Characterization of the carrier state in porcine reproductive and respiratory syndrome virus infection

by

Dennis Carl Horter

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Veterinary Microbiology

Program of Study Committee:
Jeffrey J. Zimmerman, Co-major Professor
Kyoung-Jin Yoon, Co-major Professor
Mark R. Ackermann

Iowa State University

Ames, Iowa

2003

Graduate College
Iowa State University

This is to certify that the master's thesis of

Dennis Carl Horter

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

Signatures have been redacted for privacy

TABLE OF CONTENTS

ABSTRACT

It was known for some time that PRRS virus infection resulted in persistently infected animals, but the proportion of PRRS virus carriers over time and the performance of current diagnostics in detecting carrier animals had not been described. To address this question, samples were collected periodically over 105 days from 60 3-week-old PRRS virus-inoculated pigs and 60 age-matched uninoculated controls and tested for PRRS virus by virus isolation (VI), swine bioassay, and/or reverse transcriptase-nested polymerase chain reaction (RT-nPCR). Overall, infectious PRRS virus was found in 51 of 59 (84%) necropsied animals by VI or swine bioassay. Of the samples analyzed, oropharyngeal scrapings targeting the tonsil of the soft palate yielded the highest diagnostic sensitivity for both VI (28 of 51) and RT-PCR (35 of 39). ELISA was useful for detecting animals previously infected with PRRS virus, but was unable to differentiate carrier and non-carrier animals. Overall, RT-nPCR on oropharyngeal scrapings was the combination of assay and sample that achieved the highest accuracy in detecting carriers (90%).

INTRODUCTION

Thesis Organization

The thesis is composed of two chapters. The first chapter provides an overview of the anatomy, histology, immunology, and pathology of the tonsils, as well as a discussion of the tonsil as a site of persistence for certain commensal opportunistic pathogens. The second chapter describes the thesis research on the persistence of porcine reproductive and respiratory syndrome (PRRS) virus in swine and detection of PRRS virus carrier animals. This chapter was subsequently published in Veterinary Microbiology (2002 86:213-218). Chapter One provides the foundation in which to frame the questions raised in Chapter Two regarding the role of the porcine tonsil in the persistence of PRRS virus. The final chapter of the thesis is a general discussion summarizing the results presented in the manuscript.

Review of Literature

A REVIEW OF PORCINE TONSILS IN IMMUNITY AND DISEASE

A paper to be submitted to Animal Health Research Reviews

Dennis C. Horter[1], Kyoung-Jin Yoon[2], Jeffrey J. Zimmerman[2]

Abstract

The porcine tonsils are a group of lymphoepithelial tissues located at the common openings of the gastrointestinal and respiratory tracts. The tonsils participate in a variety of functions involving innate, cellular, and humoral immunity at the local and systemic levels. Among these immunological functions is continuous surveillance for the presence of foreign antigens at the openings of the gastrointestinal and respiratory tracts. Within the pig, the movement of lymphocytes, cytokines, and chemotactic molecules from the tonsils to other lymphoid organs confers immunity to other portals of pathogen entry and facilitates an efficient and rapid systemic immune response. In spite of the immunologic nature of the tonsils, some microorganisms have acquired adaptations that allow them to circumvent the tonsillar immune defenses and utilize the tonsils as a site of entry, replication, and colonization. Several bacterial and viral pathogens persist asymptomatically within the tonsils making identification of asymptomatic carrier animals difficult for disease control and/or pathogen elimination. This paper reviews the current information on the anatomy, immunology, and pathobiology of the porcine tonsils and discusses the tonsils as a site of pathogen entry,

---

[1]Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA, USA

[2]Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA, USA

replication, and colonization using *Salmonella* spp., classical swine fever virus, and porcine reproductive and respiratory syndrome virus as examples.

Introduction

The tonsils are aggregates of lymph nodules and diffuse lymphoid tissues organized into independent lymphoid organs and positioned at the junction of the nasopharynx and oropharynx (Schummer and Nickel, 1979). Functionally, the tonsils provide a protective immunological ring at the openings of the gastrointestinal and respiratory tracts. Histologically, the tonsils are lymphoepithelial tissues composed of crypts, lymphoepithelium, lymphoid follicles, parafollicular regions, connective tissue, lymphoid cells (B- and T-lymphocytes), dendritic cells, and macrophages. Together these components provide innate, cellular, and humoral immunity at the local and systemic levels. As secondary lymphoid organs, the anatomical location of the tonsils places them in an optimal position to surveil, detect, and initiate an immune response to pathogens entering the body through the mouth and/or nares. Paradoxically, some bacterial and viral microorganisms utilize the tonsils as a site of entry, primary replication, and/or colonization. These include some of the most significant pathogens of swine, including *Salmonella* spp., classical swine fever (CSF) virus, and porcine reproductive and respiratory syndrome (PRRS) virus. This article reviews the anatomy and immune functions of the porcine tonsils and discusses the pathobiology of these tissues in the context of important pathogens that exploit the tonsils as a portal of entry and site of replication.

Gross Anatomy

In the domestic mammals, tonsils are named according to their location within the pharynx: lingual tonsils, palatine tonsil, paraepiglottic tonsils, pharyngeal tonsil, tubal tonsils, and tonsil of the soft palate. Swine possess all of the tonsils listed above except the palatine tonsils (Schummer and Nickel 1979). The lingual tonsils are located in the mucosa at the root of the tongue and are composed of a few isolated tonsillar follicles. The paraepiglottic tonsils consist of a small group of tonsillar follicles at the bottom of the *sulci tonsillares* located bilaterally at the base of the epiglottis. The pharyngeal tonsil is composed of irregularly raised tonsillar tissue located in the roof of the nasopharynx caudal to the end of the pharyngeal septum. The tubal tonsils are large, flat tonsils located in the walls of the pharyngeal openings of the auditory tubes (Schummer and Nickel 1979). The tonsil of the soft palate is large, flat, and located on the ventral surface of the soft palate (Fig. 1). (The reader should note that the tonsil of the soft palate is sometimes mis-identified as the palatine tonsil in the published literature (Schummer and Nickel 1979))

Tonsils are characterized as follicular or non-follicular based on the presence or absence of tonsillar crypts. "Follicular" is derived from the Latin "*follicu*" meaning "a little bag" (Borror 1971) and, in this instance, refers to extensively branching blind pouches (tonsillar crypts) that terminate in the underlying lymphoid tissue and function in immune surveillance (Belz and Heath 1996). In swine, the tonsil of the soft palate, tubal tonsils, and paraepiglottic tonsils are follicular, whereas the pharyngeal and lingual tonsils are non-follicular (Trautmann 1957). The openings of the tonsillar crypts are visible on the surface of the follicular tonsils as puncta 90 to 500 μm in diameter (Fig. 2). Tonsillar crypts are not

unique to swine and are also present in rabbits (Slipka 1988), cattle (Velinova 2001), and humans (Kassay and Sando 1962). Swine possess more crypts in the tonsil of the soft palate (160 to 190) (Belz and Heath 1996) than humans (10 to 30 crypts) (Kassay and Sandor, 1962) or rabbits (1 crypt) (Slipka 1988). A physiological or immunological rationale for this difference among species has not been identified.

Tonsillar crypts are filled with lymphocytes and debris. In humans, an analysis of nucleated cells within the crypts identified 82.1% as lymphocytes, 10.1% as mononuclear phagocytes, 5.6% as plasma cells, 1.7% as neutrophils, and 0.5% as mast cells (Howie 1981). Comparable information is not available for swine. The detritus within crypts consists of cells in the process of degeneration (lymphocytes and desquamated epithelial cells), food particles, and bacteria (Belz and Heath 1996). In swine, the crypt bacterial population includes commensals, such as *Streptococcus porcinus*. *Streptococcus dysgalactiae*, *Staphylococcus hyicus,* and *Staphylococcus aureus,* as well as commensal opportunistic pathogens, such as *Streptococcus suis* (Baele 2001; Devriese 1994). Since the openings are too small to allow for the random transport of foreign material into the crypts, it has been hypothesized that material is moved into the crypts by the pressure generated through the act of swallowing (Belz and Heath 1996). Removal of detritus from the crypts is facilitated by the presence of mucus secreted from goblet cells located within the crypts (Belz and Heath 1996; Ramos 1992).

Histology

The non-follicular tonsils (pharyngeal and lingual tonsils) function as secondary lymphoid organs, i.e. lymph nodes, and are covered by avascular, non-keratinzed stratified

squamous epithelium with a simple lamina of lymphatic tissue below. Connective tissue containing nerves, blood vessels, lymphatics, and striated muscle fibers lie deep to the epithelium and lymphatic tissue.

The follicular tonsils (tonsil of the soft palate, tubal tonsils, and paraepiglottic tonsils) are also covered by avascular, non-keratinized stratified squamous epithelium, beneath which lies a layer of connective tissue containing nerves, blood vessels, and lymphatic vessels. At their openings on the surface of the tonsil, the lumina of the crypts are lined with non-keratinized squamous epithelium similar to that which covers the tonsil surface (Trautman 1957). The middle and lower portions of the crypts are lined with lymphoepithelium, a stratified squamous epithelium thinner than that on the tonsil surface, with microscopic plications on the surface and occasional membranous cells (M cells) and goblet cells embedded within it. Lymphocytes, macrophages, and plasma cells are found within the crypts and migrate within and through the lymphoepithelium. M cells within the lymphoepithelium are joined to adjacent epithelial cells by desmosomes and are located in close proximity to intraepithelial lymphocytes. M cells have microvilli and microfolds in the cellular membrane to facilitate the uptake of foreign antigens (Belz and Heath 1996; Ramos 1990). Their function is to take up foreign antigens from the crypt lumen and present them to macrophages, dendritic cells, and lymphocytes in the follicular and parafollicular areas of the tonsil. This immunological surveillance system allows for continuous sampling of material within the crypts by the lymphoid tissue, but it also facilitates colonization of the tonsil by pathogens that are able to utilize M cells to gain access to the interior of the tonsil, such as *Salmonella* species (Belz and Heath 1996; Neutra 1996).

The bulk of the tissue of the follicular tonsils is composed of lymphoid tissue. Located just beneath the lymphoepithelium of the tonsil, lymphoid follicles are sites of B lymphocyte maturation and differentiation and T cell activation (Bianchi 1992). The distribution of lymphoid and non-lymphoid cells within the lymphoid follicles in the porcine tonsil is similar to that found in other species, with B cells and follicular dendritic cells located within the lymphoid follicles and T cells in the parafollicular areas (Bianchi 1992, Boeker 1999, Zuckermann 1999). The lymphoid follicles are encompassed by parafollicular areas consisting of connective tissue and T-cells, as well as a lesser number of interdigitating dendritic cells, macrophages, plasma cells, and B-cells (Bianchi 1992, Boeker 1999, Ramos 1992).

Blood and lymph circulation

The major blood supply to the tonsils is provided by the minor palatine branch of the maxillary artery (Schummer and Nickel 1979). Arterioles enter at the caudal portion of the tonsil and are drained by vessels that leave at the center and cranial portion of the tonsil.

Lymphatic pathways of the tonsil of the soft palate begin with lymph moving from the lymphoepithelium through the pores in the basement membrane to the subepithelial tonsilar lymphoid tissue (Belz 1998, Saar and Getty 1964, Williams and Rowland 1972). From the tonsil of the soft palate, lymph flows via two pathways: deep and superficial (Fig. 3). The superficial pathway drains lymph from the subepithelial lymphoid tissue to the mandibular lymph center via the lymphatics, then from the mandibular lymph center to the ventral superficial cervical lymph nodes (middle group) and accessory mandibular node. Subsequently, lymph flows to the ventral superficial lymph node and enters the lymphatic

system (Saar and Getty 1964; Williams and Rowland 1972). Lymph, via the deep pathway, flows directly from the tonsils to the medial retropharyngeal lymph node, which empties into the tracheal trunk and, eventually, the blood stream (Belz and Heath 1995).

Innate and active immunity associated with tonsillar tissue

In swine, the tonsil of the soft palate comprises the bulk of tonsil tissue (Trautmann 1957). Perhaps for that reason and because of its accessibility, the majority of the research on porcine tonsils has focused on the tonsil of the soft palate. Although the information derived from research on the tonsil of the soft palate is almost certainly applicable to the other follicular tonsils, we will not speculate on that point. Thus, the remainder of the discussion is primarily based on information published on the tonsil of the soft palate.

The immune system has two major branches, innate immunity and active immunity. Innate immunity consists of nonspecific, broad-spectrum mechanisms that provide immediate defenses against pathogens. Active immunity is an adaptive, secondary system that organizes a specific response towards a specific antigen that takes 4 to 7 days (or longer) to develop. The immune functions of the tonsils include both innate and active components.

Innate immunity consists of diverse elements, including physical barriers, mucin, complement, immune cells, and antimicrobial peptides. The epithelium lining the surface of the tonsil and lumen of the crypts provides a strong physical barrier against the entry of pathogens (Belz and Heath 1996). Mucin, a glycoprotein present in mucus sections, lines the gastrointestinal tract and upper respiratory tract, including the oral cavity and tonsils, and provides innate immunity by preventing the adherence of bacteria to epithelial cells (Wu 1994). Complement, activated through either the classical or the alternative pathway, is

probably present within the porcine tonsil (Laufer 2000; Zwirner 1989). Once activated, complement components mediate inflammation, direct phagocytes to the site of inflammation through chemotaxis, lyse bacteria, and opsonize bacteria and fungi (Cooper 1985). The research to verify the presence of specific complement components in porcine tonsils has not been done, but complement components C3 and C4, which function as opsonins and inflammatory mediators, are present within the mucosal crypts of the gastrointestinal tract of humans and C3b is present within the germinal centers of all lymphoid tissues in humans (Laufer 2000; Zwirner 1989). Macrophages and neutrophils within the tonsils, lymphoepithelium, and tonsillar crypts provide innate protection by phagocytosis of microorganisms and play a role in active immunity by presenting antigen to lymphocytes (Belz and Heath 1996; Yamamoto 1992).

The antimicrobial peptides (AMPs) are a group of small, polycationic molecules produced by neutrophils, epithelial cells, and phagocytes. AMPs work in conjunction with immune cells (neutrophils and lymphocytes) to promote and regulate the inflammatory response and provide chemotaxis for neutrophils and T cells (Hancock 2000; Zhang 2000). AMPs are also able to directly kill a variety of infectious agents, including bacteria, fungi, viruses, and parasites, by disrupting electrostatic forces within cells and causing lysis and/or coagulation of the microbial cell membranes (Zhang 2000). The first AMPs described included lactoferrin, an iron chelator (Bagby 1981), and lysozyme (Fleming 1922), an enzyme that hydrolyses bonds in the microbial cell membranes (Hancock 2000). AMPs are classified on the basis of the homology of their protein sequences and include the cathelicidin, saposin, defensin, and cecropin families. Currently, 14 distinct porcine AMPs

have been described, with the majority (11 of the 14) belonging to the cathelicidin family (Zhang 2000). Little has been done experimentally with AMPs within the environment of the tonsil itself, but it has been shown that porcine AMP epithelial beta-defensin 1 is expressed in the dorsal tongue in high concentrations (Shi 1999). In humans, human beta-defensin 1 is produced by both the mucosal epithelium and the duct cells of salivary glands and is present in saliva coating the oral mucosal surfaces, including tonsillar epithelium and lymphoepithelium (Sahasrabudhe 2000). Since porcine beta-defensin 1 is present within the epithelium of the digestive and respiratory tract, it may also be present within the lymphoepithelium of the swine tonsil. At present, information regarding the presence and functions of AMPs in the tonsils is scant.

Active/adaptive immunity is the second branch of the immune system. Tonsils are strategically positioned at the entrance of the respiratory and digestive tracts to conduct immune surveillance; possess tonsillar crypts to trap incoming materials; and are rich with T cells, B cells, and antigen presenting cells to process and respond to antigens. Foreign material that enters the tonsillar crypts is transported by M cells lining the lymphoepithelium of the tonsils across the epithelial barrier to antigen-presenting cells (macrophages, dendritic cells) and lymphocytes in the parafollicular area (Brandtzaeg 1999; Sato 1990). Lymphocytes within the lymphoid follicular and parafollicular areas constitute the majority of the immune cells within the swine tonsil. Approximately 75% of the lymphocytes within the tonsillar microenvironment are T lymphocytes and slightly less than 25% are B lymphocytes. Approximately 92% of the T lymphocytes are CD4+ (2%), CD8+ (47%), or CD4+CD8+ (43%) T cells. The remaining 8% are gamma delta T cells, whose specific

functions are unknown, but which may involve cell mediated immune activation and anti-viral immunity (Boeker 1999; Salles 2000). CD4+CD8+ T cells, concentrated primarily in the parafollicular areas of the tonsil, are postulated to constitute a functionally mature immunological memory cell population within the pig (Pescovitz 1994; Zuckermann and Gaskins 1996). In swine, it has been shown that: 1) CD4+ T cells become CD4+CD8+ T cells after antigenic stimulation (viral, allogenic, or parasitic) (Pescovitz 1994; Saalmuller 1987; Dillender 1993); 2) the tonsils of 3-day-old pigs are primarily populated with CD4+ T cells and the number of CD4+CD8+ cells increases gradually with age, repeated antigen exposures, and infection (Pescovitz 1994; Zuckermann and Husmann 1996); 3) porcine CD4+CD8+ T cells are able to respond to viral antigens previously seen by the host (Zuckermann and Husmann 1996); and 4) the population of CD4+CD8+ T cells in tonsils ranges from 1.4 to 1.7 times that of the regional lymph nodes (Zuckermann and Husmann 1996). Therefore, CD4+CD8+ T cells are most likely a memory cell population advantageously positioned within the tonsil to recognize pathogens at their point of entry.

T-cells located within the tonsil are capable of producing a variety of cytokines (interleukin-2, interleukin-4, interleukin-5, interleukin-6, tumor necrosis factor-beta, interferon-gamma, transforming growth factor-beta) that activate and regulate the functions of lymphocytes, antigen presenting cells, and neutrophils; not only within the micro-environment of the tonsil, but systemically (Zuckermann 1999, Sallusto 1998). The influence of cytokines on, and the interaction of T-cells with, intercellular adhesion molecules (ICAM-1, ICAM-2, ICAM-3, ICAM-4) determines effective function (i.e. cell mediated vs humoral

response) of the T-cells to create a directed, systemic, adaptive immune response (Zuckermann 1999; Sallusto 1998).

The remaining lymphocyte population within porcine tonsils (approximately 25%) is made up of B cells concentrated in the lymphoid follicles. The predominant antibody isotype on the surface of swine B cells is IgM (approximately 14% of total lymphocyte population) (Boeker 1999). B-cells with surface IgM are likely B-cells that have not been exposed to antigen and have not undergone isotype switching. B cells with IgA (~5%) and IgG (~5%) on their cell surface make up the remaining B-lymphocyte population, i.e., 10% of the total lymphocyte population (Boeker 1999). Immunoglobulin-secreting plasma cells are present within the tonsillar crypts of swine (Belz and Heath, 1996).

Antibody, particularly IgA within the tonsillar crypts, is important in mucosal immunity and plays an important role in protection of the host against pathogens at the mucosal barrier. In humans, secretory component, a fragment of the poly-Ig receptor that is left attached to IgA after transport across epithelial cells, is not present within tonsillar secretions. This suggests that immunoglobulins in the crypt lumen are passively transferred (Howie 1981). Similar findings have been observed in swine and it is likely that antibody, particularly IgA, reaches the crypt lumen by a combination of active secretion of antibody by B lymphocytes and passive transfer of antibody through pores within the lymphoepithelium (Belz and Heath 1996).

Lymphocyte Migration

Within the pig, the movement of lymphocytes, cytokines, and chemotactic molecules (i.e. AMPs) from the tonsils to other lymphoid organs, i.e., mucosal-associated lymphoid

tissues (MALT) and bronchus-associated lymphoid tissues (BALT) in the gastrointestinal and respiratory tracts, confers immune protection to other potential portals of pathogen entry and allows for an efficient and rapid systemic immune response to infection. In most species, lymphocytes migrate within the body by passing from the blood to the secondary lymphoid tissues by moving across specialized endothelium termed "high endothelial venules" (HEV). In swine, the flow of lymphocytes is reversed. That is, lymphocyte migration occurs via HEV from secondary lymphoid tissues, e.g., tonsil and lymph nodes, to the blood (Belz 1998). HEV are present throughout the parafollicular regions in the tonsil of the soft palate in pigs, thereby allowing lymphocytes to pass directly from the blood to the lymphoid tissue or from the lymphoid tissue directly to the blood via the HEV (Belz 1998). After leaving the tonsil, lymphocytes migrate to local lymph nodes by a primary pathway (blood stream) or a secondary pathway (lymphatic vessels) (Binns and Hall 1966; Binns and Pabst 1988). Lymphocytes then migrate to the MALT in the gastrointestinal tract and BALT in the respiratory tract, where they function in mucosal immunity (Pabst and Nowara 1984, Binns and Pabst 1988, Pabst and Binns 1989, Bennell 1981)

Examples of Infection and Colonization of Tonsils by Pathogens

The tonsils' physical location and histological organization mandates that these tissues play an important role in the immune response of the host. Ironically, a number of microorganisms are able to circumvent their immunological defenses and use the tonsils as a portal of entry into the body, a site of primary replication, and/or a nidus of persistent, chronic infection (Table 1). Bacterial agents most typically persist within the tonsillar crypts, while viral agents persist within cells of the lymphoepithelium and/or lymphoid follicles.

Particularly important examples of swine pathogens able to colonize the tonsils include *Salmonella* spp, classical swine fever (CSF) virus, and porcine reproductive and respiratory syndrome (PRRS) virus.

Although there are over 2400 serotypes of salmonella, *Salmonella choleraesuis* and *S. typhimurium* are the serotypes most commonly isolated from swine (Straw 1999). *S. choleraesuis* is a host-adapted, facultative, intracellular bacterium that causes septicemic and/or pneumonic salmonellosis and, occasionally, enterocolits (Baskerville and Dow 1973). In contrast, *S. typhimurium* more commonly causes enterocolits and, less frequently, pneumonia and septicemia in swine (Wilcock 1976). Originally identified in 1885 as the putative cause of "hog cholera" (Salmon 1889), *S. choleraesuis* has long been considered an important pathogen of swine. In contrast, *S. typhimurium* has become an emerging food safety pathogen and the most common serovar identified in human cases of salmonellosis in 2000 (CDC 2001; Rabsch 2001).

Transmission of salmonella occurs via nose-to-nose contact, fecal-oral exposure, salmonella-contaminated aerosols, and by fomites, such as feed and water (Fedorka-Cray 1994). Following oral exposure, *S. typhimurium* enters the host through colonization of tonsillar crypts, while *S. choleraesuis* typically utilizes the M cells and enterocytes for adherence and invasion in the gastrointestinal tract (Pospischil 1990; Meyerholz 2002). After resolution of clinical disease, regardless of serotype and route of inoculation, *Salmonella* spp. persist within the tonsillar crypts and superficial epithelium, resulting in asymptomatic carrier animals (Fig. 4) (Fedorka-Cray 1995). In experimentally infected animals, *S. choleraesuis* and *S. typhimurium* have been recovered from the tonsils for up to 140 days post inoculation

(Gray 1995; Wood 1989; Wilcock 1978). The presence of low numbers of microorganisms replicating within the tonsillar crypts provides continual immune stimulation, but the extracellular location of the bacteria within the microenvironment of the tonsillar crypt make it difficult for the immune system to eliminate the infection (Payne 1963; Gray 1995; Wood 1989).

Classical swine fever (hog cholera) is another important swine pathogen able to colonize the tonsils. CSF virus is primarily transmitted via direct contact (i.e. oropharyngeal and lacrimal secretions, urine, feces, semen) with infected animals (Chevelle and Mengling 1969; Choi 2003). After CSF virus exposure, entry occurs through invasion of the epithelium of the tonsillar crypts and CSF virus replicates within the surrounding tonsillar lymphoid follicles (Fig. 5) (Cheville and Mengling 1969). Following the acute phase of infection, CSF virus has been shown to persist within the lymphoepithelium of the tonsillar crypts for up to 11 months with few or no clinical signs in carrier animals (Cheville and Mengling 1969). During the persistent phase of CSF virus infection, asymptomatic carrier pigs shed infectious virus in oral secretions and are capable of transmitting CSF virus to susceptible pigs, thereby complicating eradication and control of CSF (Van Oirschot and Terpstra 1977). For this reason, a significant technological advance in the campaign to eradicate CSF from domestic swine was the development of a fluorescent antibody tissue section test (FATST) to diagnose infection (Dunne 1973). Immunofluorescence techniques were described as early as 1942 (Kwapinski 1972). As reviewed by Solorzano et al. (1966), the first fluorescent antibody tests for CSF virus were reported in 1962, with the application of the technique to cell cultures, impression smears, and tissue sections described in 1963. Since CSF virus persists

within the tonsil of the soft palate, the FATST on tonsil biopsies proved to be an effective sample / test combination for the rapid ante-mortem detection of acutely infected and carrier animals (Robertson 1965). More recently, rapid examination of tonsillar biopsies using the direct immunofluorescence test proved critical in identifying and controlling the 1997/1998 epizootic of CSF in the Netherlands (Terpstra 2000).

The previous two examples illustrated infection and colonization of tonsils by well-known and historically important pathogens, but newly emerging pathogens can exploit the tonsils, as well. For example, following catastrophic clinical outbreaks in the United States in the 1980's, then Europe and Asia in the early 1990's (Zimmerman 2003), European workers reported the isolation of a previously unrecognized arterivirus in 1991 (Terpstra 1991; Wensvoort 1991). It is now recognized that porcine reproductive and respiratory syndrome (PRRS) virus infection, as in the case of CSF virus, results in long-term colonization of tonsil tissue. Following the initial infection and subsequent viremia, PRRS virus replicates in mucosal, pulmonary, and/or regional macrophages and dendritic cells (Rossow 1996). Within the lymphatic parenchyma of the tonsil, antigen-positive cells are scattered or clustered in small groups with occasional antigen-positive cells in the subepithelial regions of the crypts (Beyer 2000) (Fig. 6). After resolution of clinical disease, PRRS virus persists within lymphoid tissue, particularly the tonsils, for an extended period of time (Horter 2002, Wills 1997, Rossow 1996). For example, PRRS virus was isolated from tonsils and oropharyngeal scrapings from experimentally infected, clinically normal animals for up to 157 days post infection (Wills 1997; Allende 2000). Horter et al. (2002) found infectious PRRS virus in the tonsils of 91% of animals (10 of 11) 105 days post inoculation.

Interestingly, both Kleiboeker et. al. (2002) and Horter et. al. (2002) reported detection of infectious PRRS virus in the tonsils of convalescent, seronegative (HerdChek® PRRS ELISA (IDEXX Laboratories Inc., Westbrook, Maine) animals. In endemically infected herds, control and/or elimination of PRRS virus in domestic swine herds is complicated by the presence of asymptomatic, persistently infected animals that transmit virus to susceptible animals as they are introduced into the herd (Zimmerman 1992; Albina et al 1994).

Persistent infections in tonsils are not unique to swine. In humans, the tonsillar crypt epithelium is a site of primary replication and then persistence of human immunodeficiency virus (HIV) (Pantaleo 1991). In cats, feline calicivirus has been shown to persist as a chronic infection within the lymphoepithelium of the feline tonsil for 75 days post infection (Dick 1989). In cattle, bovine herpesvirus 1 was consistently recovered from the tonsils of clinically normal calves 60 days post inoculation (Winkler 2000).

Although the specific mechanisms by which microorganisms persist in the tonsils are poorly described, it is recognized that these agents frequently persist in the face of a strong humoral and cell mediated immune response (Loemba 1996; Bautista 1997; Gray 1995). In general, the mechanisms of persistence can be broadly classified as: 1) immune evasion, 2) latency, 3) anergy, and 4) immunotolerance.

Ploegh (1998) have thoroughly reviewed the mechanisms of immune evasion, that is, the development of mechanisms to avoid recognition or destruction by the immune system. In brief, bacterial methods of immune evasion focus on avoiding engulfment by phagocytic cells, i.e. macrophages, and escape from the phagosome/lysosome complex. These mechanisms are exploited by *Salmonella* spp. (Fu 1999; Uchiya 1999). Viral mechanisms of

immune evasion include altering antigen presentation (inhibition of proteolysis and transport, e.g., the Epstein-Barr virus) (Levitskaya 1995; Levitskaya 1997; Sharipo 2001) or alteration of class I and II molecules (e.g., murine cytomegalovirus) (Krmpoti´c 2002), modulating and/or mimicking cytokines (e.g., human cytomegalovirus) (Kotenko 2000), inhibiting apoptosis within infected cells (e.g., gamma herpesviruses) (Senkevich 1996), and continuous mutation/recombination (i.e. equine infectious anemia virus) (Payne 1984). Latency can be described as the cessation of viral replication within a cell for a period of time with reactivation of viral replication at a later time. Establishment of a latent infection, such as the case with the alpha herpesviruses (e.g., Aujeszky's disease virus), is another mechanism of persistence (Ploegh 1998; Brown 1995; Wheeler 1991). Anergy, the exhaustion of a clonal portion of the immune system, has been suggested as a mechanism of persistence for HIV in humans (Hardy 2002). Immunotolerance, the clonal deletion or inactivation of lymphocytes, is another persistence mechanism as with *in utero* infection of calves with BVD virus (McClurkin 1984). Pathogens can utilize either a single mechanism or a combination of mechanisms. For example, *Salmonella* spp. utilize both evasion of phagocytosis and escape from the lyosome/phagosome complex. Identification of the specific mechanisms microorganisms use to establish persistent infections utilize within the tonsillar environment has not been determined. Because of the importance of many of these pathogens to both human and animal health, this information is pertinent to our understanding of the pathogenesis of disease and directly impacts our ability to control and/or eradicate these agents.

Bibliography

Ackermann MR, De Bey MC, Register KB, Larson DJ, and Kinyon JM (1994). Tonsil and turbinate colonization by toxigenic and non-toxigenic strains of *Pasteurella multocida* in conventionally raised Iowa swine. *Proceedings of the International Pig Veterinary Society* **13**: 162.

Albina E, Madec F, Cariolet R, and Torrison J (1994). Immune response and persistence of the porcine reproductive and respiratory syndrome virus in infected pigs and farm units. *Veterinary Record*. **134**: 567-573.

Alexandersen S, Oleksiewicz MB, and Donaldson AI (2001). The early pathogenesis of foot-and-mouth disease in pigs infected by contact: a quantitative time-course study using TaqMan RT–PCR. *Journal of General Virology*. **82**: 747-755.

Allende R, Laegreid WW, Kutish GF, Galeota JA, Wills RW, and Osorio FA (2000). Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. *Journal of Virology*. **74**: 10834-10837.

Andries K and Pensaert M (1980). Immunofluorescence studies on the pathogenesis of hemagglutinating encephalomyelitis virus after oronasal inoculation. *American Journal of Veterinary Research*. **41**: 1372–1378.

Baekbo P (1988). Pathogenic properties of *Pasteurella multocida* in the lung of pigs. *Proceedings of the International Pig Veterinary Society*. **10**: 58.

Baele M, Chiers K, Devriese LA, Smith HE, Wisselink HJ, Vaneechoutte M, and Haesebrouck F (2001). The gram-positive tonsillar and nasal flora of piglets before and after weaning. *Journal of Applied Microbiology*. **91**: 997-1003.

Bagby GC Jr, Rigas VD, Bennett RM, Vandenbark AA, and Garewal HS (1981). Interaction of lactoferrin, monocytes, and T lymphocyte subsets in the regulation of steady-state granulopoiesis in vitro. *Journal of Clinical Investigations*. **68**: 56-63.

Bautista EM and Molitor TW (1997). Cell.mediated immunity to porcine reproductive and respiratory syndrome virus in swine. *Viral Immunology*. **10**: 83-94.

Baskerville A and Dow C (1973). Pathology of experimental pneumonia in pigs produced by Salmonella choleraesuis. *Journal of Comparative Pathology*. **83**: 207–215.

Bennell MA and Husband AJ (1981). Route of lymphocyte migration in pig. I. Lymphocyte circulation in gut-associate lymphoid tissue. *Immunology*. **42**: 469-474.

Beyer J, Fichtner D, Schirrmeier H, Polster U, Weiland E, and Wege H (2000). Porcine reproductive and respiratory syndrome virus (PRRSV): kinetics of infection in lymphatic organs and lung. *Journal of veterinary medicine. B, Infectious diseases and veterinary public health*. **47**: 9-25.

Bianchi AT, Zwart RJ, Jeurissen SH, and Moonen-Leusen HW (1992). Development of the B- and T-cell compartments in porcine lymphoid organs from birth to adult life: an immunohistological approach. *Veterinary Immunolology and Immunopathology*. **33**: 201-221.

Binns RM and Hall JG (1966). The paucity of lymphocytes in the lymph of unanaesthetized pigs. *British Journal of Experimental Pathology*. **47**: 275-280.

Binns RM and Pabst R (1988). Lymphoid cell migration and homing in the young pig: alternative immune mechanisms in action. In: Husband A (ed). *Migration and homing of lymphoid cells*. Boca Raton: CRC Press, pp. 137-174.

Belz GT (1998). Intercellular and lymphatic pathways associated with tonsils of the soft palate in young pigs. *Anatomy and Embryology*. **197**: 331-340.

Belz GT and Heath TJ (1995). Lymphatic drainage from the tonsil of the soft palate in pigs. *Journal of Anatomy*. **187**: 491-495.

Belz GT and Heath TJ (1996). Tonsils of the Soft Palate of Young Pigs: Crypt Structure and Lymphoepithelium. *The Anatomical Record*. **245**: 102-113.

Boeker M, Pabst R, and Rothkotter J (1999). Quantification of B, T and null lymphocyte subpopulations in the blood and lymphoid organs of the pig. *Immunobiology*. **201**: 74-87.

Borror DJ (1971). Dictionary of Word Roots and Combining Forms. Palo Alto, California: Mayfield Publishing Company.

Brandtzaeg P, Baekkefold ES, Farstad IN, Jahnsen FL, Johanson FE, Nilsen EM, and Yamanaka T (1999). Regional specialization in the mucosal immune system: what happens in the microcompartments? *Immunology Today* **20**:141-151.

Brown TT Jr, Shin KO, and Fuller FJ (1995). Detection of pseudorabies viral DNA in tonsillar epithelial cells of latently infected pigs. *American Journal of Veterinary Research*. **56**: 587-594.

CDC (2001). Preliminary FoodNet Data on the Incidence of Foodborne Illnesses --- Selected Sites, United States, 2000. *Morbidity and Mortality Weekly Report*. **50**:241-246.

Cheville NF and Mengeling WL (1969). The pathogenesis of chronic hog cholera (swine fever): Histologic, immunofluorescent, and electron microscopic studies. *Laboratory Investestigations*. **20**: 261–274.

Choi C and Chae C (2003). Detection of classical swine fever virus in boar semen by reverse transcription-polymerase chain reaction. *Journal of Veterinary Diagnostic Investigations*. **15**: 35-41.

Colgrove G, Haelterman EO, and Coggins L (1969). Pathogenesis of African swine fever virus in young pigs. *American Journal of Veterinary Research*. **30**: 1343–1359.

Cooper NR (1985). The classical complement pathway. Activation and regulation of the first complement component. *Advances in Immunology*. **37**: 151-216.

de Jong MF, Wellenberg G, Schaake J, and Frik K (1988). Selection of pig breeding herds free from atrophic rhinitis by means of a bacteriological screening of pigs on DNT producing *Pasteurella multocida*: A field evaluation from 1981 until 1987. *Proceedings of the International Pig Veterinary Society*. **10**: 49.

Dekker A, Moonen P, de Boer-Luijtze EA, and Terpstra C (1995). Pathogenesis of swine vesicular disease after exposure of pigs to an infected environment. *Veterinary Microbiology*. **45**: 243-50.

Devriese LA, Hommez J, Pot B, and Haesebrouck F (1994). Identification and composition of the streptococcal and enterococcal flora of tonsils, intestines and faeces of pigs. *Journal of Applied Bacteriology*. **77**: 31–36.

Dick CP, Johnson RP, and Yamashiro S (1989). Sites of persistence of feline calicivirus. *Research in Veterinary Science*. **47**: 367-73.

Dillender MJ and Lunney JK (1993). Characteristics of T lymphocyte cell lines established from NIH minipigs challenge inoculated with *Trichinella spiralis*. *Veterinary Immunology and Immunopathology*. **35**: 301-319.

Dunne HW (1973). Hog Cholera (European swine fever). *Advances in veterinary science and comparative medicine*. **17**: 315-359.

Edwards S, Fukusho A, Lefevre P-C, Lipowski A, Pejsak Z, Roehe P, and Westergaard J (2000). Classical swine fever: The global situation. *Veterinary Micriobiology*. **73**: 103-119.

Ellis J, Hassard L, Clark E, Harding J, Allan G, Willson P, Strokappe J, Martin K, McNeilly F, Meehan B, Todd D, and Haines D (1998). Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. *The Canadian Veterinary Journal*. **39**: 44-51.

Elvinger F, Liggett AD, Tang KN, Harrison LR, Cole JR Jr, Baldwin CA, and Nessmith WB (1994). Eastern equine encephalomyelitis virus infection in swine. *Journal of the American Veterinary Medical Association*. **205**: 1014-1016.

Fedorka-Cray PJ, Whipp SC, Isaacson RE, Nord N, and Lager K (1994). Transmission of Salmonella typhimurium to swine. *Veterinary Microbiology*. **41**: 333-344.

Fedorka-Cray PJ, Kelley LC, Stabel TJ, Gray JT, and Laufer JA (1995). Alternate routes of invasion may affect pathogenesis of *Salmonella typhimurium*. *Infection and Immunity*. **63**: 2658–2664.

Fleming A (1922). On a remarkable bacteriolytic element found in tissues and secretions. *London Proc Roy Soc Series B*. **93**: 306-317

Fu Y, and Galan JE (1999). A Salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. *Nature*. **401**: 293-297.

Gram T, Ahrens P, and Nielsen JP (1996). Evaluation of a PCR for detection of Actinobacillus pleuropneumoniae in mixed bacterial cultures from tonsils. *Veterinary Microbiology*. **51**: 95-104.

Gray JT, Fedorka-Cray PJ, Stabel TJ, and Ackermann MR (1995). Influence of inoculation route on the carrier state of Salmonella choleraesuis in swine. *Veterinary Microbiology*. **47**: 43-59.

Hancock RE and Scott MG (2000). The role of antimicrobial peptides in animal defenses. *Proceedings of the National Acadamy of Science*. **97**: 8856-8861.

Hardy GA, Imami N, and Gotch FM (2002). Improving HIV-specific immune responses in HIV-infected patients. *Journal of HIV Therapy*. **7**: 40-45.

Harms PA, Sorden SD, Halbur PG, Bolin SR, Lager KM, Morozov I, and Paul PS (2001). Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. *Veterinary Pathology*. **38**: 528-539.

Horter DC, Pogranichniy RM, Chang CC, Evans RB, Yoon KJ, and Zimmerman JJ (2002). Characterization of the carrier state in porcine reproductive and respiratory syndrome virus infection. *Veterinary Microbiology*. **86**: 213-28.

Howie AJ (1981). The cells in tonsillar crypts. *Clinical otolaryngology and allied sciences*. **7**: 35-44.

Kassay D and Sandor A (1962). The crypt system of the palatine tonsils. *Archives of otolaryngology*. **75**: 144-155.

Kernkamp HCH (1961). The natural history of hog cholera. In: Minwaring GT, Sorensen DK (eds). *Symposium on Hog Cholera*. University of Minnesota, Institute of Agriculture, pp 19-28.

Kleiboeker SB, Lehman JR, and Fangman TJ (2002). Concurrent use of reverse transcription-polymerase chainreaction testing of oropharyngeal scrapings and paired serological testing for detection of porcine reproductive and respiratory syndrome virus infection in sows. *Journal of Swine Health and Production*. **10**: 251-258

Kume K, Nakai T, and Sawata A (1984). Isolation of *Haemophilus pleuropneumoniae* from the nasal cavities of healthy pigs. *Japanese Journal of Veterinary Science*. **46**: 641–647.

Kwapinski JBG (1972). *Methodology of Immunochemical and Immunological Research*. New York, New York: John Wiley and Sons, Inc., pp. 609-611.

Laufer J, Oren R, Goldberg I, Horwitz A, Kopolovic J, Chowers Y, and Passwell JH (2000). Cellular localization of complement C3 and C4 transcripts in intestinal specimens from patients with Crohn's disease. *Clinical Experimental Immunology*. **120**: 30-37.

Levitskaya J, Coram M, Levitsky V, Imreh S, Steigerwald-Mullen PM, Klein G, Kurilla MG, and Masucci MG (1995). Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen 1. *Nature*. **375**: 685–688.

Levitskaya J, Sharipo A, Leonchiks A, Ciechanover A, and Masucci MG (1997) Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein- Barr virus nuclear antigen 1. *Proceedings of the National Academy of Science*. **94**: 12616–12621.

Loemba HD, Mounir S, Mardassi H, Archambault D, and Dea S (1996). Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. *Archives of Virology*. **141**: 751-761.

Long JF (1985). Pathogenesis of porcine polioencephalomyelitis. In *Comparative Pathobiology of Viral Diseases*, vol. 1. Ed. R. A. Olsen, S. Krakowka, and J. R. Blakeslee. Boca Raton, Fla.: CRC Press, pp. 179–197.

Meyerholz DK, Stabel TJ, Ackermann MR, Carlson SA, Jones BD, and Pohlenz J (2002). Early epithelial invasion by Salmonella enterica serovar Typhimurium DT104 in the swine ileum. *Veterinary Pathology*. **39**: 712-720.

Kotenko SV, Saccani S, Izotova LS, Mirochnitchenko OV, and Pestka S (2000). Human cytomegalovirus harbors its own unique IL-10 homologue (cmvIL-10). Proceedings of the *National Academy of Science*. **97**: 1695–1700.

Krmpoti´c A, Busch DH, Bubi´c I, Gebhardt F, Hengel H, Hasan M, Scalzo AA, Koszinowski UH, and Jonji´c S (2002). MCMV glycoprotein gp40 confers virus resistance to CD8+ T cells and NK cells in vivo. *Nature Immunology*. **3**: 529–535.

McClurkin AW, Littledike ET, Cutlip RC, Frank GH, Coria MF, and Bolin SR (1984). Production of cattle immunotolerant to bovine viral diarrhea virus. *Canadian Journal of Comparative Medicine*. **48**: 156-161.

Morozov I, Sirinarumitr T, Sorden SD, Halbur PG, Morgan MK, Yoon KJ, and Paul PS (1998). Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. *Journal of Clinical Microbiology*. **36**: 2535-2541.

Murakami S, Azuma R, Koeda T, Oomi H, Watanabe T, and Fujiwara H (1998). Immunohistochemical detection for Actinomyces sp. in swine tonsillar abscess and granulomatous mastitis. *Mycopathologia*. **141**: 15-9.

Neutra MR, Pringault E, and Kraehenbuhl JP (1996). Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annual Review of Immunology*. **14**: 275-300.

Nielsen B, Heisel C, and Wingstrand A (1996). Time course of the serological response to Yersinia enterocolitica O:3 in experimentally infected pigs. *Veterinary Microbiology*. **48**: 293–303.

Pabst R and Nowara E (1984). The emigration of lymphocytes from palatine tonsils after local labeling. *Archives of Otorhinolaryngology*. **240**: 7-13.

Pabst R and Binns RM (1989). Heterogeneity of lymphocyte homing physiology: several mechanisms operate in the control of migration to lymphoid and non-lymphoid organs in vivo. *Immunology Reviews*. **108**: 83-109.

Pantaleo G, Graziosi C, Butini L, Pizzo PA, Schnittman SM, Kotler DP, and Fauci AS (1991). Lymphoid organs function as major reservoirs for human immunodeficiency virus. *Proceedings of the National Academy of Sciences*. **88**: 9838-9842.

Paton D (2002). The reappearance of classical swine fever in England in 2000. *In*: Morilla A, Yoon K-J, Zimmerman JJ (eds). *Trends in Emerging Viral Infections of Swine*. Ames, Iowa: Iowa State Press, pp. 153-158.

Payne S, Parekh B, Montelaro RC, and Issel CJ (1984). Genomic alterations associated with persistent infections by equine infectious anaemia virus, a retrovirus. *Journal of General Virology*. **65**: 1395-1399.

Payne JM and Derbyshire JB (1963). Portals of entry for bacterial infection in calves and piglets with particular reference to the tonsil. *Journal of Pathology and Bacteriology*. **85**: 171-178.

Pescovitz MD, Sakopoulos AG, Gaddy JA, Husmann RJ, and Zuckermann FA (1994). Porcine peripheral blood CD4+/CD8+ dual expressing T-cells. *Veterinary Immunology and Immunopathology*. **43**: 53.

Ploegh HL (1998). Viral strategies of immune evasion. *Science*. **280**: 248-53.

Pospischil A, Wood RL, and Anderson TD (1990). Peroxidase-antiperoxidase and immunogold labeling of Salmonella typhimurium and Salmonella choleraesuis var kunzendorf in tissues of experimentally infected swine. *American Journal of Veterinary Research*. **51**: 619-624.

Rabsch W, Tschape H, and Baumler AJ (2001). Non-typhoidal salmonellosis: emerging problems. *Microbes and Infection*. **3**: 237-247.

Ramos JA, Ramis AJ, Rabanal RM, Marco A, Domingo M, and Ferrer LM (1990). Scanning electron microscopy of swine lymphoid organs. *Histology and Histopathology*. **5**:397-406.

Ramos JA, Ramis AJ, Marco A, Domingo M, Rabanal R, and Ferrer L (1992). Histochemical and immunohistochemical study of the mucosal lymphoid system in swine. *American Journal of Veterinary Research*. **53**: 1418-1426.

Ressang AA (1973). Studies on the pathogenesis of hog cholera. I. Demonstration of hog cholera virus subsequent to oral exposure. *Zentralblatt für Veterinärmed*. **20**: 256–271.

Robertson A, Bannister GL, Boulanger P, Appel M, and Gray DP (1965). Hog cholera. V. Demonstration of the antigen in swine tissues by the fluorescent antibody technique. *Canadian Journal of Comparative Medicine and Veterinary Science*. **29**: 299-305.

Rossow KD, Benfield DA, Goyal SM, Nelson EA, Christopher-Hennings J, and Collins JE (1996). Chronological immunohistochemical detection and localization of porcine reproductive and respiratory syndrome virus in gnotobiotic pigs. *Veterinary Pathology*. **33**: 551-556.

Rutter JM (1985). Atrophic rhinitis in swine. *Advances in Veterinary Science and Comparative Medicine*. **29**: 239–279.

Saalmuller A, Redehasse MJ, Buhring HJ, Jonjic S, and Koszinowski UH (1987). Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. *European Journal of Immunology*. **17**: 1297.

Saar LI and Getty R (1964). The interrelationship of the lymph vessel connections of the lymph nodes of the head, neck, and shoulder regions of swine. *American Journal of Veterinary Research*. **25**: 618-636.

Sahasrabudhe KS, Kimball JR, Morton TH, Weinberg A, and Dale BA (2000). Expression of the antimicrobial peptide, human beta-defensin 1, in duct cells of minor salivary glands and detection in saliva. *Journal of Dental Research*. **79**: 1669-1677.

Salles MW and Middleton DM (2000). Lymphocyte subsets in porcine tonsillar crypt epithelium. *Veterinary Immunology and Immunopathology*. **77**: 133-144.

Sallusto F, Lanzavecchia A, and Mackay CR (1998). Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. *Immunology Today*. **19**: 568-574.

Sato Y, Wake K, and Watanabe I (1990). Differentiation of crypt epithelium in human palatine tonsils: the microenvironment of crypt epithelium as a lymphoepithelial organ. *Archives of Histology and Cytology*. **53**:41-54.

Schummer A and Nickel R (1979). Digestive System. In: Sack WO (ed) *The viscera of the domestic mammals, 2nd Ed.* Berlin & Parey ; Springer-Verlag, pp. 51-55.

Senkevich TG, Bugert JJ, Sisler JR, Koonin EV, Darai G, Moss B (1996). Genome sequence of a human tumorigenic poxvirus: prediction of specific host-response-evasion genes. *Science*. **273**: 813–816.

Shadduck JA, Koestner A, and Kasza L (1967). The lesions of porcine adenoviral infection in germfree and pathogen-free pigs. *Pathologia veterinaria*. **4**: 537–552.

Sharipo A, Imreh M, Leonchiks A, Brändén C-I, and Masucci MG (2001). cis-Inhibition of proteasomal degradation by viral repeats: impact of length and amino acid composition. *FEBS Letters*. **499**: 137–142.

Shi J, Zhang G, Wu H, Ross C, Blecha F, and Ganz T (1999). Porcine epithelial beta-defensin 1 is expressed in the dorsal tongue at antimicrobial concentrations. *Infection and Immunity*. **67**: 3121-3127.

Slipka J (1988). Palatine tonsils – their evolution and ontogeny. *Acta oto-laryngologica*. **454**: 18-22.

Solorzano RF, Thigpen JE, Bedell DM, and Schwartz WL (1966). The diagnosis of hog cholera by a fluorescent antibody test. *Journal of the American Veterinary Medical Association* **149**: 31-34.

Stephano AH, Gay GM, and Ramirez TC (1988). Encephalomyelitis, reproductive failure and corneal opacity in pigs, associated with a new paramyxovirus infection (blue eye). *The Veterinary Record.* **122**: 6–10.

Higgins R and Gottschalk M (1999) Streptococcal diseases. In: Straw BE, D'Allaire S, Mengeling WL, and Taylor DJ (1999). *Diseases of Swine 8th ed.* ISU Press, pp. 563-578.

Salmon DE (1889). Hog Cholera: Its History, Nature, and Treatment. The Burea of Animal Industry, U.S. Department of Agriculture. Washington Printing Office, p. 32.
Terpstra C, Wensvoort G, and Pol JMA (1991). Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. *The Veterinary Quarterly.* **13**:131-136.

Terpstra C and Wensvoort G (1988). Bovine viral diarrhea virus infections in piglets born to sows vaccinated against swine fever with contaminated vaccine. *Research in Veterinary Science.* **45**: 143–148.

Terpstra C and de Smit AJ (2000). The 1997/1998 epizootic of swine fever in the Netherlands: control strategies under a non-vaccination regimen. *Veterinary Microbiology.* **77**: 3-15.

Thibodeau V, Frost EH, Chenier S, and Quessy S (1999). Presence of Yersinia enterocolitica in tissues of orally-inoculated pigs and the tonsils and feces of pigs at slaughter. *Canadian Journal of Veterinary Research.* **63**: 96-100.

Trautmann A and Fiebiger J. *Fundamentals of the Histology of Domestic Animals*, pp. 121-123. Comstock Publishing Associates, Ithaca New York, 1957.

Uchiya K, Barbieri MA, Funato K, Shah AH, Stahl PD, and Groisman EA (1999). A Salmonella virulence protein that inhibits cellular trafficking. *The EMBO Journal.* **18**: 3924-3933.

Van Oirschot JT and Terpstra C (1977). A congenital persistent swine fever infection. I. Clinical and virological observations. *Veterinary Microbiology.* **2**: 121–132.

Velinova M, Thielen C, Melot F, Donga J, Eicher S, Heinen E, and Antoine N (2001). New histochemical and ultrastructural observations on normal bovine tonsils. *The Veterinary Record.* **149**: 613-617.

Wensvoort G, Terpstra C, Pol JM, ter Laak EA, Bloemraad M, de Kluyver EP, Kragten C, van Buiten L, den Besten A, and Wagenaar F (1991). Mystery swine disease in the Netherlands: the isolation of Lelystad virus. *The Veterinary Quarterly*. **13**: 121-130.

Wheeler JG and Osorio FA (1991). Investigation of sites of pseudorabies virus latency, using polymerase chain reaction. *American Journal of Veterinary Research*. **52**: 1799–1803.

Wilcock BP, Armstrong CH, and Olander HJ (1976). The significance of the serotype in the clinical and pathologic features of naturally occurring porcine salmonellosis. *Canadian Journal of Comparative Medicine*. **40**: 80–88.

Wilcock BP, and Olander HJ (1978). Influence of oral antibiotic feeding on the duration and severity of clinical disease, growth performance and pattern of shedding in swine inoculated with *Salmonella typhimurium*. *Journal of the American Veterinary Medical Association*. **172**: 472–477.

Williams DM and Rowland AC (1972). The palatine tonsils of the pig – an efferent route to the lymphoid tissue. *Journal of Anatomy*. **113**: 131-137.

Wills RW, Zimmerman JJ, Yoon KJ, Swenson SL, McGinley MJ, Hill HT, Platt KB, Christopher-Hennings J, and Nelson EA (1997). Porcine reproductive and respiratory syndrome virus: a persistent infection. *Veterinary Microbiology*. **55**: 231-40.

Winkler MT, Doster A, and Jones C (2000). Persistence and reactivation of bovine herpesvirus 1 in the tonsils of latently infected calves. *Journal of Virology*. **74**:5337-5346.

Wood RL, Pospischil A, and Rose R (1989). Distribution of persistent *Salmonella typhimurium* infection in internal organs of swine. American *Journal of Veterinary Research*. **50**: 1015–1021.

Wood RL and Rose R (1992). Populations of *Salmonella typhimurium* in internal organs of experimentally infected carrier swine. *American Journal of Veterinary Research*. **53**: 653–658.

Wu AM, Csako G, and Herp A (1994). Structure, biosynthesis, and function of salivary mucins. *Molecular and Cellular Biochemistry*. **17**: 39-55.

Yamamoto Y, Okato S, Nishiyama M, and Takahashi H (1992). Function and morphology of macrophages in palatine tonsils. *Advances in Otorhinolaryngology*. **47**: 107-113.

Zhang G, Ross CR, and Blecha F (2000). Porcine antimicrobial peptides: new prospects for ancient molecules of host defense. *Veterinary Research*. **31**: 277-296.

Zimmerman JJ (in press). Historical overview. In: Zimmerman JJ, Yoon K-J (eds). *The Porcine Reproductive and Respiratory Syndrome (Porcine Arterivirus) Compendium* (2nd edition). National Pork Board, Des Moines, Iowa.

Zimmerman JJ, Sanderson T, Eernisse KA, Hill HT, and Frey ML, (1992). Transmission of SIRS virus in convalescent animals to commingled penmates under experimental conditions. *American Association Swine Practitioners Newsletter*. **4**: 25.

Zuckermann FA and Gaskins HR (1996). Distribution of porcine CD4/CD8 double-positive T lymphocytes in mucosa-associated lymphoid tissues. *Immunology*. **87**:493-499.

Zuckermann FA and Husmann RJ (1996). Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. *Immunology*. **87**: 500-512.

Zuckermann FA (1999). Extrathymic CD4/CD8 double positive T cells. *Veterinary Immunology and Immunopathology*. **72**: 55-66.

Zwirner J, Felber E, Schmidt P, Riethmuller G, and Feucht HE (1989). Complement activation in human lymphoid germinal centres. *Immunology*. **66**: 270-277.

Tables and Figures

Table 1. Pathogens and their association with the tonsil during infection.

Association	Pathogens
Portal of entry or site for primary replication	*Salmonella typhimurium* (Wilcock and Olander 1978) *Salmonella choleraesuis* (Gray 1995) Porcine adenovirus (Shadduck 1967) African swine fever virus (Colgrove 1969) Blue eye paramyxovirus (Stephano 1988) Bovine viral diarrhea virus (Terpstra and Wensvoort 1988) Eastern equine encephalomyelitis virus (Elvinger 1994) Porcine enterovirus (Long 1985) Hemagglutinating encephalomyelitis virus (Andries and Pensaert 1980) Classical swine fever virus (Ressang 1973) Swine vesicular disease virus (Dekker 1995) Porcine circovirus type 2 (Ellis 1998, Harms 2001, Morozov 1998) Porcine reproductive and respiratory syndrome virus (Rossow 1996)
Site of chronic persistent infection	PRRS virus (Wills 1997, Albina 1994, Allende 2000, Horter 2002) FMDV (Alexanderson 2001) *Actinomyces* species (Murakami 1998) *Salmonella choleraesuis* (Gray 1995) *Salmonella typhimurium* (Wilcock 1978, Wood 1989, Wood 1992, Fedorka-Cray 1995) *Actinobacillus pleuropneumoniae* (Kume 1984, Gram 1996) *Erysipelas rhusiopathiae* (Straw 1999) *Yersinia enterocolitica* (Nielsen 1996, Thibodeau 1999) Classical swine fever virus (Cheville and Mengeling 1969) PRRS virus (Wills 1997, Horter 2002) *Streptococcus suis* type 2 (Devriese 1994) *Bordetella bronchiseptica* (Rutter 1985) *Pasteurella multocida* (Baekbo 1988, de Jong 1988, Ackermann 1994)

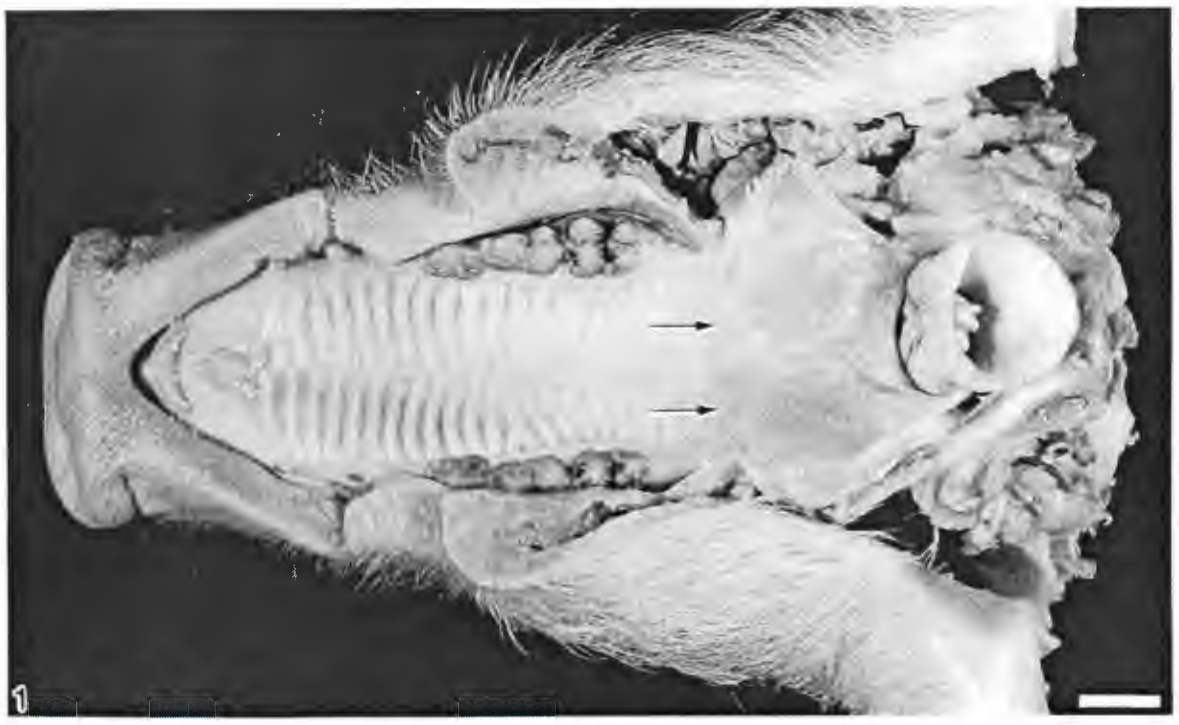

Fig. 1. Photograph of the mucosal surface of a plastinated pig's head showing the anatomical location of the tonsils of the soft palate (arrows). Bar = 10mm.

Belz GT and Heath TJ. (1996) Tonsils of the soft palate of young pigs: crypt structure and lymphoepithelium. *The Anatomical Record*. **245**: 103

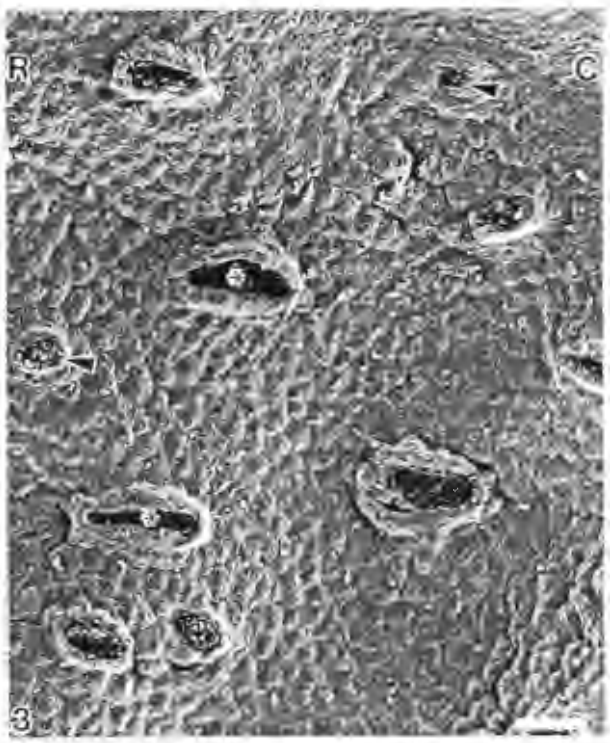

Fig. 2. Scanning electron micrograph of the oropharyngeal surface of a tonsil showing circular (arrowheads) and oval (and sometimes slit-like) (*) openings of tonsillar crypts. The openings are generally oriented in a longitudinal direction. R = rostral; C = caudal. Bar = 250 μm.

Belz GT and Heath TJ (1996). Tonsils of the soft palate of young pigs: crypt structure and lymphoepithelium. *The Anatomical Record.* **245**: 103.

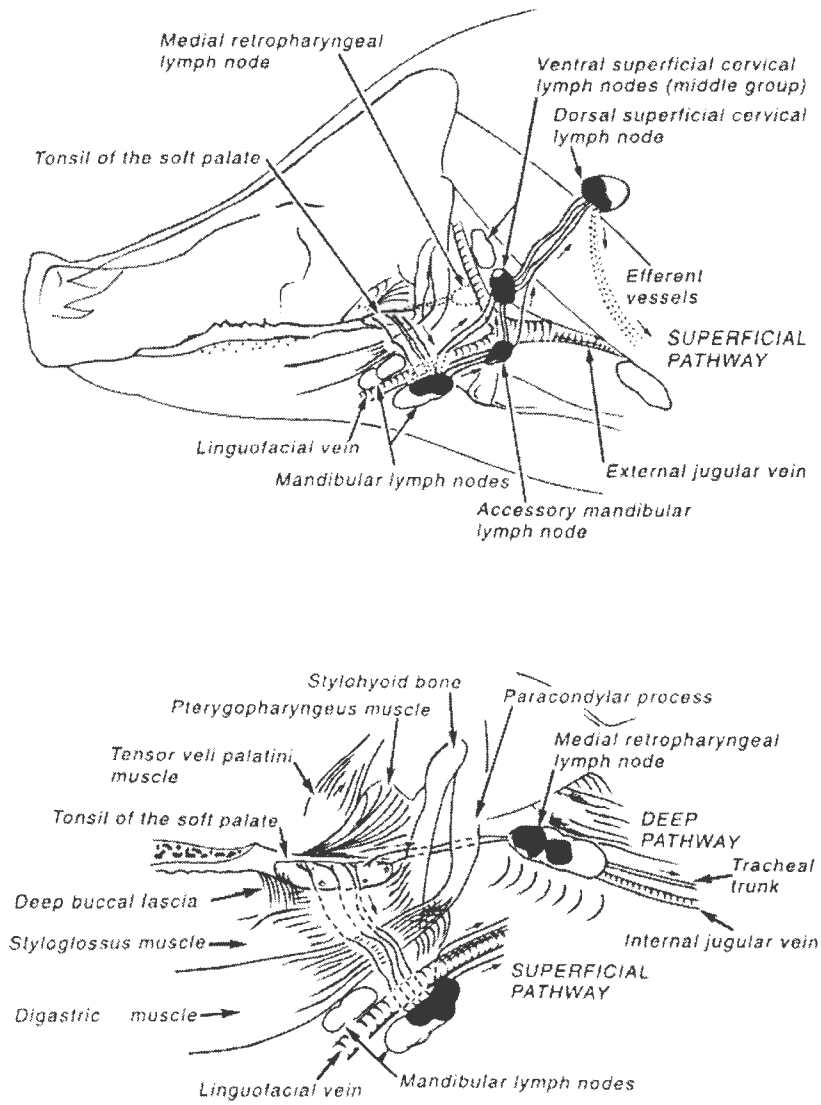

Fig. 3. Diagram of a left lateral view of a pig's head showing lymphatic drainage from the tonsil of the soft palate through the complex nodes of the head and neck comprising a superficial and a deep pathway. The deep pathway is shown in the lower, slightly magnified diagram. The drainage are to each node is indicated in black. Lymphatic vessels which course deep to structures are indicated by a broken line. The skin, cutaneous muscles, parotid node and gland, portion of the mandible and maxilla, pterygoid muscles and mandibular salivary gland have been removed.

Belz GT and Heath TJ (1995). Lymphatic drainage from the tonsil of the soft palate in pigs. *Journal of Anatomy*. **187**: 493.

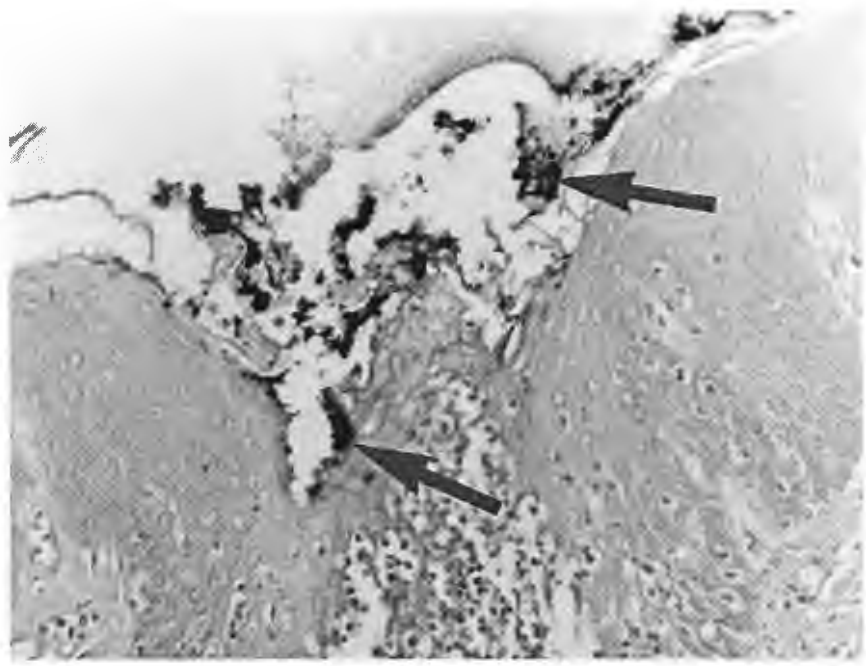

Fig. 4. *Salmonella typhimurium* immunopositive cells (arrows) on the surface of the crypt and superficial epithelium from swine.

Fedorka-Cray PJ, Kelley LC, Stabel TJ, Gray JT, and Laufer JA (1995). Alternate routes of invasion may affect pathogenesis of Salmonella typhimurium in swine. *Infection and Immunity*. **63**:2661.

Reprinted with permission of the editor of Infection and Immunity.

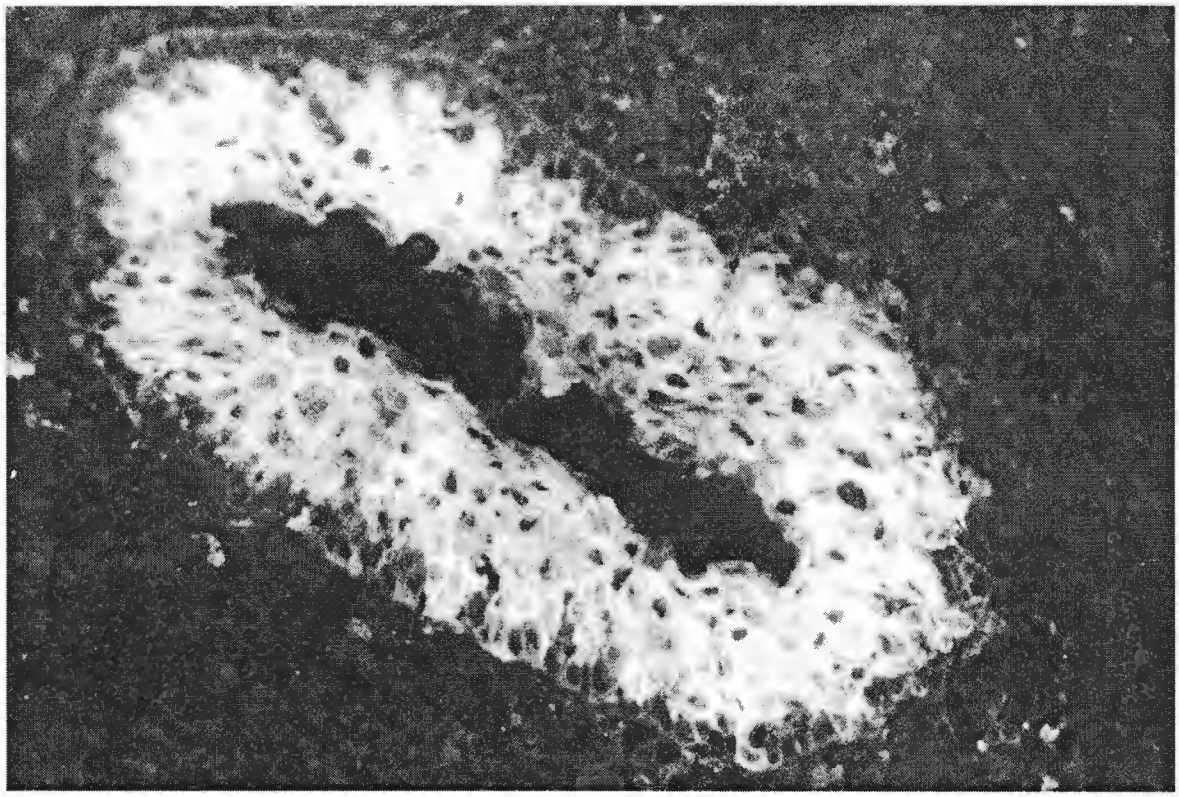

Fig. 5. Classical swine fever (CSF) viral antigen in epithelial cells of tonsillar crypts detected by immunofluorescence.

Photomicrograph courtesy of Dr. C. Terpstra. Department of Mammalian Virology, Institue for Animal Science and Health (ID-DLO), Lelystad, Netherlands.

Fig. 6. Porcine reproductive and respiratory syndrome (PRRS) viral antigen in macrophages and dendritic cells scattered or clustered in small groups throughout the tonsil and subepithelial regions of the crypts.

Photomicrograph courtesy of Dr. Pat Halbur, Veterinary Diagnostic Laboratory, Iowa State University, Ames IA, USA

CHARACTERIZATION OF THE CARRIER STATE IN PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS INFECTION

A paper published in Veterinary Microbiology

Dennis C Horter[1], Roman M. Pogranichniy[2], Chih-Cheng Chang[1], Richard B. Evans[2], Kyoung-Jin Yoon[2], Jeffrey J. Zimmerman[2]

Abstract

Porcine reproductive and respiratory syndrome (PRRS) virus infection results in clinically normal, but persistently infected, animals. An understanding of the carrier state is necessary for prevention, control, and/or elimination of PRRS virus. The objective of this experiment was to estimate the proportion of PRRS virus carriers over time and determine which combination of sample and diagnostic assay could most effectively identify persistently-infected animals. Sixty 3-week-old pigs were inoculated with PRRS virus ATCC VR-2332 and followed for up to 105 days post inoculation (PI). Sixty age-matched animals served as uninoculated controls. Samples (serum, peripheral blood leukocytes, oropharyngeal scrapings, tonsil, bronchoalveolar lavage, lung tissue, and tracheobronchial lymph nodes) were collected periodically and tested for evidence of PRRS virus infection by virus isolation (VI), swine bioassay, and reverse transcriptase-nested polymerase chain reaction (RT-nPCR). The PRRS virus-specific antibody response was monitored with a commercial ELISA.

---

[1]Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA, USA

[2]Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA, USA

Overall, PRRS virus was found in 51 of 59 (84%) necropsied animals by VI or swine bioassay between 63 and 105 days PI, including 10 of 11 (91%) of animals at day 105 PI. RT-nPCR on oropharyngeal scrapings was the most effective combination of assay and sample for detecting carriers. There was no significant difference in the antibody response of carrier vs. non-carrier animals. Infectious PRRS virus is present in most pigs the first 105 days following infection. Antibody response, as measured by a commercial ELISA, cannot be used to determine carrier status. RT-nPCR is a useful tool for detection of carriers, but diagnostic sample selection is critical if false negative results are to be avoided.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) virus is a positive sense RNA virus in the genus Arterivirus of the family Arteriviridae in the order Nidovirales (Cavanagh, 1997). Typical clinical signs of PRRS virus infection in susceptible animals include inappetence, fever, and respiratory disease in pigs of any age (Keffaber et al., 1989), often complicated by secondary bacterial or viral infections (Collins, 1991; Stevenson et al., 1993), and abortion or increased proportions of stillborn and weakborn pigs in pregnant animals (Keffaber, 1989; Terpstra et.al., 1991). Clinically, PRRS virus may produce catastrophic losses upon introduction into naïve herds or appear as intermittent re-breaks in endemically infected herds. Although first isolated and identified in 1991 (Wensvoort et al., 1991), serological evidence showed that PRRS virus was present in domestic swine in Canada as early as 1979 (Carman et al., 1995). During the 1980s, PRRS virus spread rapidly and became endemic in most of the major swine-producing areas throughout the world. Today, it

is one of the most economically important diseases of the global pork-producing community (Zimmerman et al., 1998).

Arteriviruses are enveloped viruses 40 to 60 nm in diameter with a genome 13 to 15 kilobases (kb) in size (Plagemann, 1996). Genomic replication and expression involves the reproduction of full-length genomic RNA and the transcription of a set of 6 to 9 smaller subgenomic mRNAs. In addition to PRRS virus, the genus Arterivirus includes lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV) (Plagemann and Moennig, 1992). Arteriviruses typically establish asymptomatic, persistent infections in their respective host species (Plagemann, 1996). LDV establishes a lifelong, asymptomatic infection in susceptible species of mice, i.e., Mus musculus and Mus caroli (Rowson, 1980), through the continuous production of permissive macrophages and the escape of LDV from host defense mechanisms (van den Broek et al., 1998; Stueckemann 1982). The mechanisms of persistence for SHFV and EAV are less well understood, but SHFV produces an asymptomatic persistent infection in several genera of non-macque African monkeys (London, 1977). In equids, EAV frequently localizes in the male reproductive tract and carrier stallions may shed virus in semen for months to years (Timoney and McCollum 1993; Neu et al., 1988). It has been shown that the persistence of EAV is mediated by testosterone (Little et al., 1992), but the specific mechanism by which the carrier state is established and maintained has not been described.

As shown both by transmission experiments and detection of infectious virus, PRRS virus infection results in clinically normal, but persistently infected animals (i.e., carriers). Under experimental conditions, Zimmerman et al. (1992) reported transmission from a sow

infected 99 days earlier to susceptible penmates and Albina et al. (1994) demonstrated transmission of PRRS virus by pigs infected 15 weeks earlier. Wills et al. (1997) reported isolation of virus from one of 4 pigs 157 days post inoculation (PI) and Allende et al. (2000) detected infectious virus in 2 of 5 pigs at day 150 PI.

Thus, PRRS virus produces a persistent infection in swine and carrier animals have the potential to transmit virus for months after becoming infected. Within herds, the virus maintains endemnicity through a cycle of transmission from carrier animals to susceptible animals introduced into the population through birth or purchase. Accordingly, the successful control, prevention, and/or eradication of PRRS virus requires a thorough understanding of the carrier state. This study estimated the proportion of carriers over time in an experimentally inoculated population and evaluated virus isolation (VI), reverse transcriptase-nested polymerase chain reaction (RT-nPCR), swine bioassay, and enzyme-linked immunosorbent assay (ELISA) as methods for the detection of carriers.

Materials and Methods

Virus and cells

The prototypic North American PRRS virus isolate ATCC VR-2332 was used in this study. VR-2332 was initially isolated in Minnesota, USA in association with a clinical outbreak of PRRS (Collins et al., 1992). Rossow et al. (1994, 1995) has previously described the clinical course and lesions produced by infection with VR-2332 in young pigs. The virus was purchased from a commercial vendor (American Type Culture Collection, Manassas, VA, USA) and used after passage in MARC-145 cells, a highly permissive clone derived from the African monkey kidney cell line MA-104 (Kim et al., 1993). For propagation of the

virus, 24-hour-old confluent monolayers of MARC-145 cells were prepared in 150-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY, USA) and inoculated with virus. Inoculated cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO/BRL Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma Chemical Company, St. Louis, MO, USA), 100 IU/ml penicillin (Sigma Chemical Company, St. Louis, MO, USA), 100 μg/ml streptomycin (Sigma Chemical Company, St. Louis, MO, USA), and 0.25 μg/ml amphotericin B (Sigma Chemical Company, St. Louis, MO, USA). Inoculated cells were incubated for 48 hours at 37° C in a humid 5% CO_2 atmosphere, after which the cells were subjected to one freeze-thaw cycle (minus 80° C/25° C). Cell lysates were harvested and clarified by centrifugation at 400 x g for 15 minutes at 4° C. The supernatant was saved as the source of virus and aliquoted into respective volumes. The challenge virus represented 6 in vitro passages.

Virus titer was determined using an immunofluorescence foci assay prior to animal inoculation (Yoon et al., 1999). A 1:10 dilution series was made with the cell culture supernatant containing PRRS virus, ranging from $1:10^0$ through $1:10^5$. A 24-hour-old confluent layer of MARC-145 cells prepared in a 96-well plate was inoculated in triplicate with 100 μl of the diluted virus and 100 μl of DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. After 24 hours of incubation at 37° C in a humid 5% CO_2 atmosphere, cells were fixed with 80% aqueous cold acetone. Cells were stained and evaluated with immunofluorescence microscopy using a cocktail of anti-PRRS virus monoclonal antibodies (SDOW17, JP24, SR30) (Nelson et al., 1993) conjugated with fluorescin isothiocyanate (FITC) (Rural Technologies Inc., Brookings,

SD, USA). Fluorescent foci were counted at the virus dilution giving between 10 and 100 countable foci per well. Virus titer was expressed as fluorescent foci units per ml (FFU/ml).

Experimental Design

The experiment was designed as a longitudinal study in which samples were collected from pigs over time post inoculation (PI) and assayed for PRRS virus or virus-specific antibodies, as appropriate to the sample.

Three week-old pigs (n = 120) were obtained from a herd free of PRRS virus. Throughout the study, animals were housed and cared for in compliance with the requirements set forth in Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies). Prior to initiating the study, individual pigs were tested and determined to be seronegative for PRRS virus antibodies using a commercial ELISA (IDEXX Laboratories, Inc., Westbrook, MA, USA). Subsequently, animals were randomly assigned to one of 2 treatments: PRRS virus inoculated (n = 60) or control (n = 60). Pigs in the inoculated group were housed approximately 5 per room in a bio-safety level 2 (BSL-2) animal facility. Control pigs were housed at a separate site. Pigs were acclimated at each site for 7 days after arrival. Pigs exposed to PRRS virus were intranasally (IN) inoculated with 2 ml (1 ml/naris) of clarified virus supernatant. Concentration of virus in the challenge inoculum was calculated to be 10^3 FFU/ml using the method described previously.

Selection of samples to be assayed for virus was based on previous reports of persistent infection (Benfield et al., 1997; Wills et al., 1997). Serum samples were collected from all animals on days –5, 0, 7, 14, 21, and every 14 days thereafter until animals were

euthanized. Whole blood samples for peripheral blood leukocytes were collected in ethylenediamine tetraacetic acid (EDTA) from all pigs beginning on day 63 PI and every 14 days thereafter or until euthanasia. Twelve animals from each group were euthanized on days 63, 77, 91, and 98 PI. One animal in the inoculated group expired prematurely as the result of an incarcerated umbilical hernia, thus 11 inoculated and 12 uninoculated were euthanized at 105 days PI. Humane euthanasia was performed in accordance with guidelines established by the American Veterinary Medical Association (Beaver et al., 2000). Prior to euthanasia, serum, peripheral blood leukocytes, and oropharyngeal scrapings (Wills et al., 1997) were collected. Following euthanasia, lung, bronchoalveolar lavage, tonsil, and tracheobronchial lymph node samples were collected. Lung tissue was harvested from the left cranial lobe and the entire right lung was lavaged following a procedure described by Mengling et al. (1996).

Determination of Carrier Status

PRRS virus status was determined for each individual animal. An inoculated pig was considered a carrier if infectious virus was detected in any sample by either VI or swine bioassay. Initially, all samples were screened for PRRS virus by VI. If the VI results were negative, a swine bioassay was conducted using tonsil homogenate filtrate as the inoculum. Inoculated pigs were considered 'non-carriers,' i.e., no detectable, if both VI and bioassay results were negative.

Sample Processing

Following collection, samples were processed as appropriate to the specimen. To harvest peripheral blood leukocytes (PBLs), approximately 16 ml of whole blood in EDTA was centrifuged at 4^{o} C at 1000 x g for 30 minutes. PBLs at the interface of the plasma and

red blood cells were collected, resuspended in 4 ml of 0.01M phosphate buffered solution (PBS) (pH 7.2), and then centrifuged at 1000 x g for 30 minutes at 4° C. The resulting cell pellet was resuspended in 4 ml of PBS and subjected to one freeze-thaw cycle (minus 80° C/25° C). Lysates were centrifuged at 400 x g for 30 minutes at 4° C. Each supernatant was saved and filter-sterilized through a 0.22 μm membrane filter. For tissue samples (lung, lymph nodes, tonsil), approximately 2 g of minced tissue was suspended in 10 ml of Earle's balanced salt solution (EBSS) (Sigma Chemical Company, St. Louis, MO) and homogenized in a Stomacher® 80 (Seward Limited, London, UK) for 60 seconds. The homogenate was clarified by centrifugation at 4,000 x g for 30 minutes at 4° C, then sterilized by passing through a 0.22 μm membrane filter. Lung lavage samples were centrifuged at 400 x g for 15 minutes at 4° C. The supernatant was discarded and the cell pellet was resuspended in 4 ml of EBSS and subjected to one freeze-thaw cycle. Lysates were centrifuged at 400 x g for 30 minutes at 4° C for clarification and then passed through a 0.22 μm membrane filter. Oropharyngeal scrapings, placed in 2 ml cold EBSS at the time of collection, were centrifuged at 400 x g for 30 minutes at 4° C and filter-sterilized through a 0.22 μm membrane filter. All filtrates and sera were aliquoted into appropriate volumes, coded with random numbers, and stored at minus 80° C until tested.

Enzyme-linked immunosorbent assay

A commercial ELISA kit (HerdChek® Porcine Reproductive and Respiratory Syndrome Virus Antibody Kit, IDEXX Laboratories, Westbrook, ME, USA) was used to detect PRRS virus-specific antibody in serum samples by following the procedures recommended by the manufacturer. A sample was classified as positive for PRRS virus

antibody if the sample-to-positive (S/P) ratio was equal to, or greater than, 0.4.

Virus isolation

For virus isolation (VI), approximately 24-hour-old confluent monolayers of MARC-145 cells were prepared in 48-well plates or 25-cm^2 tissue culture flasks. Filtered samples were inoculated into duplicate wells (200 µl per well) of 48-well plates or 25-cm^2 flasks (1.0 ml per flask) containing confluent cell monolayers. After a 2 hour incubation at 37° C in a humid 5% CO_2 incubator, 400 µl and 5 ml of DMEM supplemented with 5% FBS, 0.25 µg/ml amphotericin B, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamicin were placed into each well of the 48-well plates and into each 25-cm^2 flask, respectively, without discarding the inoculum. Inoculated cells were monitored for cytopathic effect (CPE) for up to 7 days PI. All inoculated cells were subjected to one round of freeze-thawing (minus 80° C/25° C) after CPE was detected or after 7 days incubation and cell lysates was harvested. The presence of PRRS virus in cell lysates was confirmed by subinoculating 200 µl of supernatant on monolayers of MARC-145 cells prepared in 48-well plates, fixing the cells in 80% aqueous cold acetone after 48 hours, and performing immunofluorescence microscopy using a cocktail of anti-PRRS virus monoclonal antibodies (SDOW17, JP24, SR30) (Nelson et al., 1993) conjugated with FITC.

Swine bioassay

The swine bioassay procedure for the detection of infectious PRRS virus in biological samples has been described previously (Swenson et al., 1994). The swine bioassay was conducted when all samples from an individual pig were negative for PRRS virus by VI. In

brief, individually-housed pigs were intramuscularly inoculated with tonsil homogenate filtrate (10 ml/pig) from a single animal, then monitored for evidence of PRRS virus infection. Bioassay pigs were bled 0, 7, and 14 days post inoculation and virus isolation and ELISA were performed on serum samples. The inoculum was considered to have contained infectious virus, that is, the pig from which the sample was collected was still infected, if the bioassay pig became viremic and seroconverted to PRRS virus.

Reverse transcriptase-nested polymerase chain reaction (RT-nPCR)

Reverse transcriptase-nested polymerase chain reaction was performed as previously described (Christopher-Hennings et al., 1995) and recommended RT-nPCR quality control measures (Christopher-Hennings, personal communication) were followed throughout. Specifically, gloves were worn at all times and were changed between each step. To avoid contamination, the extraction, RT, and PCR steps were conducted in separate rooms and siliconized tubes and aerosol-resistant pipet tips (United Laboratory Plastics, St. Louis, MO, USA) were used. Positive control (PRRS virus ATCC VR-2332 at a titer of 10^2 FFU/ml) and negative control (distilled water) were included to validate each run.

RNA was extracted by adding 500 µl of sample to 500 µl of lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7], 0.5% N-lauryl sarcosine, 0.1 M 2-mercaptoethanol) in siliconized polypropylene tubes (United Laboratory Plastics, St. Louis, MO, USA). Then 500 µl of the sample/lysis buffer mixture was added to 250 µl of phenol and 250 µl of chloroform-isoamyl alcohol (24:1), vortexed, and centrifuged at 10,000 x g for 5 minutes. The upper aqueous phase was retained and extracted again with phenol-chloroform-isoamyl alcohol and once with 500 µl of chloroform-isoamyl alcohol. Three

hundred microliters of the upper phase was added to 100 µl of 2 M sodium acetate (pH 4) and 800 µl of cold 95% ethanol. The sample was precipitated at minus 70° C for 1 h and centrifuged at 16,000 x g for 30 minutes at 4° C. Ethanol was carefully removed, then the pellet was washed twice with 100 ml of 70% ethanol, air dried, and reconstituted in 30 µl of distilled water (GIBCO/BRL, Grand Island, NY, USA).

Reverse transcriptase and PCR procedures were conducted exactly as described by Christopher-Hennings et al. (1995). Reactions were carried out using reagents from the GeneAmp® RNA PCR kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Primers utilized for reverse transcription and PCR reactions were specific for the ORF 7 (nucleocapsid) region of PRRS virus (Christopher-Hennings et al., 1995). The amplified product was electrophoresed across a 1% agarose gel (Amresco Inc., Solon, OH, USA) containing ethidium bromide, then visualized using photography under UV illumination.

Western immunoblot assay

Viral and mock (cell) antigens were prepared simultaneously, as described previously (Yoon et al., 1995) with some modifications. Cells were subjected to 2 freeze-thaw (minus 80° C/22° C) cycles. The supernatant was harvested and clarified by centrifugation at 400 x g for 10 minutes at 4° C. Viral and mock proteins were pelleted with ultra centrifugation at 35,000 x g for 90 minutes at 4° C. The pellet was resuspended in a solution of 0.01M PBS containing 1% Triton X-100 (Sigma Chemical Company, St. Louis MO, USA) then incubated at 4° C overnight. The supernatant was then assayed for protein concentration (Bio-Rad® Laboratories Inc., Hercules, CA, USA) and standardized to 2 µg/µl. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransfer of protein

from gel to nitrocellulose membrane, and western immunoblotting were performed as previously described (Yoon et al., 1995).

Data analysis

Virus isolation, RT-nPCR, and bioassay results were evaluated by sample and time (day PI) using logistic regression. Results were also expressed as proportions (number positive/number tested) and examined for patterns using linear and polynomial regressions. In addition, changes in the proportion of carriers by day PI and was analyzed using a nonparametric ANOVA (Wilcoxon/Kruskal-Wallis test). The ELISA S/P values in carrier and non-carrier animals were analyzed using an equivalence test. The null hypothesis for the equivalence test was that the difference in group means (carrier and non-carrier) was outside the limits of a defined range, i.e., significantly different, and the alternative hypothesis was that the group means were within the range and therefore, not significantly different. Since there is no generally accepted range for which ELISA S/P ratios are considered equivalent, we defined the upper and lower boundaries as the 95% confidence interval (95% CI) for the means in S/P ratios across all animals, assuming no difference between groups. This was calculated to be 0.101. The actual difference in the means of the two groups would have to be within the boundaries of the range $\pm$ 0.101 in order to reject the null hypothesis.

Results

The PRRS ELISA was used to monitor the antibody response to PRRS virus in the inoculated group and document the PRRS virus-free status of the uninoculated control group. In the inoculated group, serum samples from 19 of 60 animals had ELISA S/P values $\geq$ 0.40 at day 14 PI and 59 of 60 at day 21 PI (Figure 1). The antibody response in the inoculated

group peaked at day 49 PI with a mean S/P value of 1.65 (range 0.78 to 2.24). At day 105 PI, the 11 remaining inoculated animals had a mean S/P ratio of 1.29 (range 0.39 to 2.13).

All control animals were ELISA negative throughout the study with one exception. One animal in the control group was seronegative at all sampling points prior to day 91 PI, then showed an ELISA S/P response of 0.54 on day 91 PI, 0.69 on day 98 PI, and 0.45 on day 105 PI. All samples collected from this animal at the time of necropsy (day 105 PI), as well as serum samples collected over the course of the experiment, were assayed by VI, RT-nPCR, and/or swine bioassay. All results were negative for evidence of PRRS virus infection. Western immunoblotting on serum from days 63, 91, 98, and 105 PI detected no serum antibody reactive to any of the PRRS viral proteins of 15, 19, 23, 25, or 45 kilodaltons. On the basis of the absence of detectable PRRS virus by VI, swine bioassay, and PCR; the relatively weak ELISA antibody response; the lack of specific antibody to PRRS viral proteins; and the absence of any evidence of PRRS virus infection in penmates, the ELISA results from this animal were concluded to represent a non-specific response. Excluding this singleton false positive reactor, the S/P values in the control group ranged from 0.00 to 0.15 with a mean of 0.01 for all samples across all days in the study.

Results of virus isolation attempts on serum, PBLs, lung, tracheobronchial lymph node, bronchoalveolar lavage, oropharyngeal scrapings, and tonsil homogenate samples are summarized in Tables 1 and 2. Except for serum, samples were collected between 63 and 105 days PI. Among the negative control animals, all samples were VI negative. Among the inoculated group, PRRS virus was not isolated from any PBL, lung, tracheobronchial lymph node, or bronchoalveolar lavage samples. Overall, virus was isolated from oropharyngeal

scrapings and/or tonsil homogenate from 31 of 59 inoculated animals. If all samples from a PRRS virus-inoculated animal were negative by VI, then swine bioassay was performed using tonsil homogenate as the inoculum. Virus was detected in 20 of the 28 VI negative animals by swine bioassay. Thus, 51 of 59 animals were PRRS virus carriers at the time of necropsy on the basis of positive VI or swine bioassay results (Table 3). The estimated proportion of carriers varied between sampling points, but overlapping 95% confidence intervals suggested that the estimates were not significantly different. Further analysis of the data using the Wilcoxon/Kruskal-Wallis test showed that the median proportion of carriers did not change significantly over time ($p = 0.18$). However, although the proportion of carriers was constant, the number of VI positive carrier animals, as distinct from VI negative but bioassay positive carriers, declined over time (Table 3). Thus, virus was isolated from 20 of 23 carriers sampled on days 63 and 77 PI, but only 4 of 20 carriers were VI positive on days 98 and 105 PI. This change over time in the median proportion of VI positive carriers was statistically significant (Wilcoxon/Kruskal-Wallis test, $p = 0.003$).

RT-nPCR was performed on serum, PBLs, tonsil homogenate, and oropharyngeal scrapings (Tables 1 and 2). All uninoculated control samples ($n = 780$) were RT-nPCR negative with the exception of 4 false positive results: one serum sample collected on day 35 PI, one serum sample on day 49 PI, and 2 PBL samples on day 91 PI. In the PRRS virus-inoculated group, among 8 animals in which virus was not detected by VI or bioassay, 4 had one or more RT-nPCR positive samples. Given the exposure history of these 4 animals, it is possible that these results accurately reflected that fact that inactivated virus was recovered

from previously infected animals. Alternatively, one of the results was false. If the latter were true, it could not be determined which assay failed.

ELISA S/P ratios (Figure 2) at the time of necropsy for carrier (mean S/P = 1.44) and non-carrier (mean S/P = 1.37) animals were statistically compared with an equivalence test. The null hypothesis was that a difference in means between groups of ±0.101 or greater would represent a statistically significant difference. However, the difference of the means was 0.07, well within the boundaries of the range. Elimination of the single most extreme data point (S/P = 0.39) in the carrier group had a negligible effect on the means of the carrier group and did not affect the statistical evaluation. Thus, the ELISA S/P response in carrier animals was not different from the response in non-carriers over the period of time encompassed by the study.

Discussion

The purpose of this experiment was to estimate the proportion of PRRS virus carrier animals over time and evaluate methods for their detection. Infectious PRRS virus was detected by VI or bioassay in 51 of 59 (86%) animals between 63 and 105 days PI, including 10 of 11 (91%) animals at day 105 PI. In addition, 4 of the 8 animals negative for infectious virus were positive for viral nucleic acid by RT-nPCR. Given the high specificity of the RT-nPCR assay demonstrated in this experiment, i.e., 4 false positives among 780 samples from animals known to be PRRS virus negative, these results suggest the detection of inactivated virus in previously infected animals.. Overall, the results of this and previous studies are in agreement: PRRS virus infection results in persistently-infected swine (Allende et al., 2000; Christopher-Hennings et al., 1995; Christopher-Hennings et al., 2001; Wills et al., 1995;

Wills et al., 1999). The current study differed from previous studies in that a higher proportion of carriers was detected than previously reported. However, this difference is compatible with the design implemented in this study: 1) the larger number of inoculated animals (n = 60) provided better estimates of the proportion of carriers in the population; 2) euthanasia of pigs at regular intervals post inoculation allowed for more extensive sampling of individual pigs; and 3) the routine use of virus isolation and bioassay on multiple samples made it possible to establish the carrier status of individual animals with greater certainty.

The data provided strong, albeit indirect, evidence that the quantity of virus in pigs declined with time. Specifically, a statistically significant ($p = 0.003$) decline in the proportion of VI-positive animals occurred over time, but PRRS virus was still detected in most VI-negative animals by bioassay (Table 3). The greater analytical sensitivity of the bioassay relative to VI is due both to the low minimum infectious dose of PRRS virus for swine (Yoon et al., 1999) and the larger inoculum used in the bioassay (10.0 ml vs. 1.0 ml). In this experiment, the transition from VI-positive to VI-negative, bioassay-positive animals occurred about day 90 PI. Previous reports also noted this trend at about the same point in time post inoculation (Wills et al., 1995; Wills et al., 1999). Although the specific immune mechanism responsible for controlling the infection has not been described, it has been observed that the decline in the quantity of virus coincides temporally with an increase in the number of interferon gamma-producing T-cells (Meier et al., 2000).

Other questions related to persistent infection remain to be resolved. These include the postulated role of virus-specific factors, i.e., hypothesized differences among virus isolates relative to their ability to persist in pigs, and host-specific factors, i.e., postulated

effects of age or breed on the duration of persistent infection (Christopher-Hennings et al., 2001; Halbur et al., 1998). Although the North American prototype PRRS virus (ATCC VR-2332) was used frequently (Benfield et al., 1997; Christopher-Hennings et al., 1995), but not exclusively (Allende et al., 2000; Benfield et al., 1997; Christoper-Hennings et al., 2001; Swensen et al., 1995; Wills et al., 1997; Wills et al., 1999; Zimmerman et al., 1992), there are too many inconsistencies in experimental design to be able to compare the data and determine whether strain variation exists. This issue would best be resolved by a single experiment providing for a direct comparison among strains. The same conclusion applies to postulated host factors.

As the cumulative evidence has shown, PRRSV is capable of establishing a long-term persistent infection. It follows that reliable identification of carriers is key to the prevention and control of PRRS. Our study showed that RT-nPCR on oropharyngeal scrapings or tonsil was the most effective method of detecting carriers, particularly as the quantity of virus declined over time. This statement, however, must be tempered with the observation that PCR protocols among laboratories are non-uniform and in the midst of rapid evolution as new technology emerges. In some cases, commercially-available PCR-based assays have demonstrated less than adequate performance (Wagstrom et al., 2000). The nested PCR procedure used in this study, although labor-intensive, was validated and shown to be analytically highly sensitive (Christopher-Hennings et al., 1995). However, the nested procedure has also been recognized as presenting a higher risk of misclassification errors (false positives) due to contamination of samples during testing. For that reason, the assay must be conducted according to strict quality control procedures. In this experiment, 4 of 780

negative control samples tested by RT-nPCR were misclassified as positive, giving a diagnostic specificity of 99.5%. Consequently, although variability in performance will continue to be a concern until commercial kits are available, the PCR results reported here should be applicable to laboratories performing an equivalent assay under similar conditions.

The detection of PRRS virus in tonsil and/or oropharyngal scrapings has previously been reported (Allende et al., 2000; Benfield et al., 1997; Christopher-Hennings et al., 2001; Wills et al., 1997; Wills et al., 1999). In this study, virus was detected somewhat more frequently in oropharyngeal scraping samples than tonsil tissue samples. Presumably, PRRS virus associated with infected macrophages located within tonsillar crypts and tonsillar crypt epithelium is physically expressed and collected during the process of scraping the tonsil. We speculate that recovery of virus was enhanced by the presence of mucin in the sample, known to be highly protective of viruses (Schoenbaum et al., 1990; Wu et al., 1994), and the fact that oropharyngeal scrapings samples were minimally processed prior to being assayed for virus. On the other hand, recovery and/or detection of virus in tonsil tissue samples may be inhibited by the greater dilution of the sample, more extensive sample processing, and the presence of non-specific anti-viral compounds. Cellular factors, i.e., defensins and proteolytic enzymes capable of inactivating enveloped viruses, are released when the tonsil homogenate is processed (Daher et al., 1986).

An unexpected outcome of this experiment was the appearance of an ELISA-positive animal within the negative control group at day 91 PI. Lack of the presence of virus within any samples collected from this animal, an ELISA response weaker than that characteristic of acute PRRS virus infection, lack of antibody recognition of the major PRRS virus proteins on

western immunoblotting, and the absence of PRRS virus in penmates suggested that non-specific cross reactivity with the PRRS ELISA was responsible for the response. Field reports of non-specific reactions to the PRRS ELISA are known, but this is the first false-positive response observed under experimental conditions.

The detection of carrier animals is essential for the protection of negative herds and/or to eliminate PRRS virus from infected herds. Evaluation of the diagnostic performance of VI, ELISA, and RT-nPCR by sample (serum, peripheral blood leukocytes, oropharyngeal scrapings, lung, lung lavage, tracheobronchial lymph nodes) showed that detection of carrier animals was assay dependent, sample dependent, and time post infection dependent. RT-nPCR on oropharyngeal scapings was the best combination of sample and assay, followed by RT-nPCR on tonsil homogenate. For the observation period of this study, the serum antibody response, as measured by a commercial ELISA, did not discriminate between carrier and non-carrier animals. Relative to application of these results to the field, it may be concluded that the detection of individual carrier animals using the available diagnostic technology is currently not practical on a routine basis.

Acknowledgments

This project funded in part by the Iowa Livestock Health Advisory Council and the National Pork Producers Council (Pork Check-off Funds).

References

Albina, E., Madec, F., Cariolet, R., and Torrison, J., 1994. Immune response and persistence of the porcine reproductive and respiratory syndrome virus in infected pigs and farm units. Vet Rec. 134, 567-573.

Allende, R., Laegreid, W.W., Kutish, G.F., Galeota, J.A., Wills, R.W., Osorio, F.A., 2000. Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. J. Virol. 74(22), 10834-10837.

Beaver, B.V., Reed, W., Leary, S., McKiernan, B., Bain, F., Schultz, R., Bennett, B.T., Pascoe, P., Shull, E., Cork, L.C., Francis-Floyd, R., Amass, K.D., Johnson, R., Schmidt, R.H., Underwood, W., Thornton, G.W., Kohn, B., 2000. 2000 Report of the AVMA panel on euthanasia. J. Am. Vet. Med. Assoc. 218(5), 669-696.

Benfield, D.A., Christopher-Hennings, J., Nelson, E.A., Rowland, R.R.R., Nelson, J.K., Chase, C.C.L., Rossow, K.D., Collins, J.E., 1997. Persistent fetal infection of porcine reproductive and respiratory syndrome (PRRS) virus. In: Proc. Am. Assoc. Swine. Pract. 28, 455-458.

Carman, S., Sanford, S.E., Dea, S., 1995. Assessment of seropositivity to porcine reproductive and respiratory syndrome (PRRS) virus in swine herds in Ontario--1978 to 1982. Can. Vet. J. 36, 776-777.

Cavanagh, D., 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch. Virol. 142, 629-633.

Christopher-Hennings, J., Nelson, E.A., Nelson, J.K., Hines, R.J., Swenson, S.L., Hill, H.T., Zimmerman, J.J., Katz, J.B., Yaeger, M.J., Chase, C.C.L., Benfield, D.A., 1995. Detection of porcine reproductive and respiratory syndrome virus in boar semen by PCR. J. Clin. Microbiol. 33(7), 1730-1734.

Christopher-Hennings, J., Holler, L.D., Benfield, D.A., Nelson, E.A., 2001. Detection and duration of porcine reproductive and respiratory syndrome virus in semen, serum, peripheral blood mononuclear cells, and tissues from Yorkshire, Hampshire, and Landrace boars. J. Vet. Diagn. Invest. 13(2), 133-142.

Collins, J.E., 1991. Diagnostic notes: newly recognized respiratory syndromes in North American swine herds. Am. Assoc. Swine. Pract. Newsletter. 3, 7-11.

Collins, J.E., Benfield, D.A., Christianson, W.T., Harris, L., Hennings, J.C., Shaw, D.P., Goyal, S.M., McCullough, S., Morrison, R.B., Joo, H.S., Gorcyca, D., Chladek, D., 1992. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in

North America and experimental reproduction of disease in gnotobiotic pigs. J. Vet. Diagn. Invest. 4(2), 117-126.

Daher, K.A., Selsted, M.E., Lehrer, R.I, 1986. Direct inactivation of viruses by human granulocyte defensins. J. Virol. 60(3), 1068-1074.

Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (First Revised Edition). 1999. Federation of Animal Science Societies, Savoy IL.

Halbur, P.G., Rothschild, M.F., Paul P.S., Thacker B.J., Meng X-J., 1998. Differences in susceptibility of Duroc, Hampshire, and Meishan pigs to infection with a high virulence strain (VR2385) of porcine reproductive and respiratory syndrome virus (PRRSV). J. Anim. Breed. Genet. 115, 181-189.

Keffaber, K.K., 1989. Reproductive failure of unknown etiology. Am. Assoc. Swine Pract. Newsletter. 2, 1-10.

Kim, H.S., Kwang, J., Yoon, I.J., Joo, H.S., Frey, M.L., 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Arch. Virol. 133(3-4), 477-483.

Little, T.V., Holyoak, G.R., McCollum, W.H., Timoney, P.J., 1992. Output of equine arteritis virus from persistently infected stallions is testosterone-dependent. In: Proceedings of 6th International Conference on Equine Infectious Diseases. Plowright W, Rossdale PD, Wade JF (Eds). R & W Publications (Newmarket) Limited. 225-229.

London, W.T., 1977. Epizootiology, transmission and approach to prevention of fatal simian hemorrhagic fever in rhesus monkeys. Nature. 268(5618), 344-345.

Meier, W., Wheeler, J., Husmann, R.J., Osorio, F., Zuckermann, F.A., 2000. Characteristics of the immune response of pigs to PRRS virus. Vet. Res. 31, 41.

Mengeling, W.L., Lager, K.M., Vorwald, A.C., 1996. Alveolar macrophages as a diagnostic sample for detecting natural infection with porcine reproductive and respiratory syndrome virus. J. Vet. Diagn. Invest. 8(2), 238-240.

Nelson, E.A., Christopher-Hennings, J., Drew, T., Wensvoort, G., Collins, J.E., Benfield, D.A., 1993. Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. J. Clin. Microbiol. 31(12), 3184-3189.

Neu, S.M., Timoney, P.J., McCollum, W.H., 1988. Persistent infection of the reproductive tract in stallions persistently infected with equine arteritis virus. In: Proceedings of the 5th International Conference on Equine Infectious Diseases. The University Press of Kentucky, Lexington, KY. 149-154.

Plageman, P.G.W., Moennig, V., 1992. Lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus: a new group of positive-strand RNA viruses. Adv. Virus Res. 41, 99-183.

Plagemann, P.G.W., 1996. Lactate dehydrogenase-elevating virus and related viruses. Fields Virology, 3rd ed, Lippincott Williams & Wilkins, pp. 1105-1120.

Rossow, K.D., Bautista, E.M., Goyal, S.M., Molitor., T.W., Murtaugh, M.P., Morrison, R.B., Benfield, D.A., Collins, J.E., 1994. Experimental porcine reproductive and respiratory syndrome virus infection in one-, four-, and 10-week-old pigs. J. Vet. Diagn. Invest. 6(1), 3-12.

Rossow, K.D., Collins, J.E., Goyal, S.M., Nelson, E.A., Christopher-Hennings, J., Benfield, D.A., 1995. Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. Vet. Pathol. 32(4), 361-373.

Rowson, K.E., 1980. A new host species for lactic dehydrogenase virus. Experientia. 36(9), 1066-1067.

Schoenbaum, M.A., Zimmerman, J.J., Beran, G.W., Murphy, D.P., 1990. Survival of pseudorabies virus in aerosol. Am. J. Vet. Res. 50, 331-333.

Stevenson, G.W., Van Alstine, W.G., Kanitz, C.L., Keffaber, K.K., 1993. Endemic porcine reproductive and respiratory syndrome virus infection of nursery pigs in two swine herds without current reproductive failure. J. Vet. Diagn. Invest. 5(3), 432-434.

Stueckemann, J.A., Holth, M., Swart, W.J., Kowalchyk, K., Smith, M.S., Wolstenholme, A.J., Cafruny, W.A., Plagemann, P.G., 1982. Replication of lactate dehydrogenase elevating virus in macrophages. 2. Mechanism of persistent infection in mice and cell culture. J. Gen. Virol. 59, 263-272.

Swenson, S.L., Hill, H.T., Zimmerman, J.J., Evans, L.E., Landgraf, J.G., Wills, R.W., Sanderson, T.P., McGinley, M.J., Brevik, A.K., Ciszewski, D.K., Frey, M.L., 1994. Excretion of porcine reproductive and respiratory syndrome virus in semen after experimentally induced infection in boars. J. Am. Vet. Med. Assoc. 204(12), 1943-1948.

Terpstra, C., Wensvoort, G., Pol, J.M.A., 1991. Experimental reproduction of porcine epidemic abortion and respiratory syndrome (Mystery Swine Disease) by infection with Lelystad virus: Koch's postulates fulfilled. Vet. Q. 13(3), 131-136.

Timoney, P.J., McCollum, W.H., 1993. Equine viral arteritis. Vet. Clin. North. Am. Equine. Pract. 9, 295-309.

van den Broek, M.F., Sporri, R., Even, C., Plagemann, P.G., Hanseler, E., Hengartner, H., Zinkernagel, R.M., 1998. Lactate dehydrogenase-elevating virus (LDV): lifelong coexistence of virus and LDV-specific immunity. J. Immunol. 159(4), 1585-1588.

Wagstrom, E.A., Yoon, K-J., Zimmerman, J.J., 2000. Diagnostic performance of a reverse transcription-polymerase chain reaction test for porcine reproductive and respiratory syndrome virus. J. Vet. Diagn. Invest. 12, 75-78.

Wensvoort, G., Terpstra, C., Pol, J.M.A., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., Broekhuijsen, J.M., Moonen, P.L.J.M., Zetstra, T., de Boer, E.A., Tibben, H.J., de Jong, M.F., van't Veld, P., Groenland, G.J.R., van Gennep, J.A., Voets, M.T., Verheijden, J.H.M., Braamskamp, J.. 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Q. 13(3), 121-130.

Wills, R.W., Zimmerman, J.J., Yoon, K-J., Swenson, S.L., McGinley, M.J., Hill, H.T., Platt, K.B., Christopher-Hennings, J., Nelson, E.A., 1997. Porcine reproductive and respiratory syndrome: a persistent infection. Vet. Mircrobiol. 55, 231-240.

Wills, R.W., Doster, A.R., Galeota, J., Sur, J.R., Osorio, F.A., 1999. Infection duration and proportion of persistently infected pigs. In: Proceedings 80th Annual Meeting of the Conference of Research Workers in Animal Disease, Chicago, IL.

Wu, A.M., Csako, G., Herp, A., 1994. Structure, synthesis, and function of salivary mucins. Mol. Cell. Biochem. 137(1), 39-55.

Yoon, K-J., Zimmerman, J.J., Swenson, S.L., McGinley, M.J., Eernisse, K.A., Brevik, A., Rhinehart, L.L., Frey, M.L., Hill, H.T., Platt, K.B., 1995. Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. J. Vet. Diagn. Invest. 7(3), 305-312.

Yoon, K-J., Zimmerman, J.J., Chang, C-C., Cancel-Tredo, S., McGinley, M.J., 1999. Effect of challenge dose and route on porcine reproductive and respiratory syndrome virus (PRRSV) infection in young swine. Vet. Res. 30, 629-638.

Zimmerman, J.J., Sanderson, T., Eernisse, K.A., Hill, H.T., Frey, M.L., 1992. Transmission of SIRS virus in convalescent animals to commingled penmates under experimental conditions. Am. Assoc. Swine. Pract. Newsletter. 4, 25.

Zimmerman, J.J., Yoon, K-J., Stevenson, G., Dee, S.A., 1998. The 1998 porcine reproductive and respiratory syndrome compendium. National Pork Producers Council and National Pork Board, Des Moines, IA. pp 61-63.

TABLES AND FIGURES

Table 1. Virus isolation and PCR results on serum and peripheral blood leukocyte samples from PRRS virus-inoculated pigs

	Serum		Peripheral blood leucocytes	
DPI[a]	VI	RT-nPCR	VI	RT-nPCR
7	60/60[b]	60/60	NS [d]	NS
	– [c]	–		
14	49/60	60/60	NS	NS
	(72, 91) [e]	–		
21	38/60	60/60	NS	NS
	(51, 76)	–		
35	38/60	53/60	NS	NS
	(51, 76)	(80, 96)		
49	0/59	36/59	NS	NS
	–	(49, 73)		
63	0/59	17/59	0/59	18/59
	–	(17, 40)	–	(19, 42)
77	0/46	2/47	0/47	11/47
	–	(0, 10) [f]	–	(11, 36)
91	0/35	2/35	0/35	12/35
	–	(0, 13) [f]	–	(19, 50)
98	0/23	0/23	0/23	4/23
	–	–	–	(2, 33)
105	0/11	0/11	0/11	1/11
	–	–	–	(0, 26) [f]

[a] Day post inoculation
[b] animals positive / animals inoculated
[c] 95% confidence interval not calculable
[d] Not sampled (NS)
[e] 95% confidence interval
[f] 95% confidence interval truncated to limits of test

Table 2. Virus isolation and PCR results on oropharyngeal scraping and tonsil homogenate samples from PRRS virus-inoculated pigs

Status	Assay	Sample	Day Post Inoculation				
			63	77	91	98	105
Carriers [a]	VI [b]	Oropharyngeal scrapings	9/12 [c] (51, 100) [d]	8/11 (46, 99)	7/8 (65, 100) [e]	1/10 (0, 29) [e]	3/10 (2, 58)
		Tonsil	7/12 (30, 86)	7/11 (35, 92)	1/8 (0, 35) [e]	0/10 – [f]	1/10 (0, 29) [d]
	PCR [g]	Oropharyngeal scrapings	NS [h]	11/11 –	7/8 (65, 100) [e]	9/10 (71, 100) [e]	8/10 (55, 100) [d]
		Tonsil	10/12 (62, 100) [e]	9/11 (59, 100) [e]	3/8 (4,71)	10/10 –	5/10 (19, 81)
		PBL	6/12 (22, 78)	2/11 (0, 41) [e]	4/8 (15, 85)	2/10 (0, 45) [e]	1/10 (0, 29) [d]
Non-carriers [i]	PCR	Oropharyngeal scrapings	–	0/1	1/4	2/2	0/1
		Tonsil	–	0/1	2/4	0/2	0/1
		PBL	–	0/1	1/4	0/2	0/1

[a] By definition, 'carrier' indicates an animal in which infectious PRRS virus was detected by virus isolation or bioassay.
[b] All serum, PBL, lung, tracheobronchial lymph node, and alveolar macrophage samples VI negative for days 63 to 105 pi.
[c] animals positive / number of carrier animals
[d] 95% confidence interval
[e] 95% confidence interval truncated to limits of test
[f] 95% confidence interval not calculable
[g] Lung, tracheobronchial lymph node, and alveolar macrophage samples not assayed by PCR.
[h] Not sampled (NS)
[i] By definition, 'non-carrier' indicates an animal in which no infectious PRRS virus was detected by virus isolation or bioassay

Table 3. Determination of proportion of carriers over time

	Day Post Inoculation				
	63	77	91	98	105
Carriers [a]	12/12 [b]	11/12	8/12	10/12	10/11
	– [c]	(76,100) [d.e]	(40,93)	(62,100) [e]	(74,100) [e]
A. VI positive [f]	10/12	10/12	7/12	1/12	3/11
B. VI negative, bioassay positive [g]	2/2 [h]	1/2	1/5	9/11	7/8

[a] Proportion of carriers determined as described in Materials and Methods
[b] Animals positive / PRRS virus inoculated animals
[c] 95% confidence interval not calculable
[d] 95% confidence interval
[e] 95% confidence interval truncated to limits of test
[f] VI performed on oropharyngeal scrapings and tonsil homogenate
[g] Swine bioassay was performed on animals negative by VI
[h] Bioassay positive animals / VI negative animals

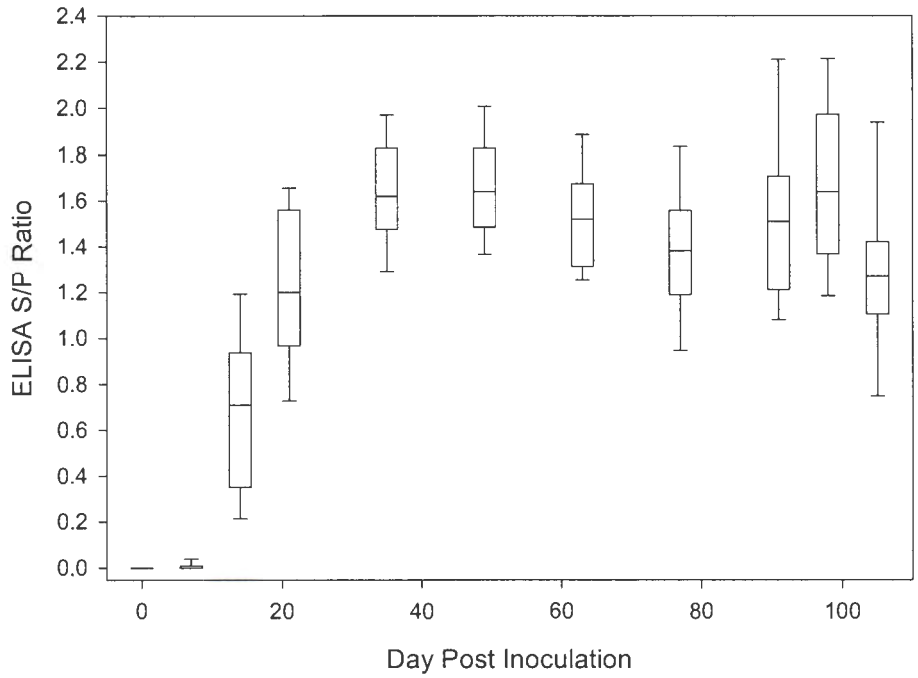

Figure 1. ELISA sample-to-positive (S/P) ratios in PRRS virus-inoculated group. Horizontal line represents mean, box represents 25th and 75th quartiles, and whisker lines represent 5th and 95th percentiles.

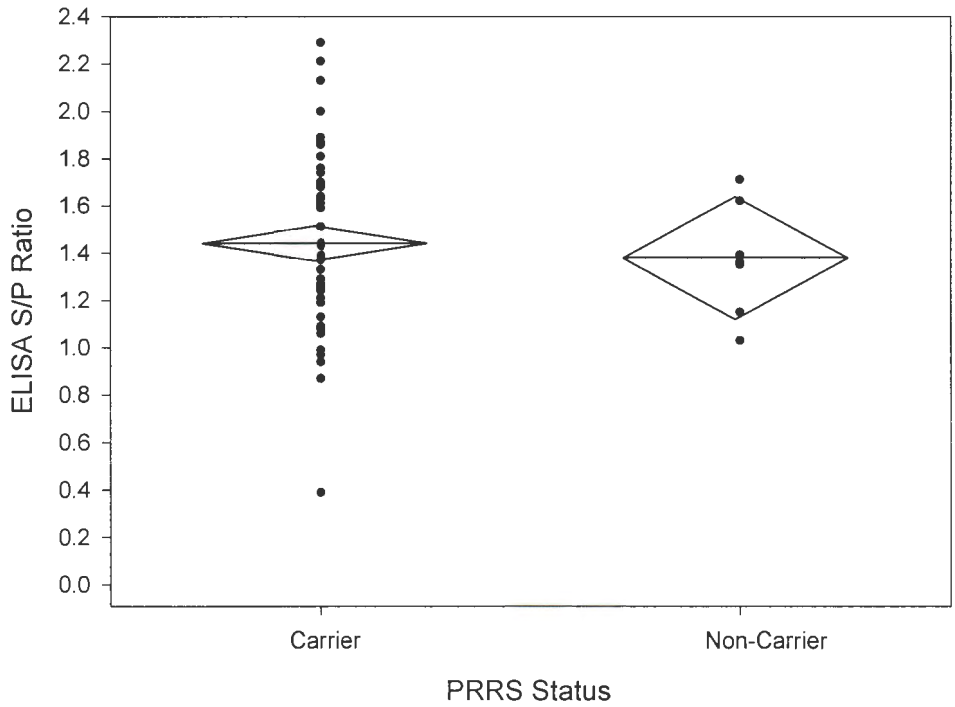

Figure 2. Scatterplot of ELISA S/P ratios for carrier vs. non-carrier animals. Horizontal line represents group means and peaks of triangles represent 95% confidence interval for group means.

GENERAL DISCUSSION

Porcine reproductive and respiratory syndrome (PRRS) virus infection results in clinically normal persistently infected carrier animals, as shown by both transmission experiments and isolation of infectious virus. It has proven to be difficult to control PRRS virus within swine herds because among other things, we do not fully understand the dynamics of the carrier state caused by PRRS virus. Prior research indicates that the tonsils are a location of persistence for PRRS virus. In spite of having strong immune components and able to initiate a strong systemic immune response, pathogens have adapted to utilize the tonsil as a location for persistence. Although the specific mechanism for persistence for PRRS virus is not currently known, we speculate that it persists within macrophages located within the tonsillar crypts and the tonsils.

Our studies in persistence, presented in this thesis in Chapter Two, characterize the duration of the carrier state and the diagnostic sensitivity and specificity of tests routinely used to for PRRS virus diagnosis. Infectious PRRS virus was detected by virus isolation (VI) or bioassay in 51 of 59 (86%) animals between 63 and 105 days PI, including 10 of 11 animals at day 105 PI. Overall, the results of this and previous studies are in agreement: PRRS virus infection results in persistently-infected swine.

Reliable identification of carriers is key to the prevention and control of PRRS. For the observation period of this study, the serum antibody response, as measured by a commercial ELISA, was useful for detecting animals infected with PRRS virus, but did not discriminate between carrier and non-carrier animals. Our study showed that RT-nPCR on oropharyngeal scrapings or tonsil was the most effective method of detecting carriers,

particularly as the quantity of virus declined over time. The nested PCR procedure used in this study, although labor-intensive, was validated and shown to be analytically highly sensitive. However, in the context of applying this to the field, it may not be a practical method for elimination of PRRS virus from a herd situation due to financial and labor considerations.

Although much as been learned from recent research, especially in the areas of immunobiology and persistence, more work is needed in determining the mechanism for persistence of PRRS virus. In the future, further research in the area of immunology and the carrier state will help to increase our knowledge as well as develop a solution for control of PRRS virus in swine.

ACKNOWLEDGMENTS

I would like to thank my major professors Jeff Zimmerman and Kyoung-Jin Yoon for all the help, support, and guidance that they have given me in both veterinary and graduate school. A big thanks to Dr. Mark Ackermann for being on my committee providing helpful support in key moments throughout the past four years. I would like to thank my friends and colleagues Roman Pogranichnyy and Michael Chang for their help and guidance in this project over the last four years. To the "bleeding crew" - Alice Heldorfer, Dusty Oedekoven, Christopher Groos, Susan Stoterau, Bryan Wheeler, Dr. Zhao, Troy Wheeler, Troy Kaiser, J. David Schneider, and Joe Hermam – Thanks for all the help on all of those Saturdays, your guys' company was the most memorable and fun time of the project. A big thanks to the Class of 2002 for the good times, the hard times, the laughter, the tears, and the memories over the last four years. I'll never forget you guys. To all the folks in the Diagnostic lab, I'm forever indebted to you for helping me find all of the things I needed, letting me invade your lab space, and for the help you gave to me. And of course, the folks at SDSU – Eric Nelson & Jane Hennings – for giving me a chance and getting me started in research. I would have never guessed that it would take me here. Thanks to Eileen & Brad Thacker for review of manuscript and for moral support throughout this time. I would also like to express my gratitude to Dr. Kline for all your help and for those great conversations that we had on Neurology.

Most importantly, I would like to thank my family and especially Deanne's family for all of their support throughout the past four years of veterinary school and graduate school. Although I could not possibly list all of my friends, I would like to thank my especially close

friends Troy Kaiser, Stacy Holzbauer, Amy Vincent, Ryan Royer, Dusty, Jenn and Morgan Oedekoven, Christopher Groos, Jason Warrington and Quentin Fixen for their friendship and support. And the biggest thank you of all to my loving wife Deanne, who for four years has been there for me, has supported me, has kept me going when the times have been tough, and who has been by my side throughout it all. I know of no greater love and I only hope that I can somehow repay you for all of your love and sacrifice.

And above all, I thank God, for without him, none of this would be possible.