of the infection and disease in a herd as a result of a continuing influx of susceptible pigs into the infected herd. Enzootic TGE is limited to the herd that has a continuous farrowing schedule or the addition of feeder pigs (Morin et al., 1983). Most sows in such herds have a variable degree of active immunity which provides sufficient protection to avoid clinical signs as well as providing a variable degree of passive immunity to suckling pigs. Weaned pigs are very susceptible to the virus because there is a sudden drop in passive immunity (Hill, 1989; Bohl, 1989). Mortality is usually around 10 to 20% (Hill, 1989; Bohl, 1989), but, if enzootic TGE persists in a herd for over a year, the economic losses may exceed those incurred from the epizootic TGE.

Intermittent enzootic TGE refers to a situation in which TGEV is reintroduced into a herd that contains immune sows, resulting in periodic episodes of disease (Pritchard, 1987).
This situation commonly occurs during the winter in the concentrated swine-rearing areas. The animals are susceptible, since the herd infection from the previous winter has been terminated during the summer and autumn. If the disease enters the farrowing house, the disease will resemble that described above for enzootic TGE. It is unclear whether the source of virus comes from reactivation of virus shedding in carrier pigs or reintroduction of the virus into the herd.

TGEV replicates mainly in the small intestine, especially in the jejunum, and, to a lesser extent in the ileum, with very little in the duodenum (Hopper and Haelterman, 1966). TGEV uses peplomer or spike proteins to bind with the receptors, aminopeptidase-N (Delmas et al., 1992) on the cell membrane of the mature absorptive enterocytes, resulting in villous atrophy, blunted, denuded villi and crypt hyperplasia (Hopper and Haelterman, 1966). There is an inverse relationship between the age of the animal infected with TGEV and the severity of the clinical signs, duration of the disease, and mortality (Bohl, 1989; Saif and Bohl, 1986). Several mechanisms have been proposed to account for the age-dependent resistance to the disease. The migration of cells from crypts of Lieberkühn to replace villous enterocytes in 3-week-old pigs is 3 times more rapid than newborn pigs (Moon, 1978). These newly replaced villous enterocytes are resistant to TGEV infection (Pensaert et al., 1970b; Shepherd et al., 1979), possibly due to the onset of an immune response, and the presence of interferon (LaBonnardiere and Laude, 1981). The lack of a tubulovascular system in pigs older than 3 weeks may contribute to the age-dependent resistance. Recently, Weingartl and Derbyshire (1994) reported a second putative receptor of 200 kDa for TGEV which is present in newborn pigs. These receptors are present only on the cell membrane of absorptive enterocytes and are absent on the cell membrane of crypt epithelial cells. Weingartl and Derbyshire (1994) speculated that this receptor may play a role in an age-dependent resistance. TGEV replicates in the cytoplasm of the mature
absorptive enterocytes (Saif & Wesley, 1994) and matures by budding through the endoplasmic reticulum (Thake et al., 1968; Pensaert et al., 1970b; Wagner et al., 1973). TGEV damages villous enterocytes and causes atrophy characterized by blunted and denuded villi of the small intestine which results in malabsorptive diarrhea. Transmission electron microscopy of infected small intestine show virus particles in cytoplasmic vacuoles of enterocytes, M cells of Peyer's patches, and macrophages (Wagner et al., 1973; Pospischil et al., 1981; Chu et al., 1982a). The crypt epithelial cells are not infected by TGEV.

TGEV has been shown to replicate to some extent in respiratory tissue (La Bonnardiere et al., 1983; Laude et al., 1993). Gnotobiotic pigs intranasally/orally inoculated with TGEV developed lung lesions but did not result in clinical pneumonia (Underdahl et al., 1975). A preliminary report indicated that TGEV was detected in the alveolar macrophages of infected neonatal pigs. However, alveolar macrophage cell cultures can support the replication of a cell culture-adapted strain but not the virulent strain (Laude et al., 1984). TGEV has also been shown to replicate in mammary tissue of lactating sows (Saif and Bohl., 1983) and virus can be shed in milk (Kemeny and Woods., 1977).

Porcine Respiratory Coronavirus Virus

Porcine respiratory coronavirus (PRCV) has been shown to be closely related to TGEV because of the antigenic and genomic similarities. PRCV replicates mainly in the respiratory tract with little or no replication in the small intestine.

PRCV was first isolated in 1984 from pigs in Belgium which were seropositive for TGEV but did not have clinical disease characteristic of TGE (Pensaert et al., 1986). There was a dramatic increase in the number of seropositive sows from about 20% to 68% (Pensaert et al., 1986). It was even more confusing because the increase in the number of
seropositive animals was not associated with clinical signs of TGE. Finally, the virus, named TLM 83, was isolated from the respiratory tract of pigs in which no enteric infection had taken place (Pensaert, 1986). The virus properties were not known at that time, but the morphology of the virus was shown to be similar to coronavirus. PRCV infection in European swine is now widespread (Pensaert, 1989). PRCV infections commonly occur in the presence of passive immunity after weaning in pigs 5-10 weeks of age and persists in closed breeding farms. PRCV has also been isolated in the United States (Hill et al., 1989; Wesley et al., 1990; Vaughn et al., 1994). PRCV causes subclinical disease to mild-moderate pneumonia in swine (Cox et al., 1990; O'Toole et al., 1989; Halbur et al., 1993b).

PRCV is believed to be a TGEV mutant, as it has been shown to be antigenically and genetically closely related to enteropathogenic TGEV but has different tissue tropism. PRCV is different from TGEV in that PRCV has a 621-681 nucleotide deletion in the 5' end of the S gene and deletions in gene 3 and 3-1 which render these mRNAs truncated or undetectable in the infected cells (Britton et al., 1991; Rassachaert et al., 1990; Sanchez et al., 1992; Laude et al., 1993; Paul et al., 1992; Paul et al., 1994; Vaughn et al., 1994; Vaughn et al., 1995; Wesley et al., 1990a; Wesley et al., 1990b). The deletion in the S gene is believed to play a role in the change of tissue tropism of PRCV. The deletion in the S gene in PRCV covers a stretch of 224 amino acids which contains one sialic acid binding site. The sialic acid binding site may contribute to the enterotropism of TGEV (Shultze et al., 1996). Sanchez et al. (1992) have proposed that four amino acid residues (92, 94, 218, and 219) in the S protein of TGEV are important in the binding of virus to enterocytes and that the tropism of certain strains of TGEV may be shifted from the intestinal tract to the respiratory tract. These amino acids are within the deletion region of PRCV.

There are several explanations for the change of tissue tropism of PRCV. Aminopeptidase-N (APN), one of the TGEV receptors, is most likely to be expressed on the
cell membrane of respiratory epithelial cells and alveolar macrophages (Laude et al., 1993) and PRCV may use APN as a receptor to enter the cells. The murine carcinoembryonic antigen gene family member 1 was the first protein to be shown to serve as receptor for MHV. MHV has also been shown to use murine carcinoembryogenic antigen gene family member as an alternate receptor to gain entry into cells (William et al., 1991; Yokomori and Lai, 1992). Therefore, there is a possibly that PRCV may use an alternative receptor other than APN to enter the cells. The N-terminal domain of the S gene may contribute to the stability of the S protein in the digestive tract (Laude et al., 1993). The deletion in this region may reduce the ability of the virus to attach and fuse with the cell membrane of the enterocytes. The deletion of gene 3 and 3-1 may play a role in the virus restriction after penetration into the enterocytes (Laude et al., 1993).

There are antigenic similarities between TGEV and PRCV and the fact that there is a dramatic reduction in the number of TGEV outbreaks (Laude et al., 1993), raises the question whether PRCV can be used as a vaccine against TGEV. There is a controversy about the ability of PRCV to induce lactogenic immunity against TGEV. In some studies, sows developed immunity against TGEV and secreted neutralizing antibodies into their milk, especially IgA after infection (Callebaut et al., 1990; Laude et al., 1993). In contrast, Paton and Brown (1990) reported that PRCV-vaccinated sows did not provide passive immunity against TGEV to their piglets. Recently several reports have shown that PRCV-vaccinated sows did provide passive immunity against TGEV to their piglets that reduced the mortality rate in piglets dramatically (Lanza et al., 1995; Sestak et al., 1996; Wesley and Woods, 1993, 1996). Therefore, sows infected with PRCV several times may develop and maintain a level of IgA in the milk (Laude et al., 1993).

The ability of PRCV to induce intestinal immunity to TGEV is unclear. PRCV-vaccinated pigs did not show reduction of viral antigen in the feces (Van Nieuwstatdt et al.,
PRCV strains may vary in their ability to cause disease. TLM83 virus was believed to be nonpathogenic as if was isolated from a clinically normal pig (Pensaert et al., 1986). ISU-1 PRCV was replicated in the lung of experimentally inoculated pigs but failed to cause significant respiratory lesions (Hill et al., 1989). However, certain strains of PRCV induced clinical disease and lung lesions in experimentally inoculated pigs (Cox et al., 1990; O’Toole et al., 1989; Halbur et al., 1993b). The lung lesions in the experimentally inoculated pigs were characterized by moderate-to-severe, tan-to-plum-colored mottled consolidated areas with irregular borders and most commonly involved the cranial, middle, and accessory lung lobes (Halbur et al., 1993b). The histopathologic changes were characterized by bronchial and bronchiolar epithelial necrosis, squamous metaplasia and proliferation, peribronchiolar and perivascular accumulation of mononuclear cells, septal infiltration with mononuclear cells, type II pneumocyte proliferation, and bronchiolar and alveolar lymphohistiocytic exudate (Halbur et al., 1993b; Jabrane et al., 1994). The other histopathologic changes were patchy nasal turbinate epithelial dysplasia with loss of cilia and mild submucosal lymphohistiocytic inflammation (Halbur et al., 1993b). The lower virulence strain induced less severe lung damage and no nasal turbinate lesions.

PRCV has been shown to replicate in several tissues and organs. In experimentally inoculated pigs, PRCV has been isolated from the lung, trachea, nasal mucosa, tonsils, stomach, small intestine, lymph nodes, thymus, spleen, liver, kidney, and leukocytes (Cox et al., 1990a; O’Toole et al., 1989). However, the primary replication site of PRCV is respiratory tissues and infected cells include bronchiolar epithelial cells, type II pneumocytes, alveolar and septal macrophages, and type I pneumocytes (Cox et al., 1990a;
PRCV antigen has also detected in unidentified cells in the lamina propria of the small intestines (Cox et al., 1990b). The replication of PRCV in the gastrointestinal tract is limited and, unlike TGEV, PRCV does not destroy villous enterocytes. Cox et al. (1990b) proposed that ingestion and/or viremia are the possible mechanisms for PRCV to spread from the respiratory tract to the gastrointestinal tract.

**Methods for the Detection of TGEV and PRCV**

TGEV can be detected by several methods. The immunofluorescence technique and the serologic diagnosis are the methods of choice because of sensitivity and specificity. The diagnosis of TGEV has become more complicated since the discovery of PRCV, because these 2 viruses have antigenic similarities. There are several methods to detect TGEV and PRCV such as electron microscopy, viral isolation, antigen detection, serology, and viral nucleic acid detection. Each of these techniques has advantages and disadvantages.

Virus isolation has been used extensively for TGEV diagnosis, however many TGE virus isolates replicate poorly in cell culture (Vaughn et al., 1993). Several cell lines have been used for the isolation of TGEV and PRCV. These include primary and secondary porcine kidney cells, continuous kidney cell lines, McClurkin swine testicular cell line (ST), primary porcine salivary gland cells, and porcine thyroid cells (Saif & Wesley, 1994). ST cells are the cells of choice for isolation and identification of TGEV and PRCV. Lungs or small intestines are homogenized in Eagle's minimal essential medium (20% w/v), clarified by centrifuge at 1,000 X g for 10 min, and filtered through a 0.22 μm filter. Nasal swabs are the samples of choice for isolation of PRCV. Three-to five-day-old ST cell monolayers in 25 cm² flasks are inoculated with 0.2 ml of filtrate for 1 hour at 37°C, and then the monolayer is washed and new medium is added. The cultures are incubated at 37°C for 48 hours and
observed daily for cytopathic effect. The cytopathic effect of TGEV and PRCV in ST cells is characterized by rounding to elongation of cells and syncytial cell formation. If no cytopathic effect is observed, the cells in the flasks are frozen and thawed 3 times, and cell lysates are inoculated onto new ST cell monolayers. Samples are passed 3 times or more. The presence of TGEV and PRCV can be confirmed by a variety of immunological and genetic techniques discussed below.

The detection of TGEV and PRCV antigen in tissue or cell culture is probably the simplest and most commonly used method. There are several antigen detection techniques for TGEV such as immunofluorescence technique, immunohistochemistry technique and immunogold technique. The fluorescence antibody (FA) technique is the most commonly used method for TGEV diagnosis because it is sensitive, very rapid and inexpensive. This technique can be done as a direct or an indirect method. The direct FA technique uses anti-TGEV antibody which is conjugated with fluorescein. The IFA technique uses anti-TGEV antibody as a primary antibody and fluorescein-labeled secondary antibody to detect the primary antibody. For both techniques, frozen tissues are sectioned using a cryostat-microtome at -20°C. Sections are placed on clean glass slides, fixed in chilled acetone, and dried. In the case of the direct FA technique, sections are incubated with anti-TGEV antibody in a humidified chamber, washed, dried, and examined by using a fluorescence microscope. For the IFA technique, sections are incubated with fluorescein-labeled secondary antibody before examining with a fluorescence microscope. TGEV antigen has been detected in the mature absorptive enterocytes but not in the crypt epithelial cells (Bohl et al., 1989; Frederick et al., 1976; Morin et al., 1973; Pensaert et al., 1970; Saif & Wesley, 1994; Shepherd et al., 1979; Woods et al., 1981). This technique is also used for detection of PRCV in tissues by using an anti-TGEV antibody (Cox et al., 1990a; Cox et al., 1990b). PRCV antigen is detected mainly in alveolar cells but also to a lesser extent in epithelial
cells of nasal mucosa, trachea, bronchi and bronchioles, alveolar macrophages, and in tonsils. The antigen is also found in unidentified cells located in the lamina propria of the small intestines. Major problems with this technique are the lack of sensitivity and specificity of the antibody, loss of infected cells due to the quality of sample or loss during processing, poor definition of tissue morphology and cell type, lack of permanent preparation, and cross-reaction with feline infectious peritonitis virus (FIPV), or canine coronavirus (CCV) (Horzinek et al., 1982; Woods et al., 1981) and PRCV (Callebaut et al., 1988).

There are several immunohistochemistry (IHC) techniques for the detection of TGEV and PRCV in tissue sections, such as immunoperoxidase and immunogold. Immunoperoxidase-base antigen detection techniques have been done using frozen tissues for TGEV (Chu et al., 1982) and PRCV (Van Nieuwstadt et al, 1989). Frozen tissues are treated as in the FA technique, except for the detection step where the peroxidase-antiperoxidase system is substituted for fluorescein labeled antibody. The result can be seen by the color development which is the result of the reaction between peroxidase, hydrogen peroxide and substrate (diaminobenzidine). The distribution of the TGEV antigen is similar to the FA technique and the distribution of the PRCV antigen is found mainly in bronchiolar epithelial cells and alveolar macrophages (O'Toole et al., 1989) which is different from the FA results reported by Cox et al. (1990). Recently, IHC can be used with formalin-fixed paraffin-embedded tissues (Shoup et al., 1996). Tissues are sectioned, deparaffinized, and rehydrated as in the immunoperoxidase technique. The primary monoclonal antibody to nucleocapsid protein is applied to the tissue sections and followed by application of biotinylated secondary antibody. Sections are treated with peroxidase labeled streptavidin (LSAB) and are incubated with hydrogen peroxide and 3- amino-9-ethylcarbazole. The cytoplasm of infected cells stain red-brown. The viral antigen distribution is similar to the FA and immunoperoxidase technique of frozen tissue. Tissue
morphology and cellular detail is far superior in the formalin-fixed tissues. This technique has several advantages over the FA technique because it can be used with formalin-fixed paraffin-embedded tissues, for retrospective study, and with a concurrent study of viral antigen distribution and histopathologic changes.

For the immunogold silver staining method (IGSS), formalin-fixed paraffin embedded sections are treated with protease, stained with anti-TGEV antibody and incubated with colloidal gold-labeled protein A (Larochelle et al., 1993). Then, the sections is incubated with a silver enhancement solution. The distribution of antigen positive cells is similar to the method mentioned above. This method has advantages compared to immunoperoxidase and immunohistochemistry in that it is safer and can be used for electron microscopy. Both IHC and immunogold techniques have a major disadvantage in that the IFA, IHC, and immunogold techniques provide no differentiate between TGEV and PRCV.

Serologic diagnosis can assist in control of TGEV at the herd level. Unfortunately, the emergence of PRCV has made the serologic diagnosis for TGEV more complicated because of the antigenic similarities of both viruses. The monoclonal antibody studies have shown that certain antigenic sites on TGEV are not present on the spike protein of PRCV (Laude et al., 1988; Callebaut et al., 1989; Sanchez et al., 1990). Thus the blocking ELISA has been developed for serologic diagnosis, to distinguish pigs infected with TGEV from those with PRCV. In the blocking ELISA, TGEV antigen is used to react with serum from pigs infected with either TGEV or PRCV and followed by the differentiating monoclonal antibody (Callebaut et al., 1989; Simkins et al., 1993). The principle is TGEV antiserum competing with the differentiating monoclonal antibody, whereas PRCV antiserum allow the differentiating monoclonal antibody to bind with the TGEV antigen. Briefly, TGEV antigens or ST cells inoculated with TGEV were coated or grown in the 96-well plate, washed, and incubated overnight at room temperature or 4°C for 17 hours with 2-fold dilution of sera
sample starting at 1:2.5 or 1:10 dilution (Callebaut et al., 1989; Simkins et al., 1993). After incubation and without removing the solution in the well, the differentiating monoclonal antibody, with or without biotin labeling, was added to each well and incubated at 37°C for 90 minutes (Callebaut et al., 1989) or 25°C for 1 to 2 hours to remove the solution in the wells (Simkins et al., 1993). In case of unlabeling differentiating monoclonal antibody, the 96-well plate was washed and incubated with goat anti-mouse IgG horseradish peroxidase. After rinsing, each well was incubated with chromogenic substrate. The results were read by an ELISA reader using the desired wave length.

TGEV in the intestinal contents and feces can be detected by using negative-staining electron microscopy (Saif et al., 1977). But this technique is not very sensitive and required well-trained personnel to conduct the test. The immuno-electron microscopy (IEM) technique has been used to detect TGEV in clinical specimens (Saif et al., 1977). This technique is as sensitive as IFA. But this technique can not differentiate between TGEV and PRCV.

Recently, the rapid progress in molecular biology techniques provide additional tools for the detection of the desired nucleic acids. These techniques are nucleic acid hybridization, a probe technology, and polymerase chain reaction (PCR). Dot and slot blot hybridization are nucleic acid-based techniques that have been developed to detect and differentiate between TGEV and PRCV (Vaughn et al., 1996). Total intracellular RNAs are isolated from infected ST cells by guanidinium isothiocyanate and centrifugation through a CsCl cushion (Wesley et al., 1990). The total RNAs are then applied on the nitrocellulose membrane (Shockley et al., 1987; Vaughn et al., 1996) or nylon membrane (Wesley et al., 1991b) by using a dot blot or slot blot apparatus. The nitrocellulose membrane is air dried and baked at 76°C for 90 min in the oven or the nylon membrane is exposed to UV light for cross-linking RNA to the membrane. The membrane is prehybridized, hybridized with 32P-labeled or nonisotopic cDNA probes, and exposed with X-ray film. By using differentiating
probe for slot blot hybridization, Wesley et al. (1991b) and Vaughn et al. (1996) have been able to differentiate TGEV from PRCV.

The reverse transcriptase polymerase chain reaction (RT-PCR) is a highly sensitive technique used to detect the desired RNA. First strand cDNA is synthesized from the total RNA of TGEV- or PRCV-infected ST cells by using avian myeloblastosis virus reverse transcriptase and random primers. The cDNA-RNA hybrid is amplified by PCR with Taq DNA polymerase and specific primers for TGEV and PRCV (Paul et al., 1994; Vaughn et al., 1996; Vaughn et al., 1995). The PCR products are run in agarose gel electrophoresis, and gels are stained with ethidium bromide and viewed with UV light. The PCR product of TGEV is about 2.4 kb but the PCR product for PRCV is smaller by 600-700 nucleotides depending on the isolate (Jackwood et al., 1993; Vaughn et al., 1994; Vaughn et al., 1995). Both slot blot hybridization and RT-PCR techniques have an advantage in that they distinguish TGEV from PRCV, but they do not provide the topology and cell type that become infected by the virus. Lai et al. (1995) reported that the use of amplication refractory mutation system PCR coupled with restriction fragment length polymorphism analysis can differentiate virulent, attenuated or vaccine strains of TGEV and PRCV.

The History of Arteriviruses

The arteriviruses are enveloped, plus-stranded RNA viruses that belong to the Arteriviridae family of viruses (Pringle, 1996), and have been shown to cause diseases in monkeys, horses, pigs and mice (Plageman, 1996). Earlier, arteriviruses were classified as a genus in the family of Coronaviridae (Holmes and Lai, 1996). The equine arteritis virus was the first arterivirus isolated from the lungs of an aborted foal in 1953 (Doll et al., 1957). Later, lactate dehydrogenase-elevating virus (LDV) was isolated as a contaminant of transplant tumors in mice (Riley et al., 1960), and simian hemorrhagic fever virus (SHFV)
was isolated from macaque monkeys in 1964 (Palmer et al., 1968). Recently, PRRSV was isolated from pigs in the United States in 1987 (Hill, 1990) and in Germany in 1990 (Wensvoort, 1993).

**Arteriviruses**

Arteriviruses are spherical, enveloped viruses with a diameter of 50-65 nm and a cubical nucleocapsid core with a diameter of 25-35 nm (Plageman, 1996). Arterivirus particles have a sedimentation coefficient of between 200 and 300 and buoyant density in sucrose or glycerol of 1.13 to 1.14 g/ml for LDV and PRRSV and 1.15-1.17 g/ml for EAV (Plageman and Moennig, 1992; Sagripanti, 1984). The arterivirus virions are not stable in solutions containing low concentrations of detergents (Britton-Darnell and Plageman, 1975; Plageman and Moennig, 1992). Arteriviruses do not agglutinate erythrocytes of any species (Plageman, 1996). Table 2 shows the members and natural host of arteriviruses.

**TABLE 2. Arteriviruses: Abbreviations, names, and hosts**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>Host</th>
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<tbody>
<tr>
<td>LDV</td>
<td>Lactate dehydrogenase Elevating virus</td>
<td>Mice</td>
</tr>
<tr>
<td>EAV</td>
<td>Equine arteritis virus</td>
<td>Horse</td>
</tr>
<tr>
<td>PRRSV</td>
<td>Porcine reproductive and respiratory syndrome virus</td>
<td>Pig</td>
</tr>
<tr>
<td>SHFV</td>
<td>Simian hemorrhagic Fever virus</td>
<td>Monkey</td>
</tr>
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The genomic RNA of arteriviruses is approximately 13 to 15 kb, linear, single-stranded, positive-sense RNA with polyadenylation (Britton-Darnell and Plageman, 1975; Meulenberg et al., 1993; Sagripanti, 1984; Van der Zeijst et al., 1975). The genomic organization and expression of the genomes of arteriviruses resemble those of coronaviruses (Plageman, 1996). At least six or seven subgenomic mRNAs are expressed in the infected cells. Each subgenomic mRNA has a common 5’ untranslated leader derived from the 5’ end of the virus genome and a common 3’ untranslated sequence which is polyadenylated. However, the mechanism of synthesis of the subgenomic mRNAs is not known. Nevertheless, it has been demonstrated that the 5’ leader is involved in the synthesis of subgenomic mRNAs. The 5’ leader has been shown to join the body of each subgenomic mRNA within or upstream of a specific heptanucleotide at its 3’ end. The 5’ leaders of EAV, PRRSV, SHFV, and LDV are 212, 211, 208, and 156 nucleotides long, respectively (Plageman, 1996). The 5’ leader junction segments of arteriviruses are similar. The 5’ leader junction segment is UAUAACC for LDV, UUUACC for PRRSV, and AUCAACU for EAV (Plageman, 1996). This process generates 5’ untranslated sequences up to 320 nucleotides in each mRNA (Plageman, 1996). The 3’ untranslated sequence of the genomes of PRRSV, LDV, SHFV, and EAV are 117-154, 80, 76, and 62 nucleotides in length, respectively (Plageman, 1996), and terminate with a poly(A) tail. The poly(A) tails for LDV vary from 40 to 80 nucleotides (Chen et al., 1994). Each of ORFs 2 to 7 overlaps to some extent which is different from coronaviruses.

The ORF 1 of arteriviruses is approximately 10 to 11 kb and encodes for two proteins: 1a and 1b. The ORF 1b protein is synthesized via a frameshift mechanism involving slippery heptanucleotide sequence (CUUAAAC) and pseudoknot (Plageman, 1996). Several putative domains are found in the ORF1a/1b proteins. These domains
include "papain-like cysteine protease and 3C-like serine protease in the ORF 1a" and "replicase, helicase, and zinc finger motif in the ORF 1b" (Plageman, 1996). The ORF 1a of LDV, PRRSV, and EAV has 11 to 12 potential transmembrane domains which flank the serine protease domain (Palmer et al., 1995).

The ORF 2 protein is a minor envelope glycoprotein which possesses a signal peptide and a C-terminal transmembrane domains with one to four potential N-glycosylation sites (Plageman, 1996). ORFs 3 and 4 encode for small glycoproteins of unknown functions.

ORF 5 encodes for the primary envelope glycoprotein which possesses a potential signal peptide and three potential internal transmembrane domains (Plageman, 1996). The ORF 5 protein has at least one to three potential N-glycosylation sites which project externally. Antibodies against the ORF 5 protein of EAV and LDV have been shown to be able to neutralize EAV and LDV infectivity (Plageman, 1996). The ORF 5 protein probably acts as a cell attachment protein. There is an internal sequence of the ORF 5 protein which has approximately 73 to 82 amino acids and may play a role in budding of the nucleocapsids into the internal space of the RER and the Golgi (Plageman, 1996).

ORF 6 encodes for a basic nonglycosylated protein which has a very short N-terminal ectodomain. The structure of the ORF 6 protein is similar to the M protein of coronaviruses in that it has three closely adjacent transmembrane domains near the N-terminal end. The ORF 6 protein has been shown to be associated with the ORF 5 protein via disulfide bonds (Plageman, 1996). Disulfide linkage between these 2 proteins is required for viral infectivity of LDV because the infectivity is decreased dramatically after treatment with dithiothreitol for 6 hours at room temperature (Faaberg et al., 1995). Therefore, the interaction of these two proteins may generate a virus receptor for attachment to the cell membrane (Plageman, 1996). ORF 7 encodes for nucleocapsid protein which is a highly basic phosphoprotein.
All arteriviruses, except EAV, replicate in primary macrophage cultures (Plageman, 1996). It has been shown that CL-2621 and MARC-145 cells are susceptible to PRRSV, EAV, and SHFV. The CRL11171 cell is also susceptible to PRRSV (Meng et al., 1996a). This finding raises the question whether PRRSV, EAV, and SHFV might recognize the same receptor. However, LDV fails to infect the CL-2621 and MARC-145 cells. The first sign of virus infection in macrophages is the formation of free and membrane-bound polyribosomes in the perinuclear region as well as double-membrane vesicles 100 to 300 nm in diameter (Plageman, 1996). Later, nucleocapsid cores bud into single membrane vesicles to form mature virus particles in the area close to double-membrane vesicles and the Golgi. The formation of double-membrane vesicles is a unique feature of arteriviruses but their origin is not known. The mature viruses appear to release from the infected cells by exocytosis (Plageman and Moennig, 1992).

Porcine Reproductive and Respiratory Syndrome Virus

Porcine reproductive and respiratory syndrome (PRRS) is a new disease characterized by severe reproductive failure (increased numbers of stillborn, mummified, and weak-born piglets), and respiratory diseases in young and growing pigs. PRRS was first recognized in the U.S. in 1987-88, and was first named “mystery swine disease” because of its unknown origin (Dial et al., 1990; Hill, 1990; Keffaber, 1989; Loula, 1991). Since then it has been reported in Canada, Germany, the Netherlands, Belgium, Britain, Spain, and Japan (Hill, 1990; Kuwahara et al., 1994; Ohlinger et al., 1992; Paton et al., 1991; Wensvoort et al., 1991; Wensvoort, 1993). The disease has been known by a number of names including porcine epidemic abortion and respiratory syndrome (PEARS), blue-eared disease (Terpstra et al., 1991), swine infertility and respiratory syndrome (SIRS) (Collin et al., 1991), or “Heko-heko” disease (Kuwahara et al., 1994).
Several pathogens were incriminated as the potential causative agents of PRRS, including encephalomyocarditis virus (EMCV), hemagglutinating encephalomyelitis virus (HEV), porcine parvovirus (PPV), atypical swine influenza virus (aSIV), porcine enterovirus (PEV), pseudorabies virus (PRV), and porcine cytomegalovirus (PCMV) (Keffaber, 1989; Joo et al., 1990; Mengeling and Lager, 1990; Woolen et al., 1990; Dea et al., 1991; Benfield et al., 1992; Meng et al., 1993). However, these viruses were proven not to be the causative agent of PRRS.

There was a general consensus at that time that the causative agent of PRRS was an unidentified virus (Wenvoort et al., 1991; Collin et al., 1991). In 1991, the causative agent of PRRS was isolated and designated as the Lelystad virus (LV). The LV was shown to be the causative agent in experimentally infected pigs (Terpstra et al., 1991). In the United States, the PRRS virus (PRRSV) was isolated in a continuous cell line (Benefield et al., 1992; Collins et al., 1992; Meng et al., 1996a) and was shown to be the causative agent of PRRS in the United States (Christianson et al., 1992). Both LV and PRRSV have been shown to have structural similarities and common antigens, however these viruses are antigenically and genetically distinct (Wensvoort et al., 1992).

PRRSV is a small pleomorphic spherical enveloped virus, 50-80 nm in diameter, with an isometric core of about 25-30 nm (Benfield et al., 1992; Dea et al., 1995; Mardassi et al., 1994a; Meng et al., 1996a; Wensvoort et al., 1991). PRRSV is a member of the family Arteriviridae (Pringle, 1996) because the morphology of the virus, the genomic organization, the strategy of gene expression, and the sequence of deduced proteins closely resemble lactic dehydrogenase virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV) (Conzelman et al., 1993; Thiel et al., 1993). All of the arteriviruses infect macrophages (Plagemann and Moennig, 1992), are capable of producing long lasting, asymptomatic infections and demonstrate strain variation. Buoyant density of the virus in a
CsCl₂ density gradient was estimated to be 1.18-1.20 g/ml (Benfield et al., 1992; Mardassi et al., 1994a; Meng et al., 1996a; Wensvoort et al., 1991). By using radioimmunoprecipitation and western immunoblotting four major viral proteins were revealed, including a 15 kDa nucleocapsid protein and 3 other proteins with molecular masses of 19, 26 and 42 kDa, which varied slightly among different PRRSV isolates (Benfield et al., 1992; Nelson et al., 1993; Mardassi et al., 1994a; Meng et al., 1995a; Meulenberg et al., 1995).

PRRSV is inactivated by a lipid solvent (chloroform), indicating the presence of a lipid containing envelope. PRRSV is stable at 4°C for one month and at -70°C for 4 months (Benfield et al., 1992; Yoon et al., 1992). The virus was inactivated after incubation at 37°C for 24 hours and at 56°C for 20 min (Benfield et al., 1992; Yoon et al., 1992). The virus infectivity titers decrease by over 90% at a pH less than 5 or higher than 7 (Benfield et al., 1992). PRRSV was recovered only on day 0 from contaminated fomites (alfalfa, wood shavings, straw, plastic, boot rubber, and stainless steel as well as saliva, urine, and fecal slurry (Pirtle and Beran, 1996). PRRSV was recovered from buffer solutions through day 6, from well water through day 9, and from city water through day 11 (Pirtle and Beran, 1996).

PRRSV persistently circulates in swine operations and may be transmitted by making contact with infected replacement animals, or via infected seed stock sold to farms where all animals are uninfected (Bilodeau et al., 1994). PRRSV can be detected in tracheal fluid, feces, saliva, and oropharyngeal fluid up to 35, 42, and 157 days (Will et al., 1997; Rossow et al., 1994a) postinoculation, respectively. It has been shown that rodents are not susceptible to infection with PRRSV and therefore are probably not a reservoir for the disease (Hooper et al., 1994). Recently, Mallard ducks have been shown to be highly susceptible to PRRSV, but guinea fowl and chickens were only marginally susceptible and Muscovy ducks were totally resistant (Zimmermann et al., 1997). Infected Mallard ducks can transmit the virus from mallard-to-mallard and mallard-to-pig.
The cellular receptor for PRRSV is not known, however, the entry process of PRRSV involves a microfilament-dependent endocytic mechanism (Kreutz and Ackermann, 1996). Viral uncoating requires a low pH environment (Kreutz and Ackermann, 1996). Infection can be inhibited by addition of heparin to PRRSV or to the MARC-145 cells before inoculation or treatment of cells with heparinase (Jusa et al., 1997). The viral RNA replication was inhibited by chloroquine, ammonium chloride, and bafilomycin A1 (Kreutz and Ackermann, 1996).

The nucleic acid of PRRSV is a single-stranded, positive-sense polyadenylated RNA genome which has a size of 15 kb (Conzelmann et al., 1993; Meng et al., 1994; Meulenberg et al., 1993). The full-length negative-sense RNA is first synthesized from the positive-sense RNA genome after infection, and is used as the template for synthesis of the 3'-terminal nested set of at least 6 subgenomic mRNAs (Conzelman et al., 1993; Meng et al., 1994, 1996b; Meulenberg et al., 1993). The subgenomic mRNAs, with the exception of the smallest one, are polycistronic in nature, and only the unique 5' open reading frame is translated (Meng et al., 1996b). The 5' end of each subgenomic mRNA does not overlap the sequence of the smaller subgenomic mRNAs. The 5' end of each subgenomic mRNA contains a 5' common leader sequence of about 200 bp in size (Meulenberg et al., 1993). The junction of that leader sequence joins with the body of each subgenomic mRNA, and the leader-mRNA junction, and has been shown to be a conservative sequence motif of six nucleotides, UCAACC, or a highly similar sequence (Meng et al., 1996b, 1995b; Meulenberg et al., 1993). In some isolates, an additional subgenomic mRNA, designated as subgenomic mRNA 3-1, is produced in the infected cells (Meng et al., 1996b).

PRRSV has eight open reading frames (ORFs) which are organized in the same manner as in coronaviruses (Lai, 1990; Meulenberg et al., 1993). These ORFs overlap each other (Conzelmann et al., 1993; Meng et al., 1994; Meulenberg et al., 1993), however, some isolates have a 10 bp noncoding region separating ORF 4 and ORF 5 (Meng et al.,
ORFs 1a and 1b are the largest of all ORFs comprising about 80% of the PRRSV genome and are thought to encode the viral replicase (Meulenberg et al., 1993). At the N-terminus of ORF 1a, replicase protein of PRRSV, there was shown to be two adjacent papain-like cysteine protease (PCP alpha and PCP beta) domains (Den Boon et al., 1995). Like PRRSV, LDV has both PCP domains but the EAV PCP alpha counterpart may be inactivated by the loss of its catalytic Cys residue (Den Boon et al., 1995).

LV has been demonstrated to contain six virion-associated proteins, one nucleocapsid protein, one integral membrane protein, and four glycoproteins (Meulenberg and Petersen Den Besten, 1996). ORFs 2, 3 and 4 are predicted to be membrane-associated proteins because the N-termini of these ORFs have a hydrophobic region, and at least one additional hydrophobic domain toward the C-termini which may function as signal sequence and membrane anchors (Meng et al., 1995b; Meulenberg et al., 1993; Morozov et al., 1995). In addition, these ORFs contain putative N-linked glycosylation sites (Conzelmann et al., 1993; Meng et al., 1995b; Meulenberg et al., 1993; Morozov et al., 1995). The predicted size of the products of these ORFs are 29.5 kD (ORF 2), 28.7 kD (ORF 3), and 19.5 kD (ORF 4) (Morozov et al., 1995). The protein of ORF 2 of LV, designated GP2, can be found in infected cell lysates by immunoprecipitation using a specific antibody. The GP2 protein has a molecular mass of 29 to 30 kD and has been shown to be N-glycosylated (Meulenberg and Petersen den Besten, 1996). The GP2 protein is sensitive to endoglycosidase H treatment in the endoplasmic reticulum but it becomes resistant to endoglycosidase H treatment during passage through the Golgi apparatus (Meulenberg and Petersen den Besten, 1996). Drew et al. (1995) using a monoclonal antibody against ORF 3 demonstrated a 45 kD protein, determined to be the product of ORF 3, in both infected cell lysates and purified virion preparations by using
radioimmunoprecipitation and Western blotting techniques. The monoclonal antibodies against ORF 3 recognize only a few PRRSV isolates, indicating that this protein is antigenically polymorphic (Drew et al., 1995). Recently, products of ORFs 3 and 4 (designated as GP3 and GP4, respectively) have been shown to be part of the virion (Van Nieuwstadt et al., 1996). By Western blotting technique, two structural proteins with molecular mass of 45 to 50 kD (ORF 3) and 31 to 35 kD (ORF 4) have been detected (Van Nieuwstadt et al., 1996). The molecular mass of GP3 and GP4 are reduced to 29 kD and 16 kD after N-glycosidase F digestion, respectively (Van Nieuwstadt et al., 1996). Monoclonal antibody against GP4 neutralized LV, which indicates that at least part of this protein is exposed at the virion surface (Van Nieuwstadt et al., 1996).

ORF 5 also contains the N- and C-terminal hydrophobic regions that may function as a signal sequence and a membrane anchor (Meng et al., 1994; Meulenberg et al., 1993) and has a molecular mass of 24 to 26 kD (Nelson et al., 1993). Recently, the product of ORF 5 of both LV and PRRSV, designated as the E protein or GP5, was found to be N-glycosylated (Mardassi et al., 1996; Meulenberg et al., 1995). The E protein is glycosylated after its association with the product of ORF 6 (M protein) in the endoplasmic reticulum (Mardassi et al., 1996). The E protein was shown to be delayed in the premedial Golgi compartment using a pulse-chase experiment (Mardassi et al., 1996). The E protein undergoes reduction in molecular mass due to trimming of the mannose-rich glycan structure. Since the E protein is transported to medial or proximal Golgi compartments, some molecules undergo complete processing of all high-mannose N-linked oligosaccharides to complex types, while in other molecules only a fraction of N-linked are terminally glycosylated. These two species of E protein were found to be incorporated into extracellular virions (Mardassi et al., 1996). The M protein is not glycosylated and is probably a type III integral membrane protein. The M protein has molecular mass of 18 to
19 kD, and has three hydrophobic domains which may be used to anchor to the viral envelope (Meulenberg et al., 1995; Nelson et al., 1993). The M protein has been shown to form heterodimeric complexes, with the E protein, by disulfide bonds (Mardassi et al., 1996). In the infected cells, the M protein was shown to be highly membrane-associated (Mardassi et al., 1996). ORF 7 encodes a highly basic, hydrophilic protein and has a molecular mass of 15 kD (Nelson et al., 1993; Meulenberg et al., 1995). The product of ORF 7 was shown to be the nucleocapsid protein (N) which elicited a strong immune response. The N protein is not glycosylated (Mardassi et al., 1996; Meulenberg et al., 1995) and is predominantly present in the cytoplasm (Mardassi et al., 1996).

Genetic variation exists not only between U.S. and European PRRSV isolates but also among U.S. and European PRRSV isolates as well (Mardassi et al., 1994a; Meng et al., 1994, 1995a; Morozov et al., 1995; Murtaugh, 1993). The amino acid sequence homology of ORFs 2 to 7 between U.S. and European PRRSV isolates was 55% (Morozov et al., 1995), 52-64% (Mardassi et al., 1995; Morozov et al., 1995), 64-66% (Kwang et al., 1994; Morozov et al., 1995), 52-54% (Mardassi et al., 1995; Meng et al., 1994), 78-81% (Meng et al., 1994, 1995a) and 57-59%, respectively (Mardassi et al., 1995; Meng et al., 1994, 1995a). Therefore, U.S. and European PRRSV isolates represent two distinct genotypes (Meng et al., 1995a; Suarez et al., 1996b). The amino acid sequence homology of ORFs 6 and 7 of the U.S. and Canadian isolates is 96-100% (Meng et al., 1995a). The amino acid sequence homology of ORFs 2 to 5 among the U.S. PRRSV isolates is 91-99%, 86-98%, 92-99%, 88-97%, respectively (Meng et al., 1995b; Andreyev et al., 1997). The amino acid sequence homology of ORFs 5 and 7 of the European PRRSV isolates is 88% and 95.3-100% (Suarez et al., 1996b). For both U.S. and European PRRSV isolates, ORFs 5 has the highest amino acid sequence variability (Kapur et al., 1996; Meng et al., 1995b; Suarez et al., 1996b) and ORF 6 has the least amino acid sequence variability (Kapur et al.,
The intragenic recombination is the mechanism behind the sequence variation of the ORFs 2, 3, 4, 5, and 7 of the U.S. isolates (Kapur et al., 1996; Meng et al., 1995a; Morozov et al., 1995). An antigenic variation has been shown between the North American and European PRRSV isolates (Le Gall et al., 1997; Magar et al., 1995, 1997; Wensvoort et al., 1992). Monoclonal antibodies against the nucleoprotein clearly discriminated between the North American and European isolates (Dea et al., 1996; Drew et al., 1995; Nelson et al., 1993; Wieczoreck-Khromer et al., 1996). The European isolates are antigenically similar to each other, but differ from the U.S. isolates. The U.S. isolates are antigenically more diverse than the European isolates (Wensvoort et al., 1992; Yoon et al., 1995a). A serologic survey by IFA found that about 20% of the sera were positive for the European isolates, about 44% were positive for VR2332, and about 20.6% were positive for both VR2332 and the European isolates (Bautista et al., 1993a). By Western blot analysis, the ORF 4 protein of isolate MN-1 reacted with only 65% of PRRSV-infected pig sera (Kwang et al., 1994). Therefore, the antigenic variation may complicate the diagnostic result and lead to misdiagnosis (Yoon et al., 1995a).

The pathogenesis of PRRSV-induced reproductive failure has remained unsolved. The aerosol inoculation of pregnant sows with LV caused anorexia, development of reddish-blue blotches on the ears, abdominal respiration, stillbirths, mummified fetus, and weak piglets (Plana Duran et al., 1992; Terpstra et al., 1991; Wensvoort et al., 1991). By experimental intranasal inoculation of pregnant sows, U.S. PRRSV strain ATCC VR-2332 was also shown to cause anorexia, premature birth, stillborn, and dead fetuses (Christianson et al., 1992). It has been shown that PRRSV causes transplacental infection after a wide period of gestation, for example, 30-90 days (Christianson et al., 1992, 1993;
Dea et al., 1992; Lager et al., 1995; Mengeling et al., 1994; Plana Duran et al., 1992; Terpstra et al., 1991; Yoon et al., 1992).

In pregnant sows infected with PRRSV, the histopathologic changes were lymphohistiocytic endometritis and placentitis with vasculitis (Stockhofe-Zurwieden et al., 1993). Similar but less severe changes were observed in the myometrium. There were also multifocal areas of microseparation, with degeneration, in the maternal-fetal contact area. At the microseparation area, there were necrotic epithelial cell and lymphohistiocytic cell infiltration. By transmission electron microscopy, there was hydropic degeneration, and sometimes necrosis and desquamation of epithelial cells of the maternal and fetal placenta, at the microseparation areas (Stockhofe-Zurwieden et al., 1993). The ultrastructural changes of the trophoblastic and maternal epithelium were characterized by dilatation of the endoplasmic reticulum, swollen mitochondria, granular material in the cytoplasm, dilatation of the intercellular channel system, and blunt villi. PRRSV-like particles were observed in the luminal cytoplasmic membrane of endothelial cells of arterioles, venules, and capillaries of the maternal placenta. The microseparation at the maternal-fetal contact area may have caused placental insufficiency and led to abortion, stillbirths, and weak born piglets.

Both U.S. PRRSV and LV have been shown to replicate in fetuses from infected sows (Christianson et al., 1992, 1993; Lager et al., 1995; Mengeling et al., 1994, 1996; Terpstra et al., 1991). It has been shown that U.S. PRRSV isolates differ in their ability to effect the reproductive tract (Mengeling et al., 1996). U.S. PRRSV strain ATCC VR-2332 caused intrauterine spread from inoculated fetuses to uninoculated fetuses (Lager et al., 1994, 1995). The umbilical cords of the infected fetuses had segmental to full length hemorrhage (Lager et al., 1996). A necrotizing umbilical arteritis with periarterial hemorrhage was present. The histopathologic changes in inoculated fetuses were marked, focally extensive hemorrhage into the mesenchymal interstitium surrounding bronchial buds,
large bronchi and blood vessels (Lager et al., 1994). The marked segmental to circumferential necrosis of smooth muscle cells and mesenchymal spindle cells subjacent to epithelial cells of bronchial buds, and moderate, focally extensive necrosis of epithelium were observed in the lungs of the inoculated fetuses. In naturally infected-cases, arteritis, myocarditis, and encephalitis were observed (Rossow et al., 1996).

Infection of susceptible boars with wild type strains or vaccine strain results in the shedding of PRRSV in semen (Christopher-Hennings et al., 1995a, 1997; Nielsen et al., 1997b; Swenson et al., 1994). Infectious virus in semen was detected after 43 days postinoculation (Swenson et al., 1994), and virus RNA in semen was detected by PCR after 92 days postinoculation (Christopher-Hennings et al., 1995b). Infected boars may have pyrexia, anorexia, coughing and loss of libido (Feitsma et al., 1992; Hopper et al., 1992). It has been shown that artificial insemination with whole or extended semen is a means of transmitting PRRSV to females (Swenson et al., 1995; Yeager et al., 1993). The source of PRRSV in semen is not known but virus has been isolated from the bulbourethral gland of experimentally inoculated boars (Christopher-Hennings et al., 1995a). The semen quality from PRRSV-inoculated boars was normal (Christopher-Hennings et al., 1997) or a temporary reduction in quality was observed (Prieto et al., 1996) but the semen quality from vaccinated boars appeared to be less than optimal (Christopher-Hennings et al., 1997). The percentage of forward movement and normal spermatozoa morphology from vaccinated boars were significantly lower than that from nonvaccinated boars. Moreover, the semen quality from vaccinated boars after challenge with PRRSV strain ATCC VR-2332 was significantly lower than that of challenged nonvaccinated boars (Christopher-Hennings et al., 1997). However, boars vaccinated with an attenuated vaccine had a significant reduction in viremia and virus shedding in semen (Christopher-Hennings et al., 1997; Nielsen et al.,
Recently, a U.S. PRRSV has been shown to replicate and to induce apoptosis in the testicular germ cells (Sur et al., 1997).

Growing pigs infected with PRRSV consistently have fever, dyspnea, abdominal respiration, lethargy, and patchy dermal cyanosis (Collins et al., 1992; Gordon, 1992; Halbur et al., 1995b, 1996b; Hopper et al., 1992, Meredith, et al., 1992). Mild to moderate consolidation of the lung and moderately enlarged lymph nodes are consistently observed in PRRSV-infected pigs (Done et al, 1995; Halbur et al., 1993a; Zeman et al., 1993). The microscopic changes in the lung are characterized by alveolar septa thickened by macrophages, type II pneumocyte hypertrophy and hyperplasia, and necrotic debris and increased number of mixed inflammatory cells in alveolar spaces (Collins et al., 1992; Halbur et al., 1995b, 1996b; Pol et al., 1991; Rossow et al., 1994a, 1995). It has been shown that PRRSV causes destruction of the bronchiolar cilia and vacuolation of bronchiolar columnar epithelium cells (Done et al., 1995). By scanning electron microscopy, the cell membrane of the infected alveolar macrophage was damaged with lost of cell integrity and collapsing of cells. The severity of the lesions in the lungs and lymph nodes vary considerably and significantly among U.S. PRRSV isolates (Halbur et al., 1995b, 1996b), and the virulent strain of U.S. PRRSV isolate (ATCC VR-2385) has been shown to induce lesions significantly more severe than that of LV (Halbur et al., 1996b).

Lymph node lesions were characterized by follicular hypertrophy, hyperplasia, lymphocyte necrosis, multiple cystic spaces, and polykaryocytes (Halbur et al., 1995b, 1996b; Rossow et al., 1994a, 1994b, 1995). Similar changes were also seen in Peyer's patches and tonsils (Halbur et al., 1995b). Lymphohistiocytic myocarditis and encephalitis have consistently been found in PRRSV-inoculated pigs (Halbur et al., 1995a, 1995b, 1996b; Rossow et al., 1994a, 1995). Lymphoplasmacytic vasculitis of the medium-sized arteries and occasionally of the veins was observed in various organs (Darbes et al., 1996; Rossow
et al., 1995). Lymphohistiocytic interstitial nephritis with interstitial edema were observed in the kidney, and was particularly most severe in the renal medulla (Cooper et al., 1997; Rossow et al., 1995). Focal, mild nonsuppurative periglomerular and peritubular infiltrates were present in renal cortices.

In the lung, the PRRSV antigen positive cells were predominantly alveolar macrophages and a few type II pneumocytes (Darbes et al., 1996; Halbur et al., 1994, 1995a, 1996a; Magar et al., 1993; Rossow et al., 1996). Moreover, endothelial cells and intravascular macrophages were also positive for viral antigen (Rossow et al., 1996). Follicular macrophages and dendritic cells in the lymph nodes were predominantly positive for viral antigen (Halbur et al., 1995a, 1996a, Rossow et al., 1996). Other tissues and cells in which virus antigen was detected included macrophages and endothelial cells in the heart, macrophages and interdigitating cells in the thymus, macrophages and dendritic cells in the spleen and Peyer's patches, and macrophages in hepatic sinusoids, renal medullary interstitium, and adrenal gland (Halbur et al., 1996a, Rossow et al., 1996).

In the lung, PRRSV RNA positive cells were predominantly alveolar macrophages (Haynes et al., 1997; Larochelle et al., 1997; Sur et al., 1996). Other tissues and cells in which viral RNA was detected include tingible body macrophages and macrophages or interdigital dendritic cells in the lymph node, macrophages at the epithelial-lymphoid interface and within the paracortical area, multinucleated cells in thymic medulla, and spindle-shaped cells within smooth muscle trabecula within the spleen (Haynes et al., 1997; Larochelle et al., 1997; Sur et al., 1996).

PRRSV-infected pigs are frequently associated with secondary infection from both bacteria and viruses. It has been shown that PRRSV predisposes pigs to infection and disease caused by Streptococcus suis (Done et al., 1995; Galina et al., 1994), Pasturella multocida (Done et al., 1995), Haemophilus suis (Done et al., 1995; Solano et al., 1997),
Mycoplasma hyopneumoniae (Zeman et al., 1993), Salmonella sp. (Zeman et al., 1993), Actinobacillus pleuropneumoniae (Zeman et al., 1993), Hemophilus parasuis (Kobayashi et al., 1996), and Mycoplasma hyorhinis (Kawashima et al., 1996; Kobayashi et al., 1996). On the other hand, there are reports that PRRSV does not increase susceptibility or severity of Mycoplasma hyopneumoniae (Van-Alstine et al., 1996), Hemophilus parasuis (Cooper et al., 1995), Streptococcus suis (Cooper et al., 1995), Salmonella cholerasuis (Cooper et al., 1995), or Pasteurella multocida (Carvalho et al., 1997) infection. It has also been shown that PRRSV potentiated swine influenza virus (Groschup et al., 1993; Van Reeth et al., 1996), porcine respiratory coronavirus (Groschup et al., 1993; Van Reeth et al., 1996), porcine paramyxovirus virus (Groschup et al., 1993), and encephalomyocarditis virus (Carlson, 1992) infection.

The mechanism of PRRSV-potentiated concurrent infection of bacteria and viruses is not known. It has been reported that there is a significant reduction of the circulating leukocytes and platelets (Christianson et al., 1992) and the percentages of CD2+, CD4+, and CD8+ cells is significantly decreased (Christianson et al., 1992; Nielsen et al., 1997a; Zhou et al., 1992). However, the reduction of these lymphocytes occurred at 3-7 days postinoculation (Nielsen et al., 1997a), or 14 days postinoculation (Zhou et al., 1992). The number of alveolar macrophages and the ability to synthesize superoxide were also significantly decreased at 7 days postinoculation but returned to normal after 28 days postinoculation (Molitor et al., 1992; Zhou et al., 1992). It has been shown that LV induces apoptosis in a continuous cell line and in swine macrophage cells (Suarez et al., 1996a). Moreover, only the product of the open reading frame 5 of the LV is enough to cause apoptosis in the continuous cell line (Suarez et al., 1996a).

Antibodies to PRRSV can be detected by indirect immunofluorescence (IFA) at 14-21 (Nelson et al., 1994) or 9-11 (Yoon et al., 1995b), by immunoperoxidase monolayer
assays (IPMA) at 5-9 (Yoon et al., 1995b), and by enzyme-linked immunoabsorbent assay (ELISA) at 9-13 (Yoon et al., 1995b) days postinoculation. The antibodies to PRRSV were not detected by IFA at 137, IPMA at 158, and ELISA at 324 days postinoculation (Yoon et al., 1995b). The antibody to a 15-kD viral protein was detected by 7 (Yoon et al., 1995b) or 7-21 (Nelson et al., 1994) days postinoculation and persisted through 105 days postinoculation. The antibodies to the 19-, 23-, and 26-kD proteins were detected from 9-35 (Yoon et al., 1995b) or 51-70 days postinoculation (Nelson et al., 1994) and persisted through 105 days postinoculation (Yoon et al., 1995). Antibodies against the E, N, and M proteins were detected as early as 7, 14, and 14 days postinoculation, respectively (Loemba et al., 1996). By IFA, specific IgM antibody against proteins N, M, and E peaked by days 14-21 postinoculation and decreased to undetectable levels by days 35-42 postinoculation (Loemba et al., 1996). The specific IgG antibody against proteins N, M, and E peaked by day 21-28 postinoculation and remained unchanged 6-9 weeks postinoculation. The N protein has been shown to be the most immunodominant PRRS virus protein (Loemba et al., 1996; Rodriguez et al., 1997; Yoon et al., 1995b).

The neutralizing antibodies were detected at 9-28 (Yoon et al., 1995b), 21-28 (Loemba et al., 1996) or 51-70 days (Nelson et al., 1994) postinoculation and were not detected at 356 or 262 (Nelson et al., 1994; Yoon et al., 1995b) days postinoculation. The antibodies to the product of ORFs 3 (Plana-Duran et al., 1997) and 5 (Pirzadeh et al., 1997; Plana-Duran et al., 1997) have been shown to produce a neutralization effect against PRRSV. Neutralizing antibodies against PRRSV have protective effect only in the continuous cell line (Christianson et al., 1993). Antisera to PRRSV failed to neutralize the infectivity of alveolar macrophage cultures or in vivo (Choi et al., 1992; Christianson et al., 1992).
The mechanism in which antibodies mediate and enhance infection rather than prevent infection is called antibody-dependent enhancement (ADE). Pigs with subneutralizing levels (neutralizing antibody titer <1:4) of PRRSV-specific antibody had higher levels and longer periods of viremia than pigs without antibodies against PRRSV, after challenge with a homologous strain (Yoon et al., 1994). There is variability in the susceptibility of PRRSV isolates to ADE (Yoon et al., 1997).

It has been shown that T cell responses coincide with the SN antibody response but rapidly decline (Bautista et al., 1997). However, immunized pigs developed memory T cells that not only respond faster on a second virus exposure but also at a higher magnitude after stimulation with PRRSV antigens (Bautista et al., 1997). Major histocompatibility molecules are required in the proliferative response to PRRSV and monocytes seem to play an important role as antigen presenting cells in PRRSV immune response (Bautista et al., 1997).

Methods for the Detection of PRRSV

There are several methods or techniques for diagnosis of PRRSV infection in addition to clinical signs and pathologic findings. These techniques are used to detect infectious virus particles, antibodies against PRRSV, PRRSV antigen, and PRRSV ribonucleic acids.

Clinical signs of PRRSV infection can be divided into two main categories: reproductive failure and respiratory disease. The clinical signs of PRRSV infection may vary from farm to farm (White et al., 1992) and subclinical infection is common (Morrison et al., 1992). Systemic signs in all age groups are anorexia, pyrexia, agalactia, lethargy and skin discoloration (Hooper et al., 1992). Respiratory signs are abdominal breathing and coughing. After 5-7 days of the clinical appearance, sows and piglets may develop transient
skin lesions which are characterized by blue (cyanosed) ears, blue vulvas, blue skin areas and erythematous plaques.

Reproductive losses involve an entire reproductive cycle within a herd and may last for 4-5 months. Premature and late farrowings are very common but abortions can be observed as early as 22 days through to 109 days post-mating. Mixtures of stillborn, mummified, decomposing piglets, and apparently normal and weak newborn piglets may be seen in a litter. Infertility problems, including delayed returns to estrus, persistent rebreeding, and persistent anestrus have been reported (Gordon, 1992; Hopper et al., 1992; Keffaber, 1989). Infected boars may show loss of libido (Fiestma et al., 1992; Hopper et al., 1992) and a temporary reduction in semen quality (Fiestma et al., 1992). The reduction in sperm output was seen by 4 weeks after the appearance of clinical signs and returned to normal by 7 weeks postinoculation (Fiestma et al., 1992).

The pathologic findings in the fetuses or placenta were segmental to full length necrotizing hemorrhagic umbilical arteritis with periarterial hemorrhage (Lager et al., 1996), marked focally extensive hemorrhage into the mesenchymal interstitium surrounding bronchial buds, large bronchi and blood vessels, necrosis of smooth muscle cells and mesenchymal spindle cells subjacent to epithelial cells of bronchial buds, and moderate focally extensive necrosis of epithelium (Lager et al., 1994), and myocarditis, and encephalitis (Rossow et al., 1996). Gross lesions typical of PRRS in young pigs include a multifocal-to-diffuse, tan mottled pneumonia, and enlarged, tan-colored lymph nodes (Done et al, 1995; Halbur et al., 1993a; Zeman et al., 1993). The microscopic changes in lungs are characterized by alveolar septa thickened by macrophages, type II pneumocyte hypertrophy and hyperplasia, and necrotic debris and increase in number of mixed inflammatory cells in alveolar spaces (Collins et al., 1992; Halbur et al., 1995b, 1996b; Pol et al., 1991; Rossow et al., 1994a, 1995). Lymph node lesions were characterized by follicular hypertrophy,
hyperplasia, lymphocyte necrosis, multiple cystic spaces, and polykaryocytes (Halbur et al., 1995b, 1996b; Rossow et al., 1994a, 1994b, 1995).

Virus isolation is the method to detect infectious virus particles in the specimens. Swine alveolar macrophages (SAM) were first used to isolate LV (Wensvoort et al., 1991). However, the difficulties and the high cost in obtaining these cells have limited their use. The first continuous cell line used to isolate U.S. PRSSV isolates was ATCC CL2621 (Benfield et al., 1992; Collins et al., 1992), however, these cells were less susceptible to PRRSV than SAM (Bautista et al., 1993b). Recently, the MARC-145 cell line (Kim et al., 1993) and ATCC CRL11171 (Meng et al., 1996a) were shown to support the replication of U.S. PRRSV isolates. The most suitable specimen for virus isolation in all ages of pigs was alveolar macrophages (Mengeling et al., 1995). It has been reported that serum is also an excellent sample for virus isolation (Stevenson et al., 1993; Van Alstine, 1993). PRRSV cannot be isolated from mummified or autolyzed fetuses (Christiansen et al., 1992).

SAM can be grown in Earle’s minimal essential medium supplemented (MEM) with 10% fetal bovine serum (FBS), 200 U/ml penicillin, 0.2 mg/ml streptomycin, 100 U/ml mycostatin, and 0.3 mg/ml glutamine (Wensvoort et al., 1991). The CL2621 cells were propagated in MEM supplemented with 10% beta propiolactone-treated FBS and 100 µg/ml of gentamicin (Bautista et al., 1993b). The MARC-145 cells can be grown in MEM supplemented with 30% FBS (Kim et al., 1993). The ATCC CRL11171 cells can be grown in Dulbecco’s minimal essential medium supplemented with 10% FBS and 1 x antibiotics (10,000 U/ml penicillin G, 10,000 mg/ml streptomycin, and 25 mg/ml amphotericin B) (Meng et al., 1996a).

There are several serologic tests to detect antibodies against PRRSV in swine sera. These tests include the immunoperoxidase monolayer assay (IPMA), indirect fluorescent
antibody (IFA), enzyme-linked immunoabsorbent assay (ELISA), and serum virus neutralization (SN).

The immunoperoxidase monolayer assay was the first serological test developed for the detection of both LV and U.S. PRRSV (Frey et al., 1992; Wensvoort et al., 1991). An amount of 100 TCID$_{50}$ of virus was added to macrophage cultures on microtiter plates and incubated for 2 days. The medium was discarded and cells were washed with 0.15 M NaCl, dried for 45 minutes at 37°C, and frozen for 45 minutes at -20°C. Cells were then fixed with 4% cold paraformaldehyde, washed, and incubated with test serum (diluted 1:10 in 0.15 M NaCl, 0.05% Tween-80, and 4% horse serum) for 1 hour at 37°C. Cells were washed and incubated with sheep anti-pig immunoglobulin conjugated to horseradish peroxidase for 1 hour at 37°C. After that cells were incubated with 3-amino-9-ethyl-carbazol. IPMA can be performed in continuous cell lines such as CL 2621 and MARC-145 as well. IPMA seems to be more sensitive than IFA, ELISA, and SN tests (Yoon et al., 1995b). Using the IPMA method, antibodies against PRRSV could be detected as early as 5-9 and as late as 324 days after infection (Yoon et al., 1995b).

The immunofluorescent antibody assay was initially developed for swine alveolar macrophage cultures, but later adapted for the use of continuous cell lines (Yoon et al., 1992). Cells were inoculated with virus and fixed with ethanol for 10-20 minutes at room temperature after exhibiting an early stage of cytopathic effect. Cells were washed and incubated with 4-fold dilution of test sera for 45 minutes at 37°C. After washing with PBS, cells were incubated with rabbit anti-swine IgG conjugated with fluorescein isothiocyanate (1:200-400). IFA seems to be equally sensitive (Yoon et al., 1992) or slightly less sensitive than IPMA (Yoon et al., 1995b). By IFA, antibodies against PRRSV could be detected as early as 7 days after infection (Yoon et al., 1992) and lasted until 158 days after infection (Yoon et al., 1995b). The IFA titer peaked at 11-21 days after infection (Yoon et al., 1992).
The indirect enzyme-linked immunosorbent assay was first developed in 1992 (Albina et al., 1992). An indirect ELISA has been performed using swine alveolar macrophage cultures (Albina et al., 1992) or continuous cell lines (Takikawa et al., 1996). MARC-145 cells were inoculated with PRRSV for 4 days at 37°C, centrifuged at low speed, resuspended in 20 ml of TE buffer (0.05 M Tris, 0.025 M EDTA, pH 7.4) containing 0.5% Triton X-100, sonicated twice for 5 minutes at 50 Watt, and stirred for 90 minutes (Takikawa et al., 1996). After low speed centrifugation, the supernatant was mixed with equal volume of 1, 1, 2-Trichlo-1, 2, 2-trifluoro-ethane for 5 minutes and centrifuged at 3,000 g for 10 minutes at 4°C. The aqueous phase was centrifuged at 10,000 g for 1 hour. One hundred microliters of the aqueous solution was used to coat a 96-well plate, stored overnight at 4°C, and washed with PBS. The unbound sites on the plate were blocked, and incubated with 100 μl of test sera (1:100) for 1 hour at 37°C. The plate was incubated with 100 μl of rabbit anti-swine IgG conjugated with horseradish peroxidase (1:4,000) for 1 hour at 37°C, washed, and incubated with substrate (0.1 M citric acid, 0.2 M phosphate buffer, pH 4.8 with 0.02% of 30% H₂O₂ and 0.4% O-phenylen-diamine dihydrochloride) for 15 minutes at room temperature. The optical densities were read at a wavelength of 492 nm. By the ELISA method, antibodies against PRRSV were detected as early as 9-13 days after infection and last as long as 137 days after infection. The ELISA was shown to have less sensitivity than IPMA and IFA.

Recently, the recombinant nucleocapsid protein of PRRSV (rPRRS) was used as an antigen source for indirect ELISA (Denac et al., 1997). The rPRRS ELISA proved to be more sensitive when compared to commercially available ELISA, IFA, and IPMA. The blocking ELISA was developed using antigen derived from PRRSV-infected swine alveolar macrophages and polyclonal antibodies as the detection system (Houben et al., 1995). The advantages of the blocking ELISA were increased sensitivity, less background, and cheaper
and higher capacity than when using IPMA and indirect ELISA (Houben et al., 1995; Sorensen et al., 1997).

Serum neutralization test was first developed in 1992 using a continuous cell line (Frey et al., 1992; Morrison et al., 1992). Four-fold diluted test sera were incubated with 100 TCID$_{50}$ of PRRSV for 2 hours at 37°C. Two hundred microliters of virus-serum mixtures was used to inoculate continuous cell line, which was checked for cytopathic effect after 3 days. The antibody titer was the highest serum dilution that blocked cytopathic effect. By the SNT, antibodies against PRRSV were detected as early as 9-28 (Yoon et al., 1995b) or 51-70 (Nelson et al., 1994) and lasted until 262 (Nelson et al., 1994) to 356 (Yoon et al., 1995b) days after infection. The SNT was considerably less sensitive than IPMA and IFA (Frey et al., 1992). The modified serum neutralization test was developed to improve sensitivity of SNT (Yoon et al., 1994) which detected antibodies as early as 9-11 that peaked at 11-21 days after infection. The SNT antibody titers were higher with the homologous PRRSV isolate than that with a heterologous PRRSV isolate.

PRRSV has been detected in the tissues of infected pigs using an immunohistochemistry technique. The first immunohistochemistry (IHC) technique developed for the detection of PRRSV was an immunogold technique (Magar et al., 1993). A streptavidin-biotin immunoperoxidase procedure for the detection of PRRSV using monoclonal antibodies was developed in 1994 (Halbur et al., 1994). To perform IHC, tissues were blocked with normal goat serum, washed and incubated with a mouse monoclonal antibody against PRRSV. After being washed, tissues were incubated with goat anti-mouse IgG-gold conjugate or biotinylated goat anti-mouse IgG. For the immunogold technique, tissues were washed and processed for silver enhancement. For streptavidin-biotin immunoperoxidase procedure, tissues were treated with peroxidase-conjugated
streptavidin (1:200), washed and incubated with substrate (3,3'-diaminobenzidine
tetrahydrochloride).

The detection of PRRSV nucleic acid is the other method for detecting PRRSV.
PRRSV nucleic acid can be detected by reverse transcription and polymerase chain (RT-
PCR) reaction and in situ hybridization (ISH).

The RT-PCR was first reported for both European and North American isolates in
1994 (Mardassi et al., 1994; Surarez et al., 1994; Van Woensel et al., 1994). RNA was
isolated from cells and used for a reverse transcription reaction. The product of reverse
transcription was used for PCR amplification and the PCR product was separated by
agarose gel electrophoresis. Recently, a multiplex PCR assay was developed to detect and
distinguish between North American and European isolates (Gilbert et al., 1997). It has
been reported that PCR for the detection of PRRSV using sperm is 10 times more sensitive
than culturing in alveolar macrophages (Gilbert et al., 1997).

ISH for the detection of PRRSV nucleic acid was developed using a cDNA probe
(Larochelle et al., 1996; Sur et al., 1996) and a RNA probe (Haynes et al., 1997). To
perform ISH, tissues were treated with proteinase K, washed and hybridized overnight with
digoxigenin-labeled DNA probes at 37°C (Larochelle et al., 1996) and 56°C (Sur et al.,
1996) or digoxigenin-labeled RNA probe at 52°C (Haynes et al., 1997). Tissues were
washed and treated with RNase A for the digoxigenin-labeled RNA probe. Tissues were
then incubated with antidigoxigenin conjugated with alkaline phosphatase, washed, and
incubated with substrate (nitroblue tetrazolium and 5-bromo-4-chloro-indoly1 phosphate
toluidinium). It has been shown that ISH is more sensitive than IHC (Larochelle et al.,
1997).
CHAPTER 3. *IN SITU* HYBRIDIZATION TECHNIQUE FOR THE DETECTION OF SWINE ENTERIC AND RESPIRATORY CORONAVIRUSES, TRANSMISSIBLE GASTROENTERITIS VIRUS (TGEV) AND PORCINE RESPIRATORY CORONAVIRUS (PRCV), IN THE FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUES

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Abstract

The *in situ* hybridization (ISH) technique was developed to detect the swine coronaviruses, transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV), in cell culture and tissue sections from TGEV-or PRCV-infected pigs. The radiolabeled RNA probes were generated from two plasmids pPSP.FP₁ and pPSP.FP₂ containing part of the S gene of TGEV. The procedure was first standardized in cell cultures. The radiolabeled pPSP.FP₂ probe detected both TGEV and PRCV in virus-inoculated cell cultures, whereas pPSP.FP₁ probe detected TGEV but not PRCV. The probe was then used to detect TGEV or PRCV in tissues of pigs experimentally infected with TGEV or PRCV or naturally infected with TGEV. Again the probes detected TGEV in intestines of experimentally and naturally infected pigs and PRCV in the lungs of experimentally infected pigs. TGEV RNA was mainly detected within the enterocytes at the
tips of villi and less often within some crypt epithelial cells. PRCV was shown to replicate mainly in the bronchiolar epithelial cells and in lesser amount in type II pneumocytes, type I pneumocytes, alveolar macrophages, and bronchial epithelial cells, respectively. ISH has potential applications as a diagnostic test for the detection and differentiation of TGEV and PRCV in tissues and in studies to gain a better understanding of the mechanism of pathogenesis of enteric and respiratory coronavirus infections.

Introduction

Coronaviruses are a common cause of enteric and/or respiratory infections in animals and man. Transmissible gastroenteritis virus (TGEV) of swine is one of the best studied coronaviruses, yet diagnostic methods for its detection are less than ideal and limited information is available on mechanisms of TGEV pathogenesis. TGEV causes transmissible gastroenteritis (TGE) in swine characterized by vomiting, severe diarrhea and high mortality in piglets under 2 weeks of age (Saif and Wesley, 1992; Bohl et al., 1989). In recent years, another coronavirus has been identified in swine and has been designated as porcine respiratory coronavirus (PRCV) which is antigenically closely related to the enteropathogenic TGEV. PRCV was first isolated in 1984 from pigs in Belgium (Pensaert et al., 1986) and is now ubiquitous in swine herds in Europe. PRCV has since been isolated from swine in the U.S. (Hill et al., 1989; Vaughn et al., 1994; Paul et al., 1994). PRCV is different from TGEV in that PRCV has 621-681 nucleotide deletion in the 5' end of S gene (Britton et al., 1991; Rassachaert et al., 1990; Sanchez et al., 1992; Laude et al., 1993; Wesley et al., 1990; Paul et al., 1992; Paul et al., 1994; Wesley et al. 1991a; Vaughn et al., 1994; Vaughn et al., 1995) and deletions in open reading frame 3 (Britton et al., 1991; Rassachaert et al., 1990; Wesley et al., 1991a). The S gene of TGEV plays an important
role for tissue tropism (Laude et al., 1993; Rassachaert et al., 1990; Parker et al., 1989). The deletion in S gene of PRCV causes virus to replicate only in the respiratory tissue rather than in both respiratory and intestinal tissues as for TGEV (Cox et al., 1990; O'Toole et al., 1989). Because PRCV is antigenically similar to TGEV, this creates a diagnostic problem. The most commonly used method for TGEV detection in diagnostic cases is immunofluorescence method on frozen sections of intestines. This technique has many problems as sections or villus enterocytes often wash off the slides during processing. Virus can be isolated in cell cultures, however many virus isolates do not replicate well in cell culture (Vaughn et al., 1993). Other methods have been developed such as dot blot hybridization (Shockley et al.; 1987, Paul et al., 1994), slot blot hybridization (Wesley et al., 1991b), immunofluorescence (Frederick et al.; 1976, Morin et al., 1973; Shepherd et al., 1979), peroxidase-antiperoxidase staining technique (Chu et al. 1982), and recently immunogold silver staining (IGSS) (Larochelle et al., 1993). All of these techniques have unique advantages and disadvantages and, except for immunohistochemistry, are not suited for detection of virus in tissue sections.

In situ hybridization (ISH) techniques can be utilized to identify specific nucleic acid in cells. Both, ISH and immunohistological staining methods have the advantages of requiring only an ordinary microscope and providing a permanent record, and they can be used for retrospective studies and have very high sensitivity and specificity. Therefore, collectively they can provide information on the presence of nucleic acid and antigen and can be used for pathogenesis studies. This study was undertaken to evaluate the ISH technique for the detection of TGEV and PRCV in infected cell cultures, to distinguish TGEV-infected cells from PRCV-infected cells, and to detect TGEV and PRCV in formalin-fixed paraffin-embedded sections from pigs experimentally or naturally infected with TGEV.
Materials and Methods

Viruses

The virulent Miller strain of TGEV (National Veterinary Services Laboratory, Ames, Iowa) and PRCV strain AR310 were used as reference viruses (Vaughn and Paul, 1993; Vaughn et al, 1994; Vaughn et al, 1995).

Cell culture

Swine testes (ST) cells were cultured on 8-well chamber slides (Nunc) as reported by Vaughn et al (1994) by growing in minimum essential medium (Gibco) supplemented with 10% heat inactivated bovine calf serum (Hazelton) and antibiotics. Four wells of 8-well chamber slides were inoculated with 200 plaque-forming units (PFU) of TGEV or PRCV per well. Another four wells served as non-inoculated controls. Twenty hours after inoculation, slides were fixed in 4% buffered paraformaldehyde at 4°C for 1 h.

Source of tissues

In experiment 1, four eight-and-one-half-week-old TGEV- and PRCV-negative pigs were divided into 2 groups. Two pigs were inoculated with 10^5 PFU of virulent Miller strain of TGEV and two pigs served as non-inoculated controls. In experiment 2, four 5-week-old TGEV and PRCV negative pigs were divided into two groups. Two pigs were inoculated with 10^7 PFU of PRCV strain AR310 and two pigs served as non-inoculated controls. Tissues from seven pigs submitted as separate diagnostic cases to the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University were also used as naturally-infected pigs and non-infected pigs. Five of the cases had frozen sections diagnosed as TGEV antigen positive and two were diagnosed as TGEV antigen negative by the standard immunofluorescence method. Histopathology on these animals supported the FA results.
Processing of tissues for in situ hybridization

TGEV inoculated pigs were euthanized and necropsied at 3 days and PRCV inoculated pigs were euthanized and necropsied at 4 days post inoculation (DPI). At the times of necropsy, one centimeter segments of the posterior part of duodenum, and the anterior and posterior part of jejunum and ileum from two TGEV-inoculated pigs and 6 pieces of lung from two PRCV-inoculated pigs were collected and fixed in 10% neutral buffered formalin. A random segment of the small intestines of the two pigs naturally infected with TGEV and the two TGEV negative pigs were placed in 10% neutral buffered formalin. The tissues were processed for 48 h in an automated tissue processor and embedded in paraffin. Sections of 5-μm-thickness were cut and placed on nuclease-free silylated slides (PGC scientific). These slides were deparaffinized and hydrated by incubating at 55°C for 30 min and subsequently passed in absolute xylene 3 times for 5 min, and 1 min each in 3 changes of absolute ethanol, 95% ethanol, 70% ethanol, and diethylpyrocarbonate (DEPC, Sigma Chemical Co.)-treated water, respectively.

Preparation of probes

RNA probes were prepared from plasmid pPSP.FP, and pPSP.FP₂ containing the left half of the S gene of TGEV cloned in phagemid vector (Vaughn et al., 1994). The plasmid pPSP.FP₁ contains S gene segment from nucleotides 1678 to 2250 (Britton and Page, 1990) which is present in TGEV but is deleted in all PRCV (Paul et al., 1994; Vaughn et al., 1994). The plasmid pPSP.FP₂ contains S gene segment from nucleotides 1678 (Britton and Page, 1990) to 2483 (Rassachaert and Laude, 1987). The plasmids pPSP.FP₁ and pPSP.FP₂ were linearized with restriction enzymes BamHI and Hpal (Promega), respectively. The in vitro transcription and labeling of the probes were performed as described previously (Angerer et al., 1992; Gibson et al., 1990) by using T7-RNA
polymerase for plasmid pPSP.FP, and T3-RNA polymerase for plasmid pPSP.FP₂ (Promega), the four NTPs (ATP, CTP, TTP, and GTP; Sigma Chemical Co.), DTT (dithiothreitol; Bio-Rad), and [³⁵S] UTPαS (Amersham). The transcription reaction was stopped by adding 1 μl (1 μg/μl) of DNase I (Worthington) and 1 μl (1 μg/μl) of RNasin (Promega) for 20 min at 37 °C. Unincorporated [³⁵S]- UTPαS were removed by passing the labeled RNA through a Sephadex G-25 spin column (Boehringer Mannheim).

In situ hybridization

De-paraffinized tissue sections and cells in 8-well chamber slides were treated with 2 ml of proteinase K (Ameresco) for 10 min at 37°C in a moist chamber. Concentration of proteinase K was 0.5 μg/ml for cell cultures and 1 μg/ml for tissue sections. Slides were washed in DEPC-treated water (0.1% DEPC) for 1 min. These slides were placed in a staining dish containing 400 ml of 0.1 M triethanolamine (pH 8.0) and 1 ml acetic anhydride and rapidly stirred for 10 min at room temperature. Slides were prehybridized in 2 × SSC for 5 min and dehydrated in graded alcohols and air dried. The hybridization solution (Angerer et al., 1992; Bohl et al, 1989) containing 50% formamide, 10% dextran sulfate, 3 × SSC, 50 mM sodium phosphate (pH 7.4), 1 × Denhardt’s solution, 0.1 mg/ml yeast tRNA, 10 mM DTT, and 1 μl of ³⁵S-labeled RNA probe (2 × 10⁶ cpm/μl). The amount of hybridization solution added was 50 μl per slide as previously described (Angerer et al., 1992), and coverslips were placed on slides. Slides were incubated at 52°C for 20 h. The coverslips were removed by soaking the slides in 2 × SSC two times for 5 min each at room temperature. The slides were treated with RNase A (Worthington) (Angerer et al., 1992; Gibson et al., 1990; Wilcox, 1993) solution (20 μg/ml) containing 10 mM Tris-HCl (pH 8.0) and 0.5 M NaCl for 30 min at 37°C. Slides were washed in 2 × SSC for 5 min at 52°C, 1 × SSC for 5 min at 52°C, 0.5 × SSC for 1 hr at 52°C, and 0.5 × SSC for 5 min at room
temperature. Slides were dehydrated, air dried, and were exposed to X-ray films (Fuji Photo film) at -70°C for 24 h. X-ray films were developed and evaluated. The slides were then coated with Kodak NTB-2 emulsion gel (Eastman Kodak Co.) which was diluted with 600 mM ammonium acetate (Angerer et al., 1992; Kohler et al., 1990) and preheated to 42°C (Angerer et al., 1992; Gibson et al., 1990; Kohler et al., 1990; Wilcox, 1993). These slides were kept in light-proof box at 4°C for 2 days. The slides were developed at 15°C for 3 min using developer (Kodak D-19) diluted 1:1 with water, followed by 20 sec rinse in deionized water, and then fixed for 3 min in fixer. The slides were then rinsed with deionized water for 5 min (Wilcox, 1993) and counterstained with hematoxylin and eosin. All of the solutions used in ISH except for the RNase A solution to remove contaminating ribonucleases were prepared by using DEPC-treated water (0.1% DEPC).

Controls included uninoculated ST cell monolayers, RNase-treated cells inoculated with TGEV, tissues from pigs experimentally inoculated with TGEV, tissues from 2 non-infected animals, RNase-A treated tissue sections from TGEV-inoculated animals, positive strand 35S-labeled RNA hybridized with sections from TGEV-infected animals, and excess amount of non-labeled RNA probe hybridized to sections from TGEV-infected animals followed by hybridization with 35S-labeled RNA probe. The positive strand 35S-labeled RNA probe was generated by using the same plasmid DNA and T7-RNA polymerase (Promega). The negative controls for PRCV inoculation experiment were similar to the TGEV inoculation experiment except RNase-A treated tissue sections from PRCV-inoculated animals and 8-well chamber slides inoculated with PRCV, 35S-labeled RNA probe produced from plasmid pPSP.FP1, and the positive strand 35S-labeled probe was generated by using the same plasmid DNA but linearized by using restriction enzyme EcoRI and T3-RNA polymerase.
Results

Detection of TGEV and PRCV in cell cultures

The in situ hybridization technique was first standardized using cell monolayers of ST cells. Uninoculated cell cultures and those inoculated with TGEV or PRCV were hybridized with the RNA probe made from plasmids pPSP.FP₁ and pPSP.FP₂. Following hybridization, slides were exposed to X-ray films. A good dark signal on X-ray films in TGEV or PRCV inoculated wells was usually observed after 24 hours exposure and inoculated wells could be differentiated from the uninoculated wells. The virus inoculated wells showed heavy dark square of silver grains, but non-inoculated wells had only pale gray color as non-specific background. The RNA probe prepared from plasmid pPSP.FP₂ detected both TGEV and PRCV whereas the probe prepared from pPSP.FP₁ reacted with TGEV but not PRCV (fig 1). The slides were then coated with emulsion gel, stored in the dark, developed after 2 days and examined under the light microscope. A heavy concentration of silver grains localized above and within the cytoplasm of the TGEV or PRCV-inoculated cells were detected (fig 2), only a very low concentration of silver grains were scattered throughout the non-inoculated cells. Positive signal was abolished by RNase A treatment of cells prior to hybridization as well as by addition of unlabeled RNA probe prior to hybridization with radiolaed probe.

In situ hybridization of sections of TGEV-infected tissues

Once the technique was standardized using cell monolayers, it was tested on intestinal sections from uninoculated and TGEV-inoculated pigs. The autoradiography results using X-ray films clearly distinguished between sections from TGEV-inoculated and non-inoculated animals as shown in Fig 3. The sections of duodenum, anterior jejunum, posterior jejunum, anterior ileum, and posterior ileum from infected animals gave heavy dark
lines of silver grains along the mucosa. No signal was detected in sections from non-infected animals, RNase-treated sections from infected animals and sections treated with excess amount of nonlabeled RNA probe demonstrating the specificity of the probe. Slides coated with emulsion gel from the infected animals had heavy concentration of silver grains along the mucosal layer, but the sections from non-infected animals did not. There were numerous silver grains over the cytoplasm of the villous enterocytes (fig 4B). A few non specific silver grains, which were not localized over the cells, were scattered throughout the sections. Viral RNA was detected in some crypt epithelial cells (fig 4D). The histopathologic changes were villous atrophy, blunted and denuded villi, and replacement of the columnar cells by flat to cuboidal cells.

Once the technique was standardized on tissues of experimentally infected pigs, we wanted to determine if it could be used on tissue sections from pigs naturally infected with TGEV. We took intestinal sections from five pigs that had been diagnosed as TGE by FA and two that had been TGE negative. As expected, sections from TGE positive pigs were positive by ISH and the TGE-negative sections were negative by ISH.

In situ hybridization of formalin-fixed paraffin-embedded sections of PRCV infected tissues

Tissues from PRCV-inoculated pigs were used to determine whether this technique could be used to detect PRCV in PRCV-inoculated pigs. The autoradiography results from X-ray films of the lung sections of the PRCV-inoculated pigs and non-inoculated control pigs distinctly illustrated the differences between these 2 groups of sections. X-ray films of the lung sections from PRCV-inoculated pigs showed the dark ring of silver grains along the small airways and dark spots scattered throughout the sections, but the lung sections from non-inoculated pigs had homogeneous pale gray color over the lung sections. The slides from the PRCV-inoculated pigs coated with emulsion gel had heavy concentration of silver
grains localized over the epithelial cells of bronchi, bronchioles, type II pneumocytes, type I pneumocytes and alveolar macrophages. The hybridization signals were also found over the necrotic epithelial cells that sloughed into the lumen of the bronchioles, and over the alveolar macrophages in the exudate in the alveoli and bronchioles. The cells that most often had the localization of the silver grains were bronchiolar epithelial cells (fig.5), especially in terminal bronchioles, followed by type II pneumocytes, alveolar macrophages and type I pneumocytes. Only a few bronchial epithelial cells were shown to have localization of the silver grains. The microscopic examination showed mild to moderate necrosis and attenuation of the bronchiolar epithelial cells, moderate lymphomacrophagic infiltration in the alveolar septa, mild type II pneumocyte hypertrophy and proliferation, and lymphomacrophagic exudation in bronchioles and alveoli.

Discussion

In this study, we have described an ISH technique for the detection and differentiation of coronaviruses TGEV and PRCV in cell cultures as well as formalin-fixed paraffin-embedded tissues of pigs. This test was first standardized in cell cultures. ISH with radiolabeled probes prepared from plasmids pPSP.FP, and pPSP.FP₂ were highly specific for the detection of TGEV and PRCV in cell culture. The TGEV-infected cells had hybridization signals with radiolabeled RNA probes produced from either of the two plasmids pPSP.FP, and pPSP.FP₂. In contrast, the PRCV-infected cells had hybridization signals with radiolabeled RNA probes produced from plasmid pPSP.FP₂ but not with probes generated from plasmid pPSP.FP₁. Non-infected cell cultures did not show hybridization signals with RNA probes generated from plasmid pPSP.FP₁ or pPSP.FP₂. Thus, we were able to distinguish TGEV-infected cells from PRCV-infected cells by using ³²S-RNA probe
produced from plasmid pPSP.FP. This plasmid contained TGEV nucleotides sequences in the S gene that were missing in PRCV (Vaughn et al., 1994). The results from X-ray films corresponded well with the results from emulsion coated slides. For rapid results, slides from suspicious case can be exposed to X-ray films after hybridization with appropriate $^{35}$S-RNA probe. For more details or to differentiate the viruses and to study pathologic changes in tissues, slides can be coated with emulsion gel.

ISH of formalin-fixed paraffin-embedded TGEV-inoculated tissues showed distribution of viral nucleic acid in intestines similar to the distribution of viral antigen reported previously (Bohl et al., 1989; Chu et al., 1982; Frederick et al., 1976; Larochelle et al., 1993; Larson et al., 1979; Morin et al., 1973; Saif and Wesley, 1992; Woods et al., 1981). However, we also detected viral nucleic acid in some crypt epithelial cells. The autoradiography results from X-ray films correlated very well with the results from the emulsion gel coated-slides, and the results were similar in both experimentally inoculated and naturally infected pigs. This result was in contradiction to previous reports where no positive cells were identified in the crypt of Lieberkuhn (Bohl et al., 1989; Chu et al., 1982; Frederick et al., 1976; Larochelle et al., 1993; Larson et al., 1979; Morin et al., 1973; Saif and Wesley, 1992; Shepherd et al., 1979). Reason for this difference is not known except previous studies have examined for viral antigen, whereas, we have looked for viral nucleic acid. TGEV enters into the cell by specific receptors on the cell membrane of mature enterocytes. Two receptors have been identified: aminopeptidase-N (CD 13) (Delmas et al., 1990) and a second putative 200 kDa receptor (Weingart et al., 1994). These receptors are found only on the absorptive enterocytes, but they are not found on the cell membrane of the crypt epithelial cells. The mechanism for TGEV infection of crypt epithelial cells is not known. One possibility is that TGEV might bind nonspecifically on the cell membrane of the
crypt epithelial cells and thus lead to infection of these cells (Weigartl and Derbyshire, 1993). Another possibility is that TGEV infection of these cells is nonproductive. In this case, there is no viral protein produced in this type of cell, and thus is not detected by immunofluorescence or immunohistological methods (Chu et al., 1982; Frederick et al., 1976; Larochelle et al., 1993; Larson et al., 1979; Morin et al., 1973; Saif and Wesley, 1992; Woods et al., 1981). ISH technique also provides for observation of histopathological lesions. The histopathologic changes were similar to previous reports (Bohl et al., 1989; Chu et al., 1982; Frederick et al., 1976; Larochelle et al., 1993; Larson et al., 1979; Morin et al., 1973; Saif and Wesley, 1992; Woods et al., 1981).

The distribution of PRCV ribonucleic acid was most intense in the epithelial cells of the bronchiolar epithelium and is compatible with the distribution of viral antigen as reported previously (O'Toole et al., 1989). However our results are incompatible with those observed by Cox et al. (1990) in which the viral replication occurred mainly in the alveolar cells. The microscopic examination showed microscopic changes in the lung tissue as previous reports (Cox et al., 1990; O'Toole et al., 1989; Halbur et al., 1993).

In this study we have described ISH for two swine coronaviruses which should be very useful for diagnostic purposes and retrospective studies. This is also the first description of this technique in TGEV and PRCV infection. Development of a non-radioactive labeled probe will make it even more user friendly. We are in the process of exploring the potential of using such a probe. This technique also has applications in elucidating mechanisms of pathogenesis of TGEV and PRCV in swine.
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References


Figure 1. Autoradiograph of cell cultures in 8-well chamber slides following hybridization with $^{35}$S-RNA probe prepared from plasmid pPSP.FP₁ (B) or pPSP.FP₂ (A and C) containing the left half of TGEV S gene. A) ST cell cultures in chambers were inoculated with TGEV (upper row) or were left as non-inoculated control (lower row). B) Cell cultures were inoculated with TGEV (upper row) or PRCV (lower row). C) Cell cultures were inoculated with PRCV (upper row) or kept as non-inoculated control (lower row). Probe from plasmid pPSP.FP₁ only reacted with TGEV whereas the probe from plasmid pPSP.FP₂ reacted with TGEV and PRCV.

Figure 2. *In situ* hybridization of uninoculated control (A) and TGEV-inoculated ST cells (B) with $^{35}$S-RNA probe produced from plasmid pPSP.FP₂ containing TGEV S gene insert. Eight-well chamber slides were exposed to autoradiographic emulsion gel for 2 days.
ST-CELL INFECTED WITH TGEV

UNINFECTED CONTROL

TGEV

PRCV

PRCV

UNINFECTED CONTROL

2a

2b
Figure 3. Photograph of an X-ray film showing autoradiographic results after 24 hours exposure of formalin-fixed paraffin-embedded sections from the various portion of small intestines of the non-inoculated pig (L) and TGEV-inoculated pig (R) after hybridization with $^{35}$S-RNA probe produced from plasmid pPSP.FPz. Small intestinal sections from non-inoculated pig showed only pale gray color but small intestinal sections from TGEV-inoculated pigs showed a dark continuous line along the mucosal surface of the small intestines.

Figure 4. Photographs of intestinal villi (A & B) and crypt of Lieberkuhn (C & D) of small intestines from uninoculated pig (A & C), or pig inoculated with TGEV (B & D) following in situ hybridization with $^{35}$S-RNA probe produced from plasmid pPSP.FPz. The histopathologic changes observed following TGEV inoculation were villous atrophy, blunted and denuded villi, and replacement of the columnar cells by flat to cuboidal cells (B). The ISH signals were specifically localized in villi (B) and over the crypt epithelial cells (D).
Figure 5. Photographs of formalin-fixed sections from lungs of uninoculated control (A) and PRCV-inoculated (B) pigs hybridized in situ with $^{35}$S-RNA probe produced from plasmid pPSP.FP$_2$ and exposed to autoradiographic emulsion for 2 days. ISH signals can be observed over the bronchiolar epithelial cells of small bronchiole of PRCV-inoculated pigs.
CHAPTER 4. RAPID IN SITU HYBRIDIZATION TECHNIQUE FOR THE DETECTION OF RIBONUCLEIC ACID IN TISSUES USING RADIOLABELED AND FLUORESCEIN-LABELED RIBOPROBES

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Abstract

In situ hybridization (ISH) is a useful diagnostic and research tool, but is also time consuming. This study was conducted to determine if a rate enhancement hybridization (REH) buffer, developed for membrane hybridization, could be used to decrease hybridization time for ISH. Tissue from swine with an enteric disease produced by a swine coronavirus, transmissible gastroenteritis virus (TGEV), was used as a model to standardize hybridization conditions for a rapid ISH technique. Small intestinal sections from pigs experimentally and naturally infected with TGEV were hybridized for various times at 52°C and 70°C with a radiolabeled or a fluorescein-labeled RNA probe in a standard hybridization or a REH buffer. Viral RNA was detected in intestines in as early as 30 min of hybridization by using both buffers with the radiolabeled probe; however, the signal was stronger with the REH buffer. With the fluorescein-labeled probe, viral RNA was detected in virus-infected cells of the intestines after 30 min of hybridization by using the REH buffer. Signal intensity was greater with the REH buffer than that with the standard hybridization buffer when compared at each hybridization time and hybridization temperature using both.
radiolabeled and fluorescein-labeled probes. With the REH buffer, hybridization signal intensity was greater at 70° C than at 52 °C for both probes. The best results were obtained when small intestinal sections were hybridized at 70° C for 2 h using a radiolabeled or a fluorescein-labeled probe diluted in the REH buffer. The fluorescein-labeled RNA probe with REH buffer resulted in minimal nonspecific signal when compared with the radiolabeled probe. These studies demonstrate that the REH buffer can be used to decrease the time of ISH for the detection of viral RNA. This rapid ISH technique should have broad applications in the utilization of probe technology in diagnostics and research for the detection of target ribonucleic acids \textit{in situ}.

\textbf{Introduction}

\textit{In situ} hybridization (ISH) is widely used for the detection of DNA and RNA in intact eukaryotic and prokaryotic cells by using radiolabeled or nonradiolabeled probes. Probes for ISH may be DNA, RNA, or oligonucleotides. A major disadvantage of ISH is that it is a time-consuming technique, taking about 2 days for nonisotopic ISH and 4 days or more for isotopic ISH. One of the rate limiting steps is hybridization step which requires hybridization of the probe with tissue for at least 16 h.\textsuperscript{1,2,3,4,5,6} There have been two reports describing a reduction of hybridization time to three hours using DNA probes\textsuperscript{7} and to two hours using riboprobes\textsuperscript{8}. Recently, a rate enhancement hybridization (REH) buffers have become commercially available. These buffers can decrease hybridization time for membrane-based procedures such as Southern, northern, and dot blot hybridizations.

For the development of rapid ISH, tissues from swine experimentally induced with an enteric disease, transmissible gastroenteritis (TGE), were used as a model. TGE is characterized by vomiting, severe diarrhea, and high mortality in piglets during the first few
weeks of life. The causative agent of TGE is a coronavirus referred to as transmissible gastroenteritis virus (TGEV). Coronavirus particles are pleomorphic and enveloped and contain a single-stranded positive-sense RNA genome. TGEV replicates in the cytoplasm of the mature absorptive enterocytes and causes villous atrophy. Here we describe a rapid ISH technique, using REH buffer, that allows to decrease hybridization time for both radiolabeled and fluorescein-labeled probes.

Materials and Methods

Virus

The virulent Miller strain of TGEV (National Veterinary Services Laboratory, Ames, Iowa) was used as a reference virus.

Source of tissues

Four 8.5 week-old TGEV-negative pigs were used. Two pigs were inoculated orally with $10^5$ plaque forming units (PFU) of virulent Miller strain of TGEV and two pigs served as non-inoculated controls. Tissues from seven pigs submitted as separate diagnostic cases to the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University were used to represent TGEV naturally-infected and non-infected pigs. Five samples were positive and two were negative for TGEV antigen by direct immunofluorescence of frozen intestinal sections.

Processing of tissues for in situ hybridization

TGEV-inoculated and non-inoculated pigs were euthanized and necropsied at 3 days post-inoculation. At the time of necropsy, one centimeter segments of the posterior end of the duodenum, and the anterior and posterior ends of the jejunum and the ileum were collected and fixed in 10% neutral buffered formalin. A segment of the small intestine
from the five pigs naturally infected with TGEV and the two TGEV negative pigs were placed in 10% neutral buffered formalin. The tissues were processed and embedded in paraffin. Consecutive sections were cut from each tissue placed on nuclease-free silylated slides (PGC scientific), deparaffinized and hydrated as previously reported.

**Preparation of probes**

RNA probes were prepared from plasmid pPSP.FP, containing the left half of the S gene of TGEV cloned in a phagemid vector. The plasmid pPSP.FP, contains nucleotides 1678 to 2250 of the S gene of TGEV, and was linearized with the restriction enzyme BamHI (Promega). The *in vitro* transcription and labeling of the probe was performed as described previously by using T7 RNA polymerase, the four NTPs (ATP, CTP, TTP, and GTP; Sigma Chemical Co.), DTT (dithiothreitol; Bio-Rad), and [³⁵S]-UTP(αS (Amersham). The transcription reaction was stopped by adding 1 μl (1 μg μl⁻¹) of DNase I (Worthington) and 1 μl (1 μg μl⁻¹) of RNasin (Promega) for 20 min at 37° C. Unincorporated [³⁵S]-UTP(αS was removed by passing the labeled RNA through a Sephadex G-25 spin column (Boehringer Mannheim). The fluorescein-labeled RNA probe was labeled by using a RNA colour kit (Amersham) and purified by using a Sephadex G-50 spin column (Boehringer Mannheim). The specificity of probes was determined by dot blot hybridization as previously reported.

**In situ hybridization**

Deparaffinized tissues were treated and hybridized as reported previously. Briefly, tissues were treated with proteinase K (1 μg ml⁻¹, Ameresco) for 15 min at 37° C and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature. Sections hybridized with a fluorescein-labeled RNA probe were incubated with 20% (v/v) cold acetic acid for 15 sec after treatment with proteinase K to destroy
endogenous alkaline phosphatase. Sections were hybridized with 50 μl of standard hybridization buffer (50% formamide, 10% dextran sulfate, 3 x SSC, 50 mM sodium phosphate, pH 7.4, 1 x Denhardt's solution, 0.1 mg ml⁻¹ yeast tRNA, and 10 mM DTT) or REH buffer (Rapid-Hyb buffer, Amersham) containing 0.5 μl of a ³⁵S-labeled RNA probe (1.43 ng μl⁻¹ or 1.6 x 10⁶ cpm μl⁻¹) or 1 μl of a fluorescein-labeled RNA probe (1 ng μl⁻¹).

Sections hybridized with a ³⁵S-labeled RNA probe were divided into 4 groups, which were incubated with the standard hybridization buffer at 52° C and 70° C, and the REH buffer at 52° C and 70° C. Sections hybridized with a fluorescein-labeled RNA probe were divided into 2 groups which were hybridized with the standard hybridization buffer at 52° C or the REH buffer at 70° C. Each group of sections were incubated with hybridization buffer for 20 h, 2 h, 1 h, or 30 min. Sections were treated with RNase A (Worthington) 20 μg ml⁻¹ at 37° C for 30 min, and washed in 2 x SSC for 5 min at 52° C, 1 x SSC for 5 min at 52° C, 0.5 x SSC for 1 h at 52° C, and 0.5 x SSC for 5 min at room temperature.

Detection of the in situ hybridization signal

For the ³⁵S-labeled RNA probe, sections were dehydrated and air dried. X-ray film (BIOMAX MR, Eastman Kodak Co.) was exposed to hybridization sections at -70° C for 24 h, developed and evaluated. Sections were then coated with Kodak NTB-2 emulsion gel (Eastman Kodak Co.) which was diluted 1:1 with 600 mM ammonium acetate¹² and preheated to 42° C¹²³⁴. These sections were kept in a light-proof box at 4° C for 2 days. The slides were developed at 15° C for 3 min by using developer (Kodak D-19) diluted 1:1 with deionized water, followed by a 20 sec rinse in deionized water and then fixed for 3 min in fixer (Eastman Kodak Co). Sections were then rinsed with deionized water for 5 min⁴ and counterstained with hematoxylin and eosin.
For the fluorescein-labeled RNA probe, sections were washed in Tris-buffered saline (TBS) containing 100 mM Tris-HCl, pH 7.5, and 400 mM NaCl for 5 min at room temperature. Sections were incubated for 1 h at room temperature in a moist chamber with a blocking solution consisting of 0.5 % (w/v) blocking agent (Amersham) in TBS. Sections were then rinsed with TBS for 1 minute and incubated with anti-fluorescein antibody conjugated with alkaline phosphatase (Amersham) diluted 1:400 in 0.5% (w/v) BSA fraction V in TBS for 1 h in a moist chamber at room temperature. Sections were washed in TBS 3 times for 5 min each, and in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 5 min at room temperature. Sections were incubated with substrate (45 μl of 4- nitroblue tetrazolium chloride and 35 μl of 5-bromo-4-chloro-3-indolyl-phosphate in 10 ml detection buffer) for 1 h in the dark, then rinsed in deionized water and counterstained with nuclear fast red for 2 min.

Controls included: tissue sections from two noninoculated control pigs and two TGEV-free pigs submitted to the Veterinary Diagnostic Laboratory; RNase-treated tissue sections from TGEV-inoculated pigs and TGEV-naturally infected pigs; sections from TGEV-infected pigs hybridized with positive strand RNA probes; sections from TGEV-infected pigs hybridized with the excess amount of non-labeled RNA probe followed by hybridization with labeled RNA probes. For the fluorescein-labeled RNA probe, we used a hybridization buffer-free probe as an additional control.

Signal Quantitation

Autoradiography results were captured on X-ray films by an AGFA Arcus II flatbed scanner and edited by using software Adobe Photoshop 3.0 on a McIntosh computer. The results were printed with a video printer (Sony Color Video Printer UP- 5000). The intensity of the signal was analyzed using software NIH Image 1.58 and reported as mean density.
and total signal area. For nonisotopic ISH, slides were scored by using semiquantitation as follows: – = negative, + = weak, ++ = moderate, +++ = strong hybridization signal.

**Results**

*In situ hybridization of sections of TGEV-infected tissues with $^{35}$S-labeled RNA probe*

**Preliminary experiment on comparison of the effect of two hybridization buffers.** Studies were first performed by using a radiolabeled riboprobe to compare the hybridization using a standard hybridization buffer with that using REH buffer. Hybridization using the standard hybridization buffer was performed at 52° C whereas hybridization with the REH buffer was performed at 70° C, both for 0.5, 1, and 2 h. Hybridization signals were detected with both hybridization buffers at all times examined; however, the signal was consistently more intense with the REH buffer than with the standard hybridization buffer. Intensity of the signal after two hours of hybridization with the REH buffer was greater than that at 20 h using the standard hybridization buffer. This experiment suggested that the hybridization buffer and temperature may impact hybridization kinetics.

**Effect of temperature on hybridization with two buffers.** To further determine the effect of the temperature and the buffers, hybridization was performed using the standard hybridization and REH buffer each at two different temperatures of 52° C and 70° C for 0.5, 1, 2, and 20 h of hybridization. Hybridization with the standard hybridization buffer was best at 52° C, whereas for the REH buffer, the optimal hybridization temperature giving the best signal was 70° C (Fig. 1 A & B). The hybridization signal at 70° C with the REH buffer after 2 h hybridization was equivalent to or better than that detected after 20 h of hybridization at 52° C with the standard hybridization buffer. Sections hybridized with the REH buffer for 2 h at 70° C gave also the best signal-to-noise ratio and the most intense signal when
compared with the standard hybridization buffer at both 52° C and 70° C, and the REH buffer at 52° C for 20 h (Fig. 1 & 2). The intensity of the hybridization signal increased with time as the hybridization signal with the REH buffer at 70° C was less intense at 2 h compared to 20 h. However, the background was also higher after 20 h hybridization than after 2 h hybridization. Viral RNA could be detected with the REH buffer at 70° C as early as 30 min.

Microscopic examination of intestinal sections hybridized with 35S-labeled RNA probe.

Sections obtained from infected pigs and coated with emulsion gel had heavy concentrations of silver grains along the mucosal layer. In contrast, sections from uninoculated control animals were negative for silver grains. The silver grains were primarily concentrated over the cytoplasm of the villous enterocytes (Fig. 3), and some were present in crypt epithelial cells. Signal intensity was similar to the autoradiographic results captured on X-ray films. Sections that were hybridized with the REH buffer at 70° C for 2 h had higher concentration of silver grains than those hybridized with the standard hybridization buffer at 52° C for 20 h (Fig. 3 A & B). Sections that hybridized with the REH buffer had higher nonspecific silver grains than standard hybridization buffer, which increased with time. The histopathologic changes observed in the small intestines from the infected animals were villous atrophy, blunted and denuded villi, and replacement of the columnar epithelial cells by flat to cuboidal cells.

In situ hybridization on sections of TGEV-infected tissues using a fluorescein-labeled RNA probe

Once the hybridization conditions were standardized with the radiolabeled probe, we compared the effect of standard hybridization buffer at 52° C with the REH buffer at 70° C by using a fluorescein-labeled RNA probe. Viral nucleic acid was detected in enterocytes of
the small intestinal sections hybridized with either buffer, mainly in villous epithelial cells (Fig. 4) and some crypt epithelial cells. Dark purple color was detected in the cytoplasm without any staining of the nucleus. Sections hybridized with the REH buffer at 70° C clearly had a higher signal intensity and a greater number of viral infected cells than that with sections hybridized with standard hybridization buffer at 52° C, at each hybridization time (Table 1). Viral infected cells were detected after 2 h of hybridization using standard hybridization buffer at 52° C. In contrast, viral infected cells were detected after 30 min of hybridization with the REH buffer at 70° C. This treatment gave an equivalent or more intense signal, and a greater number of viral infected cells than sections hybridized with standard hybridization at 52° C for 2 h. Sections hybridized with the REH buffer at 70° C for 20 h had the highest signal intensity, and the greatest number of viral infected cells. There was only a slight increase in signal intensity after 20 h hybridization with REH buffer at 70° C without an increase in the number of virus infected cells over those hybridized for 2 h. The signal intensity and the number of virus infected cells on sections hybridized with the REH buffer at 70° C for 2 h was equal to or higher than that with sections hybridized with standard hybridization buffer at 52° C for 20 h (Fig. 4 A & B). The signal intensity and the number of viral nucleic acid positive cells were less after 1 h and 30 min compared to 2 h hybridization. The intensity of the signals varied from purple after 30 min, to dark purple after 1h and to very dark purple after both 2 h and 20 h hybridization. Sections hybridized with the REH buffer containing the fluorescein-labeled RNA probe did not give higher nonspecific signal compared to the standard hybridization buffer.
Discussion

In this study, we have described a rapid ISH technique for the detection of nucleic acids in formalin-fixed paraffin-embedded tissues. Hybridization of either radiolabeled and fluorescein-labeled probes with the REH buffer decreased hybridization time from 20 to 2 h without compromising the intensity or the quality of the hybridization signal. Optimal results with strong signal and signal-to-noise ratio were obtained with both radiolabeled and fluorescein-labeled probes by using the REH buffer at 70°C for 2 h. One Minor disadvantage of using the REH buffer for isotopic ISH was the slight increase in nonspecific signal with increased hybridization time, but this increase in nonspecific signal was not a problem with 2 h hybridization and was not detected with nonisotopic ISH. Sections hybridized with the REH buffer containing a fluorescein-labeled RNA probe at 70°C detected viral infected cells as early as 30 min of hybridization whereas use of the standard hybridization buffer at 52°C only detected viral infected cells after 2 h or more of hybridization. The intensity of the signal with both types of probes decreased with shorter hybridization time of 1 h and 30 min, but the signal was still visible. Signal intensity and hybridization time are probably impacted by the copy number of the target nucleic acids. It may be possible to decrease hybridization time to 1 h or even 30 min, with a higher copy number of target nucleic acid. Morphology of the cells was not impacted with either hybridization conditions.

Standard ISH procedures require incubation of tissue sections with the probe for at least 16 h\textsuperscript{1,2,3,4}. Martinoez-Montero \textit{et al}. (1991) and Musiani \textit{et al}. (1994) have reported methods for reducing hybridization time to 2 and 3 h, respectively, by using hybridization buffer with 50% formamide. The method of Martinoez-Montero \textit{et al}. (1991) required incubation of samples at 90°C in a hot air oven for 15 minutes before hybridization with a
RNA probe of 600 nucleotides. Musiani et al. (1994) used cytospin preparations instead of formalin-fixed paraffin-embedded tissue to hybridize with a DNA probe. Both studies used higher concentrations of probes than we used in this study. We used a commercially available REH buffer with a $^{35}$S-RNA and fluorescein-labeled RNA probe with an approximate size of 600 bases. The composition of this proprietary REH buffer is not known and according to manufacturer's instructions, it was developed for membrane hybridization such as Southern blot, northern blot, and dot blot hybridization using radiolabeled DNA, RNA or oligonucleotide. The REH buffer appears to be less viscous than the standard hybridization buffer. Although, the mechanism of enhancement of hybridization using REH buffer is not known, it may be due to the unfolding of viral RNA secondary structure, resulting in dissociation of protein-nucleic acid complexes so that more target molecules are accessible for hybridization with the probe. The lower viscosity of the REH buffer may play a role in the higher rate of movement of the probe to find the target RNA. We have shown that the REH buffer can be used in ISH for both radiolabeled and fluorescein-labeled RNA probes to enhance hybridization from overnight to two h with this buffer without compromising the intensity of the signals when compared with a standard method$^{20}$. Normally, the entire procedure could be performed within one and a half days for isotopic ISH or about 7-8 h for nonisotopic ISH. Total time could be decreased to within a day using the REH buffer with nonisotopic ISH which makes this test comparable to immunohistochemistry and a good candidate for application in routine diagnosis. This method should be applicable for the detection of cellular mRNA, cellular DNA, and other microbial nucleic acids in tissues.
Acknowledgements

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References


Figure 1. Comparison of a standard hybridization (STD) buffer with the rate enhancement hybridization (REH) buffer at 2 different hybridization temperatures, showing relationship between total area of signal (mm$^2$) and hybridization times (A) and relationship between mean density of hybridization signal and hybridization times (B).
Figure 1.

A

Total signal area (mm²)

Hybridization time (hours)

STD buffer, 52 C
STD buffer, 70 C
REH buffer, 52 C
REH buffer, 70 C

B

Mean density

Hybridization time (hours)

STD buffer, 52 C
STD buffer, 70 C
REH buffer, 52 C
REH buffer, 70 C
Table 1. Effect of hybridization buffer and temperature on detection of viral RNA in small intestinal sectionsa by nonisotopic ISH.

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<tr>
<th>Buffer</th>
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<td>Standard hybridization</td>
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<td>REH buffer</td>
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a Small intestinal sections from 2 inoculated pigs infected with 105 PFU of virulent Miller strain of TGEV and 5 TGEV-positive cases by direct immunofluorescence were used in ISH.

b Hybridization results: - = negative, + = weak, ++ = moderate, +++ = strong hybridization signal
Figure 2. Photograph of an X-ray film showing autoradiographic results of the effect of hybridization buffers and temperatures on *in situ* hybridization (ISH). Sections of formalin-fixed paraffin-embedded small intestines of TGEV-inoculated pigs (B, C, E, and F) and uninoculated control (A and D) were hybridized with $^{35}$S-RNA probe with standard hybridization and rate enhancement (REH) buffer at 52° C and 70° C. Sections in panels A and B were hybridized using standard hybridization buffer at 52° C and those in panel C were hybridized using standard hybridization buffer at 70° C. Sections in panels D and F were hybridized using rate enhancement buffer at 70° C and those in panel E were hybridized using REH buffer at 52° C. The results showed hybridization signal as dark continuous lines along the mucosal surface of the TGEV-inoculated small intestinal sections. Such hybridization signal was not detected in small intestinal from uninoculated pigs. Best results were obtained with REH buffer after 2 h hybridization at 70° C.
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Figure 3. Photographs of intestinal villi of small intestines from a transmissible gastroenteritis virus-inoculated pig following *in situ* hybridization with $^{35}$S-RNA probe in the standard hybridization buffer at 52° C for 20 h (A), or the rate enhancement hybridization (REH) buffer at 70° C for 2 h (B).

Figure 4. Photographs of intestinal villi of small intestines from a transmissible gastroenteritis virus-inoculated pig following *in situ* hybridization with fluorescein-labeled RNA probe using the standard hybridization buffer at 52° C for 20 h (A), or the rate enhancement (REH) buffer at 70° C for 2 h (B).
CHAPTER 5. TRANSMISSIBLE GASTROENTERITIS VIRUS INDUCED APOPTOSIS IN SWINE TESTES CELL CULTURES

A full manuscript submitted to Archives of Virology

Theerapol Sirinarumitr, John P. Kluge, and Prem S. Paul

Abstract

Transmissible gastroenteritis virus (TGEV) is a coronavirus which causes severe gastroenteritis in piglets. TGEV has been extensively studied, but the mechanism of TGEV-induced cell death is not known. In this study, we tested our hypothesis that TGEV induces cell death by apoptosis. Swine testes (ST) cells were inoculated with the virulent Miller strain of TGEV and examined for apoptosis by analysis of DNA fragmentation pattern by agarose gel electrophoresis, ultrastructural changes by electron microscopy and in situ detection of apoptosis by terminal deoxytransferase digoxigenin-dUTP nick end labeling (TUNEL). TGEV-induced DNA fragmentation was observed as early as 24 hours postinoculation (h.p.i.) and peaked at 48 h.p.i. At the ultrastructural level, changes characteristic of apoptosis such as nuclear chromatin and cytoplasmic condensation with intact nuclear and cell membranes were detected in several cells but viral particles were not detected in these cells. In addition, viral particles were detected in the golgi apparatus and endoplasmic reticulum of several lytic cells. Analysis by TUNEL technique revealed that several cells undergoing apoptosis in TGEV-inoculated cultures and the percentage of apoptotic cells was higher in TGEV-inoculated wells than those in uninoculated cultures at each time point postinoculation. Interestingly, most of the apoptotic cells were bystander
cells as they were negative for TGEV nucleic acids by *in situ* hybridization and confirmed the result from electron microscopy. Results of this study indicate that TGEV induces apoptosis *in vitro* and that most of the cells undergoing apoptosis are bystander cells, thus amplifying the cytopathic effect of TGEV.

**Introduction**

Transmissible gastroenteritis virus (TGEV), a coronavirus, is a pleomorphic enveloped virus with a positive-sense single-stranded RNA genome [29]. TGEV is associated with an economically important disease, transmissible gastroenteritis (TGE), and causes severe vomiting, diarrhea, and high mortality in piglets during the first few weeks of life [29]. TGEV replicates in and destroys the enterocytes of the villous epithelium in the small intestine, which results in the malabsorption and dehydration characteristic of TGE. TGEV can be grown in several cell lines but the swine testicle (ST) cells are most commonly used [29] for TGEV isolation and propagation. TGEV induces extensive cytopathic effect (CPE) in ST cells characterized by rounding of cells, and syncytial formation. TGE has been recognized since 1946 [6] and has been extensively studied. However, the mechanism of TGEV-induced cell death is still unclear but it is believed that TGEV kills cells by cell lysis.

Recently, several DNA [9, 22, 25, 27, 28] and RNA viruses [10, 11, 17, 24, 32, 37, 42] have been shown to induce cell death by apoptosis. We investigated whether TGEV kills cells by apoptosis, whether there is a correlation between cytopathic effect (CPE) and apoptosis, and whether infected, bystander or both infected and bystander cells undergo apoptosis.
Materials and methods

Virus

The virulent Miller strain of TGEV (National Veterinary Services Laboratory, Ames, IA), referred to as CHV, was used as a reference virus [38].

Cell cultures

Swine testes (ST) cells were cultured on 6-well plates or 8-well chamber slides in minimum essential medium (Gibco-BRL, Gaitherburg, MD), supplemented with 10% heat inactivated fetal bovine serum (Summit Biotechnology, CO). The cells were inoculated with TGEV when a complete monolayer was observed. Medium containing 2% heat inactivated fetal bovine serum was replaced at the time of infection. TGEV (10^5 TCID_{50}/ml) was used to inoculate swine testes (ST) cells in 6-well plates (Corning Costar Corporation, Oneonta, NY) and four wells of 8-well chamber slides (Nunc, Naperville, IL) were inoculated with TGEV at a multiplicity of infection of 0.01. Another four wells of 8-well chamber slides served as uninoculated controls.

Analysis of DNA fragmentation by agarose gel electrophoresis

Uninoculated and inoculated cells were observed for cytopathic effect (CPE) at 12, 24, 32, 40, and 48 hours postinoculation (h.p.i.). The cells were then scraped with a cell scraper (Fisher, PA), transferred to 1.5 ml microfuge tube, and centrifuged at 1100 x g for 3 minutes. Cells were washed, and resuspended in 200 μl of cold phosphate buffer saline (PBS). Total cell DNA was obtained using a Qiagen tissue kit (Qiagen, CA). DNA was precipitated and resuspended in 60 μl of TE buffer. Finally, 10 μl of the total 60 μl cell DNA was electrophoresed on a 1.2% agarose gel (Amresco, Solon, OH). DNA bands were stained with ethidium bromide, visualized by UV illuminator, and recorded on Polaroid type
57 film (Polaroid Corporation, Cambridge, MA). This assay was repeated three times using 3 different sets of cells.

**Electron Microscopy**

Uninoculated and TGEV-inoculated cells were washed twice with cold phosphate buffer saline (PBS), and fixed with 3% glutaraldehyde for 3 min at 12, 24, 32, 40, and 48 hours postinoculation (h.p.i.). Cells were scraped, collected in 1.5 ml microfuge tube, and pelleted at 6000 x g for 2 minutes at 4° C. The pellets were washed three times with 0.1 M sodium cacodylate buffer (pH 7.2) for 15 minutes each, post fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hours at room temperature, dehydrated in an acetone series, and embedded in plastic.

**In situ detection of DNA fragmentation in ST cell culture**

Uninoculated and TGEV-inoculated ST cells on 8-well chamber slides at 12, 24, 32, 40, and 48 h.p.i. were fixed in 4% neutral buffer formalin for 2 hours at 4° C and washed twice, 5 minutes each, with PBS. The TUNEL method was performed using an ApopTag kit (Oncor, Gaitherburg, MD) according to the manufacturer’s instructions with slight modifications. Briefly, 8-well chamber slides were subjected to the enzymatic incorporation of digoxigenin-labeled nucleotide with enzyme terminal deoxynucleotidyl transferase, washed and incubated with either anti-digoxigenin peroxidase and 3,3'-diaminobenzidine (DAB) or (1:400) anti-digoxigenin alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) and 45 µl of 4-nitroblue tetrazolium (NBT), and 35 µl of 5-bromo-4-chloro-3-indolyl-phosphatase (BCIP) in detection buffer. These slides were counterstained with either nuclear fast red for 2 minutes or full strength of hematoxylin for 4 dips. The cells with the signal localized within the nucleus without diffuse staining in the cytoplasm or extracellular area because of intact nuclear and cell membranes were interpreted as being
apoptotic cells [5]. The number of apoptotic cells was determined by counting 1,000 cells in each of 5 different areas in each well of the 8-well chamber slides. Cells were counted in four separate wells and the number of apoptotic cells determined by counting 5,000 cells. The mean percentage of apoptotic cells was determined using the percentage of apoptotic cells in 20,000 cells counted in four inoculated, and four uninoculated wells. The results are shown as the mean percentage of apoptotic cells, and one standard deviation from the mean.

In situ hybridization

Fluorescein-labeled RNA probe was prepared from plasmid pPSP.FP, as reported previously [30] which contains an TGEV S gene segment from nucleotides 1678 to 2250 [3]. Specificity of the probe was determined by dot blot hybridization [39]. Briefly, uninoculated and TGEV-inoculated ST cells on 8-well chamber slides at 12, 24, 32, 40, and 48 h.p.i. were used for in situ hybridization (ISH) as described previously [31]. The hybridization signal was detected by incubating with either anti-fluorescein peroxidase (1:50) or anti-fluorescein alkaline phosphatase (1:400) (Boehringer Mannheim, Indianapolis, IN). Slides were counterstained with either hematoxylin or nuclear fast red. Controls included uninoculated ST cells, RNase-treated cells inoculated with TGEV, positive-sense fluorescein-labeled RNA probe hybridized with TGEV-inoculated cells, and hybridized TGEV-inoculated ST cells with hybridization buffer free of probe.

Double labeling for the detection of viral nucleic acid and apoptosis

Uninoculated and TGEV-inoculated ST cells on 8-well chamber slides at 12, 24, 32, 40, and 48 h.p.i. were used for ISH followed by TUNEL labeling. ISH was performed as described above and hybridization signal was detected using anti-fluorescein alkaline phosphatase. Then slides were used to perform TUNEL labeling as described above and
the TUNEL signal was detected by anti-digoxigenin peroxidase. Slides were counterstained with nuclear fast red for 2 minutes.

Results

Analysis of DNA fragmentation by agarose gel electrophoresis

Cytopathic effect (CPE) characterized by rounding and sloughing of cells was detected at 24 h.p.i. in TGEV-inoculated ST cell cultures. The extent of CPE increased by 48 h.p.i. (Table 1). Apoptosis was observed at 24 h.p.i. and was characterized by fragmentation of DNA to approximately 180 base pair ladder. Both CPE and relative amounts of low molecular weight (LMW) DNA were evident at 24 h.p.i. and peaked at 48 h.p.i. (Table 1 and Fig. 1).

Ultrastructural changes

Examination of thick sections of TGEV-inoculated ST cells revealed several cells with condensation and margination of nuclear chromatin (Fig. 2B). In contrast, these changes were not observed in uninoculated ST cells (Fig. 2A). Ultrastructural changes characteristic of cell lysis and apoptosis were detected in TGEV-infected cells. Lytic cells were characterized by disrupted nuclear and cytoplasmic membranes, and degenerate cell organelles (Fig. 3B). There were several cells undergoing lysis, and most of these cells had several viral particles budding from the endoplasmic reticulum (Fig. 3B). Apoptotic cells were characterized by condensation and sharp margination of nuclear chromatin, formation of large, electrolucent vacuoles in the cytoplasms, intact cell organelles, and intact nuclear and cytoplasmic membranes (Fig. 3C). Viral particles were not detected in apoptotic cells. In contrast, cells in the uninoculated wells were normal (Fig. 3A).
In situ determination of DNA fragmentation in ST cell cultures

ISH was performed on cells in four TGEV-inoculated and four uninoculated wells. The cells in the TGEV-inoculated wells were positive for TGEV and cells in the uninoculated wells were negative. The TGEV positive cells had brown color localized in the cytoplasm without any color in the nucleus (Fig. 4B). In contrast, cells in the uninoculated wells were negative (Fig. 4A). Cells in the remaining 8-well chamber slides were used to detect *in situ* DNA fragmentation. The apoptotic cells had purple nuclei and no cytoplasmic staining (Fig. 5B). Several apoptotic bodies were blebbing out of the cytoplasm and were scattered in the intercellular space. There were a few TUNEL-positive cells in the uninoculated wells (Fig. 5A). TUNEL-positive cells in TGEV-inoculated wells were detected as early as 12 h.p.i. and their number peaked at 48 h.p.i. There was a higher percentage of apoptotic cells in the inoculated wells than that in the uninoculated wells at each time point, and the percentage increased with time (Fig. 6).

Double labeling for the detection of viral nucleic acid and apoptosis

TGEV-inoculated cells in TGEV-inoculated wells had purple cytoplasm whereas apoptotic cells had brown nuclei (Fig. 7B and 7C). In contrast, the ST cells in uninoculated wells had neither purple cytoplasm nor brown nucleus (Figure 7A). Both the TGEV-positive and apoptotic cells were detected as early as 12 h.p.i. (Fig. 7B) and their numbers increased with time. The majority of the labeled cells was positive for either TGEV nucleic acid, or apoptosis. Most of the apoptotic cells lacked TGEV RNA whereas most of the TGEV-positive cells did not exhibit apoptosis. At 48 h.p.i. there were, however, a few double positive cells which were positive for TGEV RNA and had round TUNEL-positive particles in the cytoplasm (Fig. 7C). These particles were apoptotic bodies that were phagocytized from the apoptotic cells rather than the nuclei of the double positive cells.
Discussion

This is the first study to show that TGEV is capable of inducing apoptosis. In this study several procedures were utilized to identify apoptosis including total cellular DNA extract to detect LMW DNA by agarose gel electrophoresis, TUNEL labeling for detection of DNA fragmentation in situ, and transmission electron microscopy for studying nuclear morphology. There was general agreement in the results from the different techniques. In the DNA fragmentation assay, DNA fragmentation bands were detected at 24 h.p.i. and peaked at 48 h.p.i. in the TGEV-inoculated cells. The DNA fragmentation pattern in multiplication of 180-200 base pairs strongly supports the presence of an apoptosis process in the cell culture [35]. There was a correlation between appearance of apoptosis and CPE that began at 24 h.p.i. and peaked at 48 h.p.i.

Using electron microscopy, we have found several cells from inoculated wells having sharp nuclear chromatin condensation along the nuclear membrane with intact nuclear and cell membranes [13], which is the characteristic of apoptosis. There were lytic cells that contained viral particles but the apoptotic cells did not, suggesting that infected cells may be undergoing cell lysis. McClurkin and Norman [23] also reported the changes in the nuclei of the ST and swine kidney cell cultures infected with several isolates of TGEV stained with acridine orange which were characterized by shrunken, marked clumping and margination of the chromatin material along the nuclear membrane [23]. The description of the changes and presented photographs were suggestive of apoptotic changes. However, the characteristics of apoptosis were not recognized at that time.

By using TUNEL labeling, we were able to identify apoptotic cells in the TGEV-inoculated cell culture and the number of apoptotic cells increased with time and peaked at 48 h.p.i. However, TUNEL labeling is more sensitive than using a DNA fragmentation assay.
because it can detect apoptotic cells earlier. The TUNEL positive cells were individual and syncytial cells. We did a double-labeling experiment to determine whether infected or bystander cells were undergoing apoptosis. Surprisingly, the uninfected bystander cells were undergoing apoptosis but infected cells were not.

At this point, the mechanism of TGEV induced apoptosis in bystander cells is not known. Mouse hepatitis virus and feline infectious peritonitis virus, coronaviruses, have also been shown to induce apoptosis in bystander cells [8, 10]. Several cytokines, such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) [12, 14, 43] and transforming growth factor-\(\beta\) (TGF-\(\beta\)) [36, 41] have been shown to promote apoptosis in several cells. Interferon (IFN)-\(\gamma\) and TNF-\(\alpha\) [26, 34] have been shown to induce apoptosis by upregulating Fas antigen expression. It is known that rat testicular cells are highly capable of expressing Fas ligand [33]. The mice testicular cell lines are also capable of expressing Fas antigen and can be upregulated by IFN-\(\gamma\) [20]. The rat testicular cells are capable of expressing both interferon-\(\alpha\) and -\(\gamma\) [4].

TGEV has been shown to be a good type I IFN inducer [16, 18]. It has been shown that TGEV-infected pigs had high titer of type I IFN in the intestine, blood, urine, and other organs (18). Type I IFN inhibits TGEV replication in vitro but has little or no effect in vivo [16, 21]. Type I IFN also has cytotoxicity effect for both porcine intestinal explant and porcine cell lines (ST, PK-15 porcine kidney and intestinal epithelial cells) either infected or uninfected with TGEV [2, 19, 40]. Therefore, the antiviral activity against TGEV of type I IFN may partly be due to cytotoxicity effect [40]. Furthermore, cytopathic strain of bovine viral diarrhea virus has been shown to be able to prime uninfected cells to undergo apoptosis by type I IFN [1]. Therefore, it is possible that cytotoxicity effect of type I IFN may be mediated through apoptotic process. It has also been proposed that the mechanisms responsible for type I IFN inducing TGEV-infected ST cells destruction may contribute to the
pathogenesis of TGEV infection in vivo [2]. Therefore, type I IFN might play a role in inducing apoptosis in bystander cells and thus amplifying the cytopathic effect of TGEV.

At this time, we do not know if type I IFN, other cytokines, some soluble factors released from infected cells or viral products are responsible for inducing apoptosis in bystander cells. In this study, we have shown that TGEV induces apoptosis in ST cell culture, and infected cells undergo cell lysis. Most of the apoptotic cells were bystander cells whereas most of the productively infected cells did not undergo apoptosis. However, whether TGEV is capable of inducing apoptosis in enterocytes in vivo is still unresolved.

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Figure 1. Analysis of DNA fragmentation as an evidence of apoptosis by agarose gel electrophoresis. A 10 µl of total DNA extracted from TGEV-inoculated or uninoculated ST cell cultures was loaded on to each lane of a 1.2% agarose gel. A DNA ladder of bands in multiplication of about 180 nucleotides in size were detected in the DNA of TGEV-inoculated cell culture at 24 h.p.i. with highest concentration at 48 h.p.i.

Figure 2. Thick sections of uninoculated (A) and TGEV-inoculated ST cell culture (B) at 24 h.p.i. The section of TGEV-inoculated ST cell culture showed sharp condensation and margination of nuclear chromatin along the nuclear membrane of the apoptotic cells (arrow).
Table 1. The percentage of cytopathic effect and concentration of low molecular weight DNA in inoculated ST cell cultures at 12, 24, 32, 40, and 48 hours postinoculation\(^a\).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Hours</th>
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<tr>
<td>% CPE</td>
<td>12</td>
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<td>Relative amount of</td>
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<td>LMW DNA(^b)</td>
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\(^a\) The percentage of CPE and amount of LMW DNA was done by using 3 replications of uninoculated and inoculated ST cells culture.

\(^b\) Relative amount of LMW DNA was determined by comparing the intensity of the DNA bands in agarose gels as follows: - = no band, ± = very weak, + = weak, ++ = moderate, +++ = strong.
Figure 3. Electron micrograph of uninoculated (A) and TGEV-inoculated ST cell culture (B, C) at 32 h.p.i. The virus inoculated cells in TGEV-inoculated ST cell culture (B) had several virus particles in vesicles of the endoplasmic reticulum (inset) and were undergoing cell lysis. In contrast to uninoculated ST cells in TGEV-inoculated ST cell culture (C) had no virus particles in the endoplasmic reticulum (inset) and exhibited shrinking of cells and nuclei, condensation of nuclear chromatin, and intact cell and nuclear membranes.
Figure 4. *In situ* hybridization of uninoculated (A) and TGEV-inoculated ST cell culture (B) at
32 h.p.i. with fluorescein-labeled RNA probe produced from pPSP.FP1. Infected
ST cells exhibited dark brown color specifically localized in the cytoplasm but
uninoculated cells were negative.

Figure 5. TUNEL analysis for the detection of *in situ* DNA fragmentation in uninoculated (A)
and TGEV-inoculated ST cell culture (B) at 40 h.p.i. Several apoptotic cells in
TGEV-inoculated cell culture showed dark purple staining localized in the nucleus.
Figure 6. The mean percentage of apoptotic cells from uninoculated and inoculated ST cell cultures. The mean percentage of apoptotic cells increased with time in TGEV-inoculated ST cell cultures and was higher than that in uninoculated ST cell cultures at each time point.
Figure 7. Double labeling analysis of viral RNA and apoptotic cells in uninoculated (A) and TGEV-inoculated ST cell culture at 12 h.p.i (B) and 48 h.p.i. (C). Several cells from TGEV-inoculated ST cell culture were positive for TGEV RNA as shown by specific purple color cytoplasm. Several cells were also positive by TUNEL technique as shown by dark brown nuclei (arrow) and there were several apoptotic bodies scattered throughout the slides. However, there was no double-staining positive cells for both TGEV RNA and apoptosis.
CHAPTER 6. A PNEUMO-VIRULENT U.S. ISOLATE OF PORCINE
REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV)
INDUCES APOPTOSIS IN BYSTANDER CELLS BOTH IN VITRO AND IN VIVO

A full manuscript submitted to Journal of General Virology

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and Prem S. Paul

Abstract

Lelystad virus (LV), the European prototype strain of porcine reproductive and
respiratory syndrome virus has been shown to induce apoptosis in monkey kidney and
alveolar macrophage’s cultures and this has been attributed to open reading frame (ORF) 5
product. Apoptosis has also recently been detected in testicular germ cells for a U.S.
 isolate. Here we show that a virulent isolate of PRRSV induces apoptosis in vitro and in vivo in the lungs and lymph nodes and that majority of the apoptotic cells were uninfected
bystander cells. The ATCC CRL11171 cells inoculated with U.S. PRRSV strain ATCC
VR2385 and tissues of pigs infected with the same strain were examined for evidence of
apoptosis by agarose gel electrophoresis, transmission electron microscopy, and terminal
deoxytransferase dUTP nick end labeling (TUNEL) techniques. By electron microscopy and
double labeling techniques, apoptosis was detected primarily in uninfected bystander cells
in continuous cell line rather than the PRRSV-infected cells. Apoptotic cells were also
detected in lungs and lymph nodes. In the lungs, the apoptotic cells were predominantly
alveolar macrophages, pulmonary intravascular macrophages, lymphocytes, and type I and
II pneumocytes. In the lymph nodes, the apoptotic cells were predominantly lymphocytes and macrophages. By a double-labeling technique, we found that most of the apoptotic cells were negative for PRRSV antigen or nucleic acid. Apoptosis in a large number of macrophages and lymphocytes appear to be a mechanism of PRRSV pathogenesis and might be the reason for a dramatic reduction in the number of alveolar macrophages and circulating lymphocytes and monocytes in PRRSV-infected pigs leading to their susceptibility to secondary infection.

Introduction

Porcine reproductive and respiratory syndrome (PRRS), recognized in the U.S. since 1987 (Hill, 1990), is characterized by reproductive failure (stillborn, mummified and weak-born piglets), and respiratory disease in young and growing pigs (Christianson et al., 1992a; Halbur et al., 1995; Terpstra et al., 1991). The quality of semen is also reduced in boars infected with PRRS virus (PRRSV) (Prieto et al., 1996). Growing pigs infected with PRRSV are often infected or co-infected with bacteria and other viruses (Done et al., 1995; Galina et al., 1994; Groschup et al., 1993; Kawashima et al., 1996; Van Reeth et al., 1996) resulting in major decrease in production efficiency and increased death loss.

The etiologic agent of PRRS was first isolated in 1991 in Netherlands (Wensvoort et al., 1991), and then in 1992 in the United States (Collins et al., 1992). The prototype European isolate is known as the Lelystad virus (LV). PRRSV is a member of the family Arteriviridae (Pringle, 1996) because of the similarity in morphology, in genomic organization, and in strategy of gene expression to lactate dehydrogenase elevating virus, equine arteritis virus, and simian hemorrhagic fever virus (Conzelman et al., 1993; Theil et al., 1993). PRRSV is a small pleomorphic spherical enveloped virus, 50-80 nm in diameter,
with an isometric core of about 25-30 nm (Benfield et al., 1992; Dea et al., 1995; Mardassi et al., 1994; Meng et al., 1996; Wensvoort et al., 1991). The nucleic acid of PRRSV is a single-stranded, positive-sense polyadenylated RNA genome which has a size of 15 kb (Conzelman et al., 1993; Meulenberg et al., 1993) and contains eight open reading frames (ORF). At least six of the mRNAs arranged as 3'-terminal nested sets have been shown to be produced in the infected cells (Conzelman et al., 1993; Meulenberg et al., 1993). The subgenomic mRNAs, with the exception of the smallest one, are polycistronic in nature, and only the unique 5' ORF is translated.

Both the LV and U.S. PRRSV isolates are structurally related but antigenically and genetically distinct (Wensvoort et al., 1992). The amino acid and nucleotide sequence homology in ORFs 2 to 7 of the North American and the European isolates is about 60% (Conzelman et al., 1993, Mardassi et al., 1994; Meng et al., 1995 a, b; Meulenberg et al., 1993; Morozov et al., 1995) with the exception of ORF 6 which has the highest homology of about 80% (Meng et al., 1995 b). However, the amino acid and nucleotide sequence homology among different U.S. or different European isolates is about 90% (Meng et al., 1995 a, b; Suarez et al., 1996 b).

Lelystad virus has been shown to induce apoptosis in both MA-104 cells and swine alveolar macrophage cultures. This has been attributed to the ORF 5 as the product of the ORF 5 of LV induced apoptosis directly in monkey BSC40 cells (Suarez et al., 1996 a). Recently, a U.S. PRRSV isolate has been shown to induce apoptosis in germ cells in testes and the investigators have hypothesized that it may contribute to the pathogenesis of PRRSV in infected boars (Sur et al., 1997). However, several questions remain unanswered. It is not known whether U.S. PRRSV induces apoptosis in virus-infected cells or uninfected bystander cells, and whether PRRSV isolates induce apoptosis in vivo in
alveolar macrophages in the lungs, and macrophages and lymphocytes in lymph nodes which are the primary sites for virus replication. This information is needed to fully understand mechanism of pathogenesis of PRRSV in infected growing pigs. In this paper, we report that a highly pneumo-virulent U.S. PRRSV isolate (VR2385) kills cells by apoptosis both in vitro in a continuous cell line and in vivo in alveolar macrophages of the lungs and macrophages and lymphocytes of the lymph nodes of PRRSV-inoculated pigs. We also show that the primary cells that undergo apoptosis are the uninfected bystander cells but not the infected cells. Thus, this may result in amplification of viral cytocidal effects and may serve as a mechanism of viral virulence.

Materials and Methods

Virus and Cells

The U.S. PRRSV isolate ATCC VR2385 was used as a reference virus (Meng et al., 1996). The ATCC CRL11171 cells were cultured on 8-well chamber slides or 25 cm² flasks in Dulbecco's minimal essential medium (Gibco BRL, Gaitherburg, MD), supplemented with 10% heat inactivated fetal bovine serum (Gibco BRL, Gaitherburg, MD) until a complete monolayer was observed (Meng et al., 1996). Medium containing 2% heat inactivated fetal bovine serum was replaced at the time of infection. The ATCC VR2385 strain of PRRSV (10⁶.⁵ TCID₅₀/ml) was used to inoculate ATCC CRL11171 cells in four wells of 8-well chamber slides or 25 cm² flasks, at a multiplicity of infection of 0.0001. Four wells of the 8-well chamber slides served as uninoculated controls. Slides were fixed in 4% paraformaldehyde at 1, 2, 3, 4, 5, and 6 days postinoculation (d.p.i). For pig inoculation, ATCC VR2385 was thawed and diluted to a final inoculation dose titer of 10⁴.⁵ TCID₅₀/5 ml.
Pigs were inoculated intranasally. The negative control pigs were mock-inoculated with normal cell culture fluids.

**Tissues**

Twenty four 5-week-old, cesarean-derived, colostrum-deprived (CDCD) pigs were randomly divided into PRRSV- and mock-inoculated groups of 12 pigs each. Two pigs from each group were necropsied at 1, 3, and 5 d.p.i., and six pigs from each group were necropsied at 10 d.p.i. Tissues were taken from all lung lobes, and mediastinal and tracheobronchial lymph nodes. All tissues were fixed in 10% buffered formalin for at least 24 hours and routinely processed and embedded in paraffin.

**Analysis of DNA fragmentation by agarose electrophoresis**

Uninoculated and inoculated cells were observed for cytopathic effect (CPE) at 1, 2, 3, 4, 5, and 6 d.p.i. The cells were then scraped with a cell scraper (Fisher, PA) and centrifuged at 1100 x g for 3 minutes. Cells were washed, and resuspended in 200 μl of cold PBS. Total cell DNA was isolated using a Qiagen tissue kit (Qiagen, CA). DNA was precipitated and resuspended in 50 μl of TE buffer. Finally, 10 μl of the total cell DNA was electrophoresed on a 1.2% agarose gel (Amresco, Solon, OH). DNA bands were stained with ethidium bromide, visualized by UV illuminator, and recorded photographically (Polaroid Corporation, Cambridge, MA). This assay was repeated three times using 3 different sets of cells.

**Electron Microscopy**

Uninoculated and inoculated cells were washed twice with cold PBS, and fixed with 3% glutaraldehyde for 3 min at 1, 2, 3, 4, 5, and 6 d.p.i. Cells were scraped, pelleted at 6,000 x g for 2 minutes in 1.5 ml microfuge tube at 4° C, and processed and embedded in plastic for electron microscopy.
Immunohistochemistry (IHC)

Sections of lung, and mediastinal and tracheobronchial lymph nodes were cut at 5 µm, deparaffinized, rehydrated, and processed for immunohistochemistry as described earlier (Halbur et al., 1996). Briefly, sections were incubated with a primary monoclonal antibody (1:1,000) to the nucleocapsid protein for 16 h at 4°C and followed by incubation with biotinylated goat anti-mouse antibody (Dako Corp., Carpinteria, CA) for 30 min at room temperature. Sections were then washed 3 times, and incubated with a 1:200 peroxidase-conjugated streptavidin for 40 min at room temperature. Sections were washed and incubated with fresh 3,3'-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA) for 8-10 min at room temperature, and were counterstained with 4 dips in full strength hematoxylin, or 2 minutes in nuclear fast red.

In situ hybridization (ISH)

A fluorescein-labeled RNA probe was prepared from plasmid pPSP λ345 containing the entire sequence from 50 nucleotides upstream of the ORF 5 stop codon to the end of ORF 7 (Haynes et al., 1997). Specificity of the probe was determined by dot blot hybridization. Briefly, cells on 8-well chamber slides and tissue sections were used to perform in situ hybridization (ISH) as described earlier (Haynes et al., 1997). The hybridization signal was detected using (1:400) anti-fluorescein alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) and incubated with 45 µl of NBT (4- nitroblue tetrazolium; Boehringer Mannheim, Indianapolis, IN) and 35 µl of BCIP (5-bromo-4-chloro-3-indolyl-phosphate; Boehringer Mannheim, Indianapolis, IN). Slides were counterstained with nuclear fast red. Controls included uninoculated ATCC CRL1171 cells, mock-inoculated pig tissues, and RNase-treated ATCC CRL1171 cells inoculated with PRRSV, and tissues from PRRSV inoculated pigs.
In situ detection of DNA fragmentation in ATCC CRL11171 cells and inoculated tissues

Cells on 8-well chamber slides and sections of pig tissues were used to perform TdT-mediated X-dUTP nick end labeling (TUNEL) using an ApopTag kit (Oncor, Gaitherburg, MD) according to the manufacturer's instructions with slight modifications. Briefly, 8-well chamber slides and deparaffinized sections were subjected to an enzymatic incorporation of digoxigenin-labeled nucleotide with enzyme terminal deoxynucleotidyl transferase (TdT), washed and incubated with anti-digoxigenin peroxidase or anti-digoxigenin-alkaline phosphatase. After washing, slides were incubated with either DAB for anti-digoxigenin peroxidase incubated slides or NBT and BCIP for anti-digoxigenin alkaline phosphatase incubated slides. Slides were counterstained with either hematoxylin or nuclear fast red.

Double labeling for the detection of viral antigen or viral nucleic acid and apoptosis

Cells on 8-well chamber slides and deparaffinized sections were used to perform either IHC or ISH as described above. These slides were then used to perform TUNEL labeling as described above. TUNEL signal was detected using either anti-digoxigenin peroxidase or anti-digoxigenin alkaline phosphatase on slides previously processed for IHC or ISH, respectively.

Results

Analysis of DNA fragmentation by agarose gel electrophoresis

Cytopathic effect (CPE) characterized by degeneration, cell rounding, clumping of cells, and cell detachment was observed at 2 d.p.i. in PRRSV-inoculated cell cultures. About 30%, 80%, and > 98% of the PRRSV-inoculated cells detached at 4, 5, and 6 d.p.i.,
respectively. Multiple nucleosomal-sized DNA fragments increasing by 180 bases in size were observed at 2, 3, and 4 d.p.i. (Fig. 1).

**Ultrastructural changes in cell cultures**

Ultrastructural changes characteristic of both cell lysis and apoptosis were detected in PRRSV-inoculated cells. Apoptotic cells were characterized by condensation and sharp margination of nuclear chromatin, intact cell organelles, and intact nuclear and cell membranes (Fig. 2b). Lytic cells were characterized by disrupted nuclear and cell membranes, degenerate cell organelles, and lack of sharp margination of nuclear chromatin (Fig. 2c). Most of the lytic cells contained viral particles whereas apoptotic cells did not.

**In situ determination of DNA fragmentation and viral nucleic acid in cell cultures**

The TUNEL-positive cells were found in both uninoculated and virus inoculated wells, however, the number of TUNEL-positive cells in inoculated wells was higher than that in the uninoculated wells. The TUNEL-positive cells were detected as early as 1 d.p.i., and increased until 4 d.p.i. No data was collected on day 5 and 6 postinoculation because most of the cells had detached from the wells. The apoptotic cells had brown nuclei and no cytoplasmic staining (Fig. 3a). There were several apoptotic bodies scattered in the intercellular space and appeared to be phagocytized by the neighboring cells. The remaining 8-well chamber slides were used to perform ISH. Cells in the PRRSV-inoculated wells were positive for PRRSV nucleic acids and the number of virus positive cells increased with time. The cells in the uninoculated wells remained negative. The cells positive for PRRSV nucleic acid were recognized by the presence of purple staining localized only in the cytoplasm (Fig 3a). In contrast, cells in the uninoculated wells were negative for PRRSV nucleic acids by ISH (Fig. 3b).
**Double labeling for the detection of viral nucleic acid and apoptosis in vitro**

In order to determine whether the apoptotic cells were infected or uninfected bystander cells, we did a double labeling experiment. ISH was done to detect virus nucleic acid positive cells and TUNEL technique was done on the same slides to detect apoptotic cells. PRRSV-infected cells in the virus-inoculated wells of CRL11171 cells had purple cytoplasmic staining whereas apoptotic cells had a brown staining nuclei (Fig. 3a). In contrast, cells in uninoculated wells had no purple cytoplasmic staining but a few cells had brown nuclear staining (Fig. 3b). The number of both PRRSV-positive and apoptotic cells increased with time. Most of the labeled-cells were either positive for PRRSV nucleic acid or apoptosis but not both. Only a few cells were positive for both PRRSV and apoptosis at 4 d.p.i.

**Histopathologic changes and in situ determination of DNA fragmentation in vivo**

Based on our results of in vitro studies, we speculated that U.S. PRRSV may be capable of inducing apoptosis in vivo. Therefore, we infected pigs with U.S. PRRSV isolate VR2385 and collected tissues at different times postinoculation. Lung sections from the PRRSV-inoculated pigs showed moderate multifocal interstitial pneumonia at 3 and 5 d.p.i. which progressed to severe diffuse interstitial pneumonia at 10 d.p.i. The interstitial pneumonia was characterized by septal infiltration by mixed mononuclear cells, type II pneumocyte hypertrophy and hyperplasia, and accumulation of normal and necrotic macrophages in alveolar spaces. Lung sections from the mock-inoculated pigs were normal.

Prior to performing the TUNEL techniques, IHC and ISH techniques were performed on lung tissues from PRRSV- and mock-inoculated pigs to ensure that the techniques were working well. The cells positive for viral nucleic acid had purple staining localized in the
cytoplasm (Fig. 4a) and the PRRSV antigen positive cells had brown color in the cytoplasm (Fig. 4b). The viral antigen and nucleic acid positive cells were primarily alveolar macrophages and lesser numbers of mononuclear cells in the alveolar septa. The viral antigen or nucleic acid positive cells were detected as early as 1 d.p.i. and the number of positive cells increased with time until 10 d.p.i. The TUNEL positive cells were alveolar macrophages, lymphocytes, and mononuclear cells which were morphologically consistent with pulmonary intravascular macrophages (Fig. 4c). Lung sections from mock-inoculated pigs were negative for viral antigen and nucleic acids (Fig. 4d). The number of TUNEL positive cells were also detected as early as 1 d.p.i. and peaked at 10 d.p.i. A few cells in the lung of mock-inoculated pigs were positive by TUNEL labeling (Fig. 4d) representing the normal apoptosis process in the tissues.

Lymph node sections from PRRSV-inoculated pigs were hyperplastic compared to those from mock-inoculated control pigs. By day 3 and 5 postinoculation, PRRSV-induced lymphadenopathy was characterized by lymphoblastic follicular hyperplasia, swollen and vacuolated follicular macrophages and dendritic cells, and mononuclear cell infiltration in the subcapsular region. At 10 d.p.i., lymphadenopathy was characterized by follicular necrosis and increase in numbers of tingible body macrophages. PRRSV nucleic acid and antigen positive cells in the germinal centers resembled tingible body macrophages (Fig. 5a, b). Positive cells in the paracortical areas were either macrophages or interdigitating dendritic cells. The PRRSV antigen and nucleic acid positive cells in the lymph nodes were detected as early as 1 d.p.i. and peaked at 10 d.p.i. The apoptotic cells in the lymph nodes were detected as early as 1 d.p.i. and peaked at 10 d.p.i. The TUNEL-positive cells were mostly lymphocytes and tingible body macrophages (Fig. 5c). Lymph node sections from mock-inoculated pigs were negative for viral antigen and nucleic acids, and a few cells were
positive for apoptosis (Fig. 5d). The viral antigen or nucleic acid positive cells in both lungs and lymph nodes were multifocal in distribution. In contrast to viral antigen or nucleic acid positive cells, the TUNEL positive cells were scattered throughout the sections.

Double labeling for the in vivo detection of PRRSV antigen or nucleic acid, and apoptosis in vivo

By using ISH and TUNEL techniques, PRRSV-infected cells had purple color localized in the cytoplasm whereas apoptotic cells had a brown nucleus (Fig. 4a). By using IHC and TUNEL techniques, PRRSV-infected cells had brown cytoplasmic staining whereas apoptotic cells had purple nuclear staining (Fig. 4b). In lung sections, the positive cells were either positive for PRRSV antigen, nucleic acid or apoptosis. Most of the apoptotic cells were negative for PRRSV antigen or nucleic acid. It should be noted that the virus-positive alveolar macrophages were frequently seen next to the apoptotic alveolar macrophages. In the lymph nodes, we also found that most of the labeled-cells were positive for either PRRSV nucleic acid and antigen or apoptosis (Fig. 5a, b). There were a few cells in lymph nodes, especially tingible body macrophages, which were positive for both PRRSV antigen or nucleic acid and apoptosis. Evidence of apoptosis by TUNEL was present in tingible bodies in the cytoplasm rather than the nucleus of the double positive tingible body macrophages. Interestingly in both lungs and lymph nodes, there were many areas that had many virus positive cells but had a few apoptotic cells and vice versa.

Discussion

Several viruses have been shown to induce apoptosis either directly (Lu et al., 1996; Suarez et al., 1996 a; Yamada et al., 1994; Zhuang et al., 1995) or indirectly (Finkel et al., 1995; Godfraind et al., 1995; Momoi et al., 1996; Ramiro-Ibanez et al., 1996). Apoptosis
has been demonstrated in vitro for a European Lelystad isolate of PRRSV (Suarez et al., 1996), and recently in testicular germ cells for a U.S. PRRSV isolate (Sur et al., 1997). In this study, we showed that the virulent U.S. PRRSV strain ATCC VR2385 induces apoptosis both in vitro and in vivo in the lungs and lymph nodes of PRRSV-infected pigs. Evidence for apoptosis was demonstrated by three separate techniques: electrophoresis, electron microscopy, and TUNEL with good correlation among results from different techniques. We also found that the U.S. PRRSV strain ATCC VR2385 induced apoptosis in the continuous cell line much earlier than that reported for the European PRRSV isolate. The nucleosomal-sized DNA fragments were detected 2 days after inoculation with PRRSV strain VR2385 compared to 5 days after inoculation with LV (Suarez et al., 1996). Whether this difference is related to the higher virulence of VR2385 is not known at this time. The VR2385 strain has been shown to produce more severe pneumonia, and replicate to higher titer in pigs than LV (Halbur et al., 1996). Another major finding was that most of the apoptotic cells in both in vitro and in vivo studies were uninfected bystander cells. In in vitro studies, PRRSV appeared to kill infected cells by cell lysis and induced apoptosis in uninfected bystander cells. The only double-positive cells in vivo were a few tingible body macrophages in the lymph nodes. However, the tingible bodies in the cytoplasm and not the nuclei of these macrophages were positive by TUNEL. The apoptosis in uninfected bystander cells in the continuous cell line was different from that of the European isolate which has been shown to directly induce apoptosis in vitro by the ORF 5 product (Suarez et al., 1996). At the present time, we do not know the reason behind the difference in mode of apoptosis induction between these two viruses. Virulence and amino acid and nucleic acid homology of these viruses are quite different (Conzelman et al., 1993, Mardassi et al., 1994; Meng et al., 1995
a, b; Meulenberg et al., 1993; Morozov et al., 1995) and may account for the differences between these viruses.

In the lungs and lymph nodes, we found that a large number of cells were TUNEL positive. Morphologically, the TUNEL-positive cells in the lungs were alveolar macrophages, pulmonary intravascular macrophages, lymphocytes, and type I and type II pneumocytes. In the lymph nodes, the TUNEL-positive cells were predominantly lymphocytes in the germinal centers and macrophages throughout the lymph nodes. These results demonstrated that a large number of alveolar macrophages in the lungs and lymphocytes in the lymph nodes were destroyed by apoptosis during the course of infection. The ability of PRRSV to induce apoptosis in macrophages and lymphocytes might be the reason that PRRSV-infected pigs have a dramatic reduction in the numbers of the alveolar macrophages (Molitor et al., 1992) and the circulating lymphocytes and monocytes (Christianson et al., 1992 b; Zhou et al., 1992). This may in part explain why PRRSV-infected pigs appear to be prone to secondary infections with both bacteria (Galina et al., 1994; Kawashima et al., 1996; Done et al., 1995) and viruses (Groschup et al., 1993; Van Reeth et al., 1996).

At this point, the mechanism of PRRSV-induced apoptosis in bystander cells is not known. Macrophages are capable of producing several mediators of apoptosis such as tumor necrosis factor-α (Kizaki et al., 1993; Kolesnick et al., 1994), nitric oxide (Cui et al., 1994; Messmer et al., 1994), reactive oxygen species (Martin et al., 1993; Hansson et al., 1996), and interleukin-1β (IL-1β) (Kolesnick et al., 1994, Onozaki et al., 1985). The alveolar macrophages from pigs infected with a U.S. isolate of PRRSV have also been shown to express high levels of IL-1β (Zhou et al., 1992), which may play a role in apoptosis induction. Interestingly, the pathologic changes in the lungs and lymph nodes are often
diffuse and a large number of apoptotic cells are observed diffusely in both lungs and lymph nodes. This is in contrast to the multifocal distribution of PRRSV antigen or nucleic acids in infected cells in these tissues. This suggests that cytokines, especially IL-1β, reactive oxygen species, or nitric oxide might have a role in PRRSV pathogenesis. Therefore, PRRSV-induced apoptosis in bystander cells might be the mechanism for amplifying the cytopathic effect of PRRSV. Differences in virulence of PRRSV isolates have also been demonstrated (Halbur et al., 1996). Since the virulence of PRRSV varies considerably among isolates, it is possible that the ability of PRRSV to induce apoptosis in bystander cells may be related to viral virulence.

Our study demonstrated that U.S. PRRSV isolate VR2385 induces apoptosis both in vitro and in vivo. Most of the apoptotic cells were uninfected bystander cells and the infected cells were undergoing cell lysis. The apoptosis of alveolar macrophages in the lungs, and macrophages and lymphocytes in the lymph nodes may contribute to the PRRSV pathogenesis in infected growing pigs. Whether release of IL-1β or other cytokines is responsible for inducing apoptosis in PRRSV-infected pigs, and whether there is any correlation between viral virulence and the ability of PRRSV isolates to induce apoptosis need further study.

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Figure 1. Analysis by agarose gel electrophoresis of DNA fragmentation of total cell DNA extracted from uninfected (lanes 2 and 9) or PRRSV-infected cells (lane 3-8) as an evidence of apoptosis. Multiple nucleosomal-sized DNA bands increasing in multiplication of about 180 nucleotides in size were detected in the DNA of PRRSV-inoculated cell cultures at 2, 3, and 4 d.p.i.
100 bp
0 d control
1 d.p.i.
2 d.p.i.
3 d.p.i.
4 d.p.i.
5 d.p.i.
6 d.p.i.
6 d control
100 bp
Figure 2. Electron micrograph of uninoculated (a) and PRRSV-inoculated ATCC CRL11171 cell culture (b, c) at 3 d.p.i. The virus-infected cells (b) in PRRSV-inoculated CRL11171 cell cultures were undergoing cell lysis and had several viral particles in the cytoplasm (inset). In contrast, the uninfected cells (c) in PRRSV-inoculated CRL11171 cell cultures demonstrated shrinking of cells and nucleus, condensation of nuclear chromatin, and intact nuclear and cytoplasmic membranes.
Figure 3. Analysis by double labeling for virus RNA and apoptotic cells in inoculated (a) and uninoculated CRL11171 cell culture (b) at 1 d.p.i. Several cells in PRRSV-inoculated CRL11171 cell culture (b) were positive for PRRSV RNA with purple cytoplasmic staining. Several apoptotic cells and apoptotic bodies with dark brown nuclei were also present.

Figure 4. Photographs of lung sections from PRRSV-inoculated (a, b, c) and mock-inoculated pigs (d) analyzed by double labeling for the presence of viral antigen by immunohistochemistry, nucleic acid by in situ hybridization, and apoptosis by terminal deoxytransferase dUTP nick end labeling. ISH and TUNEL labeling on same sections of lungs from PRRSV-inoculated pigs at 5 d.p.i. (a) showed PRRSV nucleic acid positive cells with dark purple staining in the cytoplasm and apoptotic cells with brown staining nuclei. IHC and TUNEL staining on same sections of lungs from PRRSV-inoculated pigs at 3 d.p.i. (b) showed PRRSV-antigen positive cells with specific brown staining in the cytoplasm and apoptotic cells with dark purple nuclei. The PRRSV antigen and nucleic acid positive cells were predominantly alveolar macrophages and mononuclear cells in the alveolar septa. There were several apoptotic cells with brown staining nuclei in the lungs of PRRSV-inoculated pigs at 3 d.p.i (c) which were alveolar macrophages, pulmonary intravascular macrophages and lymphocytes. Tissues from mock-inoculated pigs (d) were negative for virus antigen and nucleic acids but a few cells were positive for apoptosis.
Figure 5. Photographs of lymph node sections from PRRSV-inoculated (a, b, c) and mock-inoculated pigs (d) analyzed by double labeling for the presence of viral antigen by immunohistochemistry, nucleic acid by *in situ* hybridization, and apoptosis by terminal deoxytransferase dUTP nick end labeling. ISH and TUNEL labeling on same sections of lymph nodes from PRRSV-inoculated pigs at 5 d.p.i. (a) revealed PRRSV nucleic acid positive cells with dark purple color localized in the cytoplasm and apoptotic cells with brown staining nuclei. IHC and TUNEL staining on same sections of lymph nodes from PRRSV-inoculated pigs at 3 d.p.i. (b) revealed PRRSV antigen positive cells with specific brown staining in the cytoplasm and apoptotic cells with dark purple nuclei. The PRRSV antigen and nucleic acid positive cells were either tingible body macrophages, macrophages or interdigitating dendritic cells. There were several apoptotic cells with brown staining nuclei in the lymph nodes of PRRSV-inoculated pigs at 5 d.p.i. (c) which were predominantly lymphocytes and macrophages. Tissues from mock-inoculated pigs (d) were negative for virus antigen and nucleic acid and a few cells were positive for apoptosis.
CHAPTER 7. GENERAL CONCLUSIONS

In the first study, we developed an in situ hybridization (ISH) technique for the detection and differentiation of TGEV and PRCV in swine testes (ST) cell culture-infected with TGEV and PRCV and in formalin-fixed paraffin-embedded tissues. ISH technique was first standardized using ST cells-infected with TGEV or PRCV using radiolabeled RNA probes prepared from plasmids pPSP.FP₁ and pPSP.FP₂. The plasmid pPSP.FP₂ contained the left half of the S gene of TGEV which presented in both TGEV and PRCV (Vaughn et al., 1994). The plasmid pPSP.FP₁ contained the S gene segment which is present in TGEV but is deleted in PRCV (Vaughn et al., 1994). The TGEV-and PRCV-infected cells had hybridization signals with radiolabeled RNA probe prepared from plasmid pPSP.FP₂. Only the TGEV-infected cells had hybridization signals with radiolabeled RNA probe prepared from plasmid pPSP.FP₁. Thus, it was possible to distinguish TGEV-infected cells from PRCV-infected cells by using RNA probe prepared from plasmid pPSP.FP₁.

Once the technique was standardized using cell monolayers, ISH technique was performed using small intestinal sections from experimentally TGEV-inoculated pigs. The virus nucleic acid positive cells were mostly enterocytes at the tips of villi. The virus nucleic acid distribution in small intestines was similar to the viral antigen distribution reported previously (Bohl and Pensaert, 1989; Chu et al., 1982; Frederick et al., 1976; Larochelle and Magar, 1993; Larson et al., 1979; Morin et al., 1973; Saif and Wesley, 1994; Woods et al., 1981). However, some crypt epithelial cells were positive for TGEV nucleic acid. This result was in contradiction to previous reports where no positive cells were found in the crypts of Lieberkuhn (Bohl and Pensaert, 1989; Chu et al., 1982; Frederick et al., 1976; Larochelle and Magar, 1993; Larson et al., 1979; Morin et al., 1973; Saif and Wesley, 1992; Woods et al., 1981). The reason for this difference is not known except previous studies have examined for viral antigen, whereas, we examined for virus nucleic acid. The receptors for
TGEV are not found in crypt epithelial cells (Delmas et al., 1992; Weingartl and Derbyshire, 1994) and the mechanism for TGEV infection of crypt epithelial cells is not known. One possibility is that the infection might be caused by nonspecific binding of virus on the cell membrane and thus lead to infection of these cells (Weingartl and Derbyshire, 1993). Another possibility is that infection of crypt epithelial cells leads to non-productive infection. In this case, viral proteins are not produced in the cells, and thus no signal is detected by immunofluorescence or immunohistochemistry. ISH was performed successfully to detect TGEV in the small intestines of naturally infected pigs submitted for diagnosis.

Lung tissues from experimentally PRCV-infected pigs were used to perform ISH. PRCV was shown to replicate mainly in the bronchiolar epithelial cells and in lesser amount in type I and type II pneumocytes, alveolar macrophages, and bronchial epithelial cells. The distribution of PRCV nucleic acid is compatible with the distribution of viral antigen as reported previously (O'Toole et al., 1989). However, these results are different from those observed by Cox et al. (1990a) in which viral replication occurred mainly in the alveolar cells.

ISH technique developed in this study was able to detect and differentiate TGEV and PRCV in tissues from experimentally and naturally infected pigs. Moreover, the sensitivity of ISH technique is equally or more sensitive when compared with immunofluorescence technique. However, ISH using radiolabeled RNA probes developed in these study is not appropriate as a diagnostic tool because it is very time-consuming and is not a user friendly technique. Thus, there is a need to improve ISH technique to be more user friendly and to consume less time.

In the second study, we developed a rapid ISH technique for the detection of TGEV in tissues using radiolabeled and fluorescein-labeled RNA probes. Small intestines from experimentally infected pigs were used to standardize ISH technique using fluorescein-labeled RNA probe prepared from plasmid pPSP.FP1, according to the protocol from the first
experiment. The nonisotopic ISH technique provided results which were as good as those using isotopic ISH. We then developed a rapid ISH technique using a rapid hybridization buffer (REH). Small intestinal sections from pigs experimentally and naturally infected with TGEV were hybridized for various times at 52°C and 70°C with a radiolabeled or a fluorescein-labeled RNA probe in a standard hybridization or with REH buffer. Hybridization of formalin-fixed paraffin-embedded tissues with either radiolabeled or fluorescein-labelled RNA probes with the REH buffer decreased hybridization time from 20 hours to 2 hours without compromising the intensity or the quality of the hybridization signal. Virus RNA was detected as early as 30 minutes hybridization using the REH buffer at 70°C or the standard hybridization buffer at 52°C with radiolabeled probe, however, the signal was stronger with the REH buffer at 70°C. Using fluorescein-labeled probe, virus RNA was detected as early as 30 minutes hybridization using the REH buffer at 70°C whereas use of the standard hybridization buffer at 52°C only detected viral infected cells after 2 hours or more of hybridization. Optimum results with strong signal and signal-to-noise ratio were obtained with both radiolabeled and fluorescein-labeled RNA probes using the REH buffer at 70°C. The signal intensity decreased with shorter hybridization using the REH buffer with both types of probes. The disadvantage of using the REH buffer for isotopic ISH was the slight increase in non-specific signal with increased hybridization time, but this was not a problem with 2 hours hybridization and was not detected with fluorescein-labeled RNA probes. The mechanism of enhancement of hybridization using REH buffer is not known, it may be due to the unfolding of viral RNA secondary structure at higher temperature (70°C) which may dissociate protein-nucleic acid complexes so that more target molecules are accessible for hybridization with the probe. The high temperature and lower viscosity of the REH buffer may play a role in the higher rate of movement of probe to find the target RNA. Using the
REH buffer with fluorescein-labeled RNA probe, the entire process could be performed within 7-8 hours which is more appropriate and more user friendly for diagnostic laboratory to use as diagnostic tool.

Although extensive studies have been conducted on TGEV, the mechanisms of TGEV-induced cell death remain unclear. In the third study, we showed that the virulent Miller strain of TGEV induced apoptosis in ST cell cultures, and the apoptotic cells were mostly uninfected bystander cells. Apoptosis in ST cell cultures was determined using agarose gel electrophoresis, electron microscopy, and TUNEL technique. The DNA fragmentation assay from TGEV-infected cells using agarose gel electrophoresis showed multiple nucleosomal-sized (180-200 base pairs) DNA fragments which were detected as early as 24 h.p.i. and peaked at 48 h.p.i. The nucleosomal-sized DNA fragments strongly support the presence of an apoptosis process in the cell culture (Tanuma and Shiokawa, 1996). Furthermore, there was a correlation between the development of CPE and DNA fragmentation.

Using transmission electron microscopy, we found both lytic and apoptotic cells in TGEV-inoculated wells. The lytic cells had virus particles in the endoplasmic reticulum. The apoptotic cells had cell and nuclear morphology typical of apoptotic cells characterized by sharp nuclear chromatin condensation along the nuclear membrane, shrinkage of cell, and intact nuclear and cell membranes. Surprisingly, these apoptotic cells did not have any virus particles. McClurkin and Norman (1966) also reported the changes in the nuclei of ST and swine kidney cell cultures infected with several isolates of TGEV. However, the authors did not understand the significance of these changes at that time. Using TUNEL technique, we were able to identify apoptotic cells in the TGEV-inoculated cell culture, and the number of apoptotic cells increased with time and peaked at 48 h.p.i. The TUNEL positive cells were individual and syncytial cells.
The double-labeling experiment was performed to confirm the electron microscopy results. Most of the labeled-cells were positive for either TGEV nucleic acid, or apoptosis. Thus the double-labeling experiment results also confirmed that uninfected bystander cells were undergoing apoptosis but infected cells were not. It has been shown that mouse hepatitis virus (Godfraind et al., 1995) and feline infectious peritonitis virus (Haagmans et al., 1996) also induce apoptosis in uninfected bystander cells. The mechanism of TGEV-induced apoptosis in bystander cells is not known at this time.

However, several cytokines, such as TNF-α (Itoh et al., 1991; Kizaki et al., 1993; Zheng et al., 1995) and TGF-β (Thompson, 1995; Weller et al., 1994), have been shown to induce apoptosis in several types of cells. Some cytokines, such as IFN-γ and TNF-α (Oyaizu et al., 1994; Takizawa et al., 1995), have been shown to induce apoptosis by upregulating Fas antigen expression. TGEV has been shown to be a good type I IFN inducer (La-Bonnardiere and Laude, 1981; Laude et al., 1984). The cytopathic strain of bovine viral diarrhea virus has been shown to prime uninfected cells to undergo apoptosis. Type I IFN has been shown to have cytotoxicity effect for both porcine intestinal explant and porcine cell lines (ST, PK-15 porcine kidney and intestinal epithelial cells) either infected or uninfected with TGEV (Bostworth et al., 1989; Laude and La-Bonnardiere, 1984; Weingartl and Derbyshire, 1991). Therefore, type I IFN might play a role in inducing apoptosis in uninfected bystander cells and amplifying the cytopathic effect of TGEV. However, whether type I IFN or other cytokines or virus products are responsible for inducing apoptosis in bystander cells or whether TGEV is capable of inducing apoptosis in vivo need further study.

In the fourth study, the mechanism of PRRSV-induced cell death was determined both in vitro and in vivo. The nucleosomal-sized DNA fragments were detected in DNA from ATCC CRL 11171 cell line infected with PRRSV strain ATCC VR2385 after 2 to 4 d.p.i. Using electron microscopy, both lytic and apoptotic cells were found in PRRSV-infected
wells. Lytic cells had several virus particles in the cytoplasmic vesicles. There were several cells that had cell and nuclear morphology characteristic of apoptotic cells, however, virus particles could not be found in these cells. Apoptotic cells were also demonstrated by TUNEL technique. Apoptotic cells were detected as early as 1 d.p.i. and increased with time until 3 or 4 d.p.i. Surprisingly, most of the positive cells were either positive for virus nucleic acid or apoptosis. According to these results, most of the apoptotic cells were uninfected bystander cells. This result was different from the European isolate which directly induces apoptosis in vitro by the ORF 5 product (Suarez et al., 1996a).

Then, we determined that PRRSV was able to induce apoptosis in vivo. The apoptotic cells were detected in the lungs and the lymph nodes from the PRRSV-infected pigs. In the lungs, the TUNEL-positive cells were alveolar macrophages, lymphocytes, pulmonary intravascular macrophages, and type I and type II pneumocytes. In the lymph nodes, the TUNEL-positive cells were predominantly lymphocytes in the germinal centers and macrophages throughout the lymph nodes. Again, the apoptotic cells in the lungs and the lymph nodes were also uninfected bystander cells. These results demonstrated that a large number of alveolar macrophages in the lung and lymphocytes in the lymph nodes were destroyed by apoptosis during the course of infection. This might be the reason that PRRSV-infected pigs have a significant decrease in the numbers of the alveolar macrophages (Molitor et al., 1992) and in the numbers of circulating lymphocytes and monocytes (Christianson et al., 1992; Zhou et al., 1992). This may in part explain why PRRSV-infected pigs frequently have secondary infections by both bacteria (Done et al., 1995; Galina et al., 1994; Kawashima et al., 1996) and viruses (Groschup et al., 1993; Van Reeth et al., 1996). At the present time, the reason behind the difference in the mode of these 2 strains inducing apoptosis is not known. In a study where LV and U.S. PRRSV VR2385 isolates were used to infect CDCCD pigs, marked differences in clinical diseases,
lesion severity, and amount of antigen in tissues was demonstrated (Halbur et al., 1996a). Amino acid and nucleic acid homology is also quite different and may account for the differences between these viruses.

At this point, the mechanism of the U.S. PRRSV induced apoptosis in bystander cells is not known. However, activated macrophages are known to be capable of producing several apoptosis mediators such as TNF-α (Kizaki et al., 1993; Kolesnick et al., 1994), nitric oxide (Cui et al., 1994; Messmer et al., 1994), reactive oxygen species (Martin et al., 1993; Hansson et al., 1996), and IL-1β (Kolesnick et al., 1994; Onozakin et al., 1985). It has been shown that alveolar macrophages from PRRSV-infected pigs expressed high levels of IL-1β (Zhou et al., 1992). Interestingly, the pathologic changes in the lungs and in the lymph nodes are often diffuse and a large number of apoptotic cells are observed diffusely in both lungs and lymph nodes. This suggests that cytokines, especially IL-1β, reactive oxygen species or nitric oxide might have a role in the pathogenesis of PRRSV. Therefore, PRRSV-induced apoptosis in uninfected bystander cells might be the mechanism amplifying the cytopathic effect of PRRSV. Whether release of IL-1β or other cytokines or soluble products is responsible for inducing apoptosis in PRRSV-infected pigs and whether PRRSV isolates difference in virulence have different ability in inducing apoptosis needs further study.


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