

ABSTRACT

MCPHATTER, LISA. The Piglet as a Model of Norwalk Gastroenteritis. (Under the direction of Dr. Jack Odle)

Norwalk-like viruses (NLV) are the most important cause of acute gastroenteritis in humans. However, little is known about their pathogenesis since no cell culture or animal model is available yet. Therefore, we have investigated if young piglets would be a suitable model to study NLV pathogenesis. Sixteen ~21-d-old piglets were orally gavaged with 3 doses of the NLV prototype strain (Norwalk virus; strain 8FIIb) previously confirmed to be infective for human volunteers. Stool samples were collected daily to assess viral shedding. Exposure to Norwalk virus had no detectable effect on pig growth. Using RT-PCR, viral NV-RNA was detected in two (high dose) of the twelve pigs gavaged with virus. In a second study, colostrum-deprived newborn piglets were orally gavaged with either the human strain (8FIIb, n=4), swine calicivirus (n=6) or a saline control (n=5). Neither swine calicivirus nor Norwalk virus could be detected by RT-PCR in any stool samples. At day 4 post infection intestinal samples were collected for histological and biochemical evaluation. Differences in diarrhea score and intestinal lactase activity were not detected, but weight gain of pigs infected with the swine calicivirus (158 g/d) was lower ($P<0.05$) than control pigs (280 g/d). In addition, there was a 49% reduction in ileal villus height in the swine calicivirus group ($P<0.05$). These results are consistent with swine calicivirus-induced villous atrophy, and attendant reduction in nutrient absorption.

THE PIGLET AS A MODEL OF NORWALK GASTROENTERITIS

by

LISA A. McPHATTER

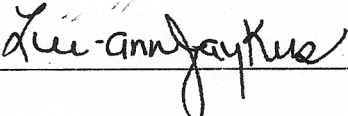
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Masters of Science

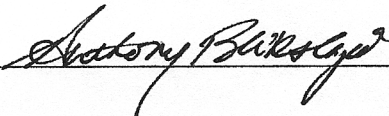
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BIOGRAPHY

I grew up on my grandfather's farm in the Blue Springs district outside Hoke County in North Carolina. As a child I loved helping to take care of the animals and was "in charge" of the piglets. As I grew older, folks around the district referred to me as "Dr. Lisa" because I'd care for stray and ill cats and dogs. My first stray was a black Labrador puppy that had been abused. Left by a stranger in a cardboard box on the front porch of our home, he presented with a swollen jaw, severe hunger, and whimpering. Weeks later after having nursed him back to health, the puppy began to bark and snap at me. I named him "Snapper" and he became a best friend to a lonely southern black girl.

These experiences from my life's early years have largely shaped the great interest I have in biomedical science. I graduated from Western Carolina University in the summer of 1989 with a BS in biology and spent the next two years in treatment and recovery following reconstructive bilateral eye surgery. In 1991 I took a job at Duke Medical Center as a research technician at the Alzheimer's Disease Research Center. There, I worked in the research laboratory developing techniques in immunohistochemistry on brains donated by patients that had died following complications with neurodegenerative disease. I co-authored two publications, *Diffuse plaques in children (1992). J Neuropathol Exp Neurol 51: 317* and *Argyrophilic plaque-like deposits in children (1995). Acta Neuropathol 89: 42-49*. In 1996 I enrolled in North Carolina State University taking courses and rotating in different research laboratories of primary investigators. In 2000, however, I was officially enrolled in the Master of Science

graduate program in the nutritional sciences. I also gained valuable teacher education, leadership skills and personal confidence when I was selected as a graduate teaching assistant in the biological sciences interdepartmental program during my Master's work.

My Master of Science studies during which I have been able to study the nutritional sciences in depth using a porcine model, motivated by a desire for investigating the pathology of such an animal model in disease, has been one of the most important educational experiences of my life. Continuously gaining knowledge is a cornerstone of science and will likely be my life-long quest.

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Chapter 1

LITERATURE REVIEW

Lisa A. McPhatter

INTRODUCTION

The Norwalk group of viruses (NLV) or small round structured viruses (SRSVs), a name given to describe NLV genera, were recently classified as belonging to the family Caliciviridae. Their taxonomy, which was originally based on morphology, has been refined due to recent data based on genetic sequencing and phylogenetic analysis (Dingle et al., 1995; Jiang et al., 1993; Lambden et al., 1993; Liu et al., 1995). The NLV genus branches into two distinctive genogroups (GGI and GGII) based on genetic divergence in the polymerase and capsid regions (Ando et al., 2000). Norwalk and Southampton viruses are reference strains included in GGI. A third genogroup (GGIII), contains animal-infecting NLV.

Norwalk virus infection is a major cause of epidemic non-bacterial gastroenteritis in humans (Graham et al., 1994), appearing in either family or community-wide outbreaks. It was also the first viral agent identified as a cause of gastroenteritis and humans do not possess the ability to develop long-term immunity. Individuals most susceptible to the virus are infants and young children, the elderly, and those with compromised immune systems. The Norwalk virus was first detected during an outbreak of epidemic gastroenteritis in an elementary school in Norwalk, Ohio, 1968. During this outbreak, students and teachers alike, became ill and the virus was spread to family contacts (Adler et al., 1969). Gastroenteritis is an important public health concern in both developed and developing countries and can be clinically debilitating and result in economic losses as a result of reduced ability to work. According to the

Centers for Disease Control and Prevention, an estimated 180,000 people a year become infected with a Norwalk-like virus, thus developing gastroenteritis. This inflammation of the mucous membranes involving the stomach and intestines has a distinct seasonality. In a review by Mounts et al., (2000), NLV is predominately seen during colder months of the year. Zahorsky, (1929), described viral gastroenteritis as a 'winter vomiting disease'.

Norwalk viruses replicate in the intestinal tract of the infected host and are excreted in the feces. They are transmitted via several routes that include person-to-person transmission, foodborne transmission, and waterborne transmission. With foodborne transmission, human caliciviruses typically cause outbreaks via contaminated products eaten raw such as fruits and vegetables used in salads or products not cooked after handling like sandwich fillings. Person-to-person transmission has two routes, fecal-oral and aerosol formation following projectile vomiting. Person-to-person spread is the most common mode of transmission in outbreaks. In an investigation by Becker et al., (2000), Norwalk virus was documented as being spread via person-to-person transmission among several players on North Carolina and Florida football teams presenting with acute diarrhea and vomiting. Because caliciviruses can survive the pH variations and digestive enzymes present in the human gastrointestinal tract, they are considered highly environmentally stable and are able to withstand a wide variety of food processing conditions. Therefore, a wide range of different types of foods can serve as vehicles for transmission. For example, an

international gastroenteritis outbreak could be traced to raspberries from Slovenia (Loopman et al., 2002).

Caliciviruses are single-stranded RNA viruses. Investigation has been difficult because the virus cannot be amplified by *in vitro* cell culture or animal models and electron microscopy (EM) is often not sensitive enough to detect virus in stool samples (Loopman et al., 2002). Recent advances in molecular diagnostic techniques such as reverse transcription-polymerase chain reaction (RT-PCR), as well as advances in molecular engineering techniques involving the expression of an NLV baculovirus system, have both increased sensitivity in viral detection and highlighted the clinical and public health importance of caliciviruses.

A History of Human Calicivirus

During the 1940s and 1950s, it was assumed by exclusion from other antigens that viruses were responsible for a major portion of diarrheal illnesses. However, because these presumed viral etiologic agents could not be recovered and propagated *in vitro*, volunteer studies were initiated to determine whether bacteria-free stool filtrates derived from outbreaks of gastroenteritis were capable of reproducing the illness.

In several human volunteer studies spanning the years 1945 through 1957, enteric illnesses were induced in volunteers both by aerosolization and oral administration of bacteria-free fecal suspensions. The induction of an afebrile diarrheal illness in volunteers by oral administration of bacteria-free fecal filtrates or throat washings obtained from patients with epidemic gastroenteritis was

described. This infectious inoculum was taken from pooled diarrheal stools obtained from two individuals who became ill during an outbreak of gastroenteritis at Marcy State Hospital, Utica, New York. This agent, designated the Marcy strain was successfully passaged serially seven additional times in volunteers, indicating that it had multiplied in vivo and was not merely a filterable toxin (Fields Virology). In a report by Gordon et al., (1947), clinical studies where volunteers were exposed to fecal extracts that had been filtered to remove all bacteria, confirmed the hypothesis that a viral agent was the likely cause.

Twenty years later, in the autumn of 1968, fifty percent of students and teachers at an elementary school in the town of Norwalk, Ohio were struck with an illness characterized principally by nausea, vomiting, and abdominal cramps (Adler et al., 1969). During this outbreak thirty-two percent of family contacts of primary cases fell ill. The average incubation time lasted approximately 48 hours. 'Norwalk agent', named after its place of discovery, was characterized using passage of agent through volunteers. A small particle, less than 36 nm diameter and having resistance to ether, acid, and moderate heating (Dolin et al., 1971, 1972), was revealed. However, all attempts to culture the agent in organ cells or to transmit it to an animal other than humans were unsuccessful (Dolin et al., 1971). In 1972, after extensive attempts by a number of researchers (Dolin et al., 1972; Jordan et al., 1953), Kapikian et al., (1972), found a 27nm virus particle by immune electron microscopy (IEM).

Epidemiology

In the United States, acute gastroenteritis is one of the most commonly noted illnesses on hospital discharge records and death certificates, yet few of these have an etiologic diagnosis. The application of new molecular diagnostic methods has shown caliciviruses (referred to as the Norwalk family of viruses or small round structured viruses) to be the most common cause of acute gastroenteritis (AGE) outbreaks in the United States, and they may emerge as a common cause of sporadic cases of AGE among both children and adults (Glass et al., 2000). Surveys in the United States suggest that nearly every American will have an average of about 1 episode of gastroenteritis each year (Mead et al., 1999), and the rate is higher for children <5 years of age and for the elderly. From the 250-350 million episodes that occur each year, ~450,000 adults and 160,000 children are hospitalized (Jin et al., 1996 and Lew et al., 1991), resulting in >4000 deaths. Berne et al. (1992), reported that the 140 million children born each year will experience, on average, 7 to 30 episodes of diarrhea before they reach the age of 5.

Diarrhea has been considered primarily a problem of poor children in less developed countries where hygiene is poor, sanitation is inadequate, and water is contaminated. Nonetheless, children in developed countries with clean water and food and good sanitation often become ill with diarrhea as well, though with less incidence of fatality (Gangarosa et al., 1985 and Ho et al., 1988). Monroe et al. (2000), wrote that in more developed countries, mortality is rare, but it is nonetheless an important cause of morbidity and economic loss. In

England and Wales, one out of every five people has a case of Infectious Intestinal Disease (IID) annually (Wheeler et al., 1999) and there are over 300 deaths and 35,000 hospital admissions every year (Djuretic et al., 1996). In the Netherlands, the incidence of gastrointestinal diseases was also found to be very high, with 283 episodes per 1000 person-years (de Wit et al., 2001). The burden of illness is highest in the young and elderly (Hedlund et al., 2000; Wheeler et al., 1999). In two Swedish studies conducted in the 1980s, potential enteropathogens were identified in 68% of children and 38% of adults with gastroenteritis (Svenungsson et al., 2000). In a study involving the epidemiology of calicivirus infections in Sweden, Hedlund et al. (2000) reported that usually Norwalk-like viruses (NLVs) were found in 407 (89%) of 455 outbreaks. The high incidence of enteric virus-related infections is also a threat among acute diarrheic patients in Jakarta, Indonesia (Subekti et al., 2002).

Norwalk-like viruses (NLV) are important economically as a cause of both sporadic gastroenteritis in the community and large outbreaks in hospitals and other institutional settings (Hale et al., 2000). Calicivirus infections typically come to the attention of the health care system as a result of outbreaks in settings such as nursing homes, hospitals, schools, cruise ships, and restaurants (Djuretic et al., 1996; Le Guyader et al., 2000). Because living quarters in both nursing homes and hospitals are confined and personal hygiene may become compromised as a result of frail health and incontinence, transmission by way of person-to-person is particularly problematic. Since illness will strike the elderly or those already ill, human calicivirus is more likely to cause severe illness or death

in these settings (Centers for Disease Control and Prevention, 2001). The virus can be transmitted by food or water, airborne droplets, or person-to-person contact (Hedberg and Osterholm, 1993; Kapikian et al., 1996; Maguire et al., 1999; Koopmans et al., 2000). It is for this reason that NLVs pose a particular health risk in hospitals and residential homes and nursing homes. NLVs were detected by EM in fecal specimens from 706 outbreaks of non-bacterial gastroenteritis in residential institutions (Hale et al., 2000).

Norwalk virus infection is the epidemiologic prototype for outbreaks of food-borne and waterborne gastroenteritis (Hedberg et al., 1993). Every year, in the United States food-borne infections cause millions of illnesses and thousands of deaths; most infections go undiagnosed and unreported (Tauxe 1997). Mead et al., (1999), estimated that food-borne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year. Known pathogens account for an estimated 14 million illnesses, 60,000 hospitalizations, and 1,800 deaths. For instance, during the course of one week in August 1982, four outbreaks of acute gastroenteritis associated with separate social events were reported to state and local public health officials in Minnesota. Epidemiologic investigation of each outbreak implicated cakes from a single bakery as the source of the outbreak. All implicated cakes used frosting made by one bakery worker who was ill with vomiting and diarrhea during his work shift. Immune electron microscopy of stool samples from outbreak-associated cases revealed 27nm virus particles. Infection with Norwalk virus was confirmed by serology (Deneen et al., 2000).

Around the world, Norwalk virus and Norwalk-like viruses appear to be major causes of food-borne and waterborne illness (Hedberg et al., 1993). Ponka et al., (1999), reported an outbreak of gastroenteritis among employees in a large company in Helsinki Finland. Their data suggested that the primary source of the outbreak was due to imported frozen raspberries contaminated by calicivirus. In a subanalysis, 108 (53%) of the employees met with the case definition of gastroenteritis, presenting with diarrhea and/or vomiting.

Epidemiologic methods

Prior to the discovery of the Norwalk agent by Kapikian et al., (1972), laboratory confirmation for a vast majority of infectious outbreaks had not been possible. This was because of the relative insensitivity of EM such that the etiology of most outbreaks could not be confirmed. Presently, the etiology of many outbreaks remain unconfirmed because sensitive immuno- and molecular diagnostics are only available to reference laboratories and many times, samples are not appropriately collected. Furthermore, the spreading of human calicivirus can be rapid. Thus, implementation of infection control measures often can not await diagnosis in the laboratory. In this absence of diagnosis, epidemiologic traits must be employed to identify a viral cause. Criteria include negative stool cultures for bacterial pathogens, a mean incubation period of 24-48 hours, a mean duration time of 12-60 hours, and vomiting in greater than fifty percent of cases (Kaplan et al., 1982). Hedberg and Osterholm, (1993), proposed that the criteria be extended to include increased frequency of vomiting relative to fever instead of vomiting which occurs in greater than >50% of cases. Though this

method of assessment can not be used for determining a specific NLV agent, it can be utilized as a public health tool for assessing the overall burden of viral gastroenteritis.

Immunity

The immune system is a complex organization of cells and molecules with specialized roles that protect against infections by microbes such as bacteria, viruses, fungi, and parasites. It exists to distinguish between self and non-self. When accosted by an infectious agent, the immune system will mount two fundamentally different types of responses, one being a nonspecific innate or natural immune response whereby protection is achieved in the absence of cellular recognition; and the second, a specific immune response dependent upon specific recognition by lymphocytes. The specific immune response is acquired or adaptive immunity that depends on specific recognition by lymphocytes of foreign (non-self) substances (antigen). Molecules recognized by receptors on lymphocytes are generically referred to as antigens (Delves et al., 2000). This response mounted against the antigen is specific for that antigen. For example, a lymphocyte that recognizes an influenza viral antigen will not recognize leprosy antigen. Lymphocytes differentiate into effector cells known as B cell and T cells. B cells secrete immunoglobulins, the antigen-specific antibodies responsible for eliminating extracellular microorganisms. In contrast, T cells help B cells make antibody and can also eradicate intracellular pathogens by activating macrophages and by killing virus infected cells (Delves et al., 2000)

Because only a few lymphocytes are specific for a given antigen, T cells and B cells need to migrate throughout the body to increase the probability that they will encounter that particular antigen. In their travels, lymphocytes spend only about 30 minutes in the blood during each cycle around the body (Pabst et al., 1998). Immune responses to blood-borne antigens are usually in the spleen, and responses to microorganisms in tissues are generated in local lymph nodes, but most pathogens are encountered after they are inhaled or ingested (Delves et al., 2000). Antigens entering the body through mucosal surfaces activate cells in the mucosa-associated lymphoid tissues (Delves et al., 2000). Antigens from the gut are taken up by specialized epithelial cells, the microfold (or M) cells (E Pringault., 1999). These cells transport the antigen across the epithelium to Peyer's patches, the chief sites for the induction of mucosal responses to ingested antigen (Delves et al., 2000).

The pig represents a valuable experimental model for the study of mucosal immune response and lymphocyte migration (Binnes et al., 1988). Saif et al. (1996), used the gnotobiotic piglet as a model for studies of disease pathogenesis and immunity and found the results of these studies indicated that the magnitude of the immune response was greatest in lymphoid tissues adjacent to the local site of viral replication in the small intestine. In addition, they reported a direct correlation between the degree of protection induced and the level of the intestinal immune response. For instance, significantly higher local immune responses and complete protection were induced only after primary exposure to disease by heterologous rotavirus, including human rotavirus under

experimental conditions. Brandtzaeg et al. (1999), reported that after an immune response is induced in Peyer's patches, the lymphocytes enter the blood and travel to mucosal effector sites, such as lamina propria, where large amounts of secretory IgA are produced. VanCott et al.(1994), described a model of mucosal immunity in pigs which compares two antigenically-related porcine coronaviruses that replicate at distinct mucosal sites. Their results suggested that a virus-specific IgG- secreting cell precursor derived in the bronchus-associated lymphoid tissues of porcine respiratory-primed pigs may migrate in the gut in response to transmissible gastroenteritis virus challenge, and contribute to the partial protection observed. Also, the presence of IgA antibody-secreting cells in the gut lamina propria of transmissible gastroenteritis virus-primed pigs at the time of the challenge correlated with complete protection against transmissible gastroenteritis virus challenge.

Monroe et al., (1993) described a recombinant-expressed Norwalk virus (rNV) capsid protein in enzyme immunoassays to quantitatively measure immunoglobulins IgG and IgA to Norwalk virus in serum from patients involved in outbreaks of gastroenteritis and found NLV to induce both. As reported by Ball et al., (1998), nonreplicating rNV virus like particles are immunogenic when administered orally in the absence of any delivery system or mucosal adjuvant, demonstrating that rNV-like particles are an excellent model to study the oral delivery of antigen and may provide a potential mucosal vaccine for Norwalk virus infections.

In a study by To et al., (1998) involving human rotavirus in gnotobiotic pigs, antibodies to human rotavirus such as IgA and IgG were detected in serum and intestinal contents of pigs of all groups after virus inoculation or challenge and the antibody titers in intestinal contents showed similar kinetics to the serum responses. A positive correlation was found to exist between protection and serum IgA. Intestinal IgA and intestinal IgG antibody titers denoting that serum IgA antibodies to human rotavirus could act as an indicator of IgA antibodies in the intestine after rotavirus infection.

Seroprevalence methods

Several methods for detecting antibody (Ab) to human calicivirus have been developed to allow for the screening of large numbers of specimens (Atmar and Estes, 2001). The immune adherence hemagglutination assay (IAHA), was among the first. In this methodology, purified viral particles from stool specimens are used as antigen (Ag), and form antigen-antibody complement interactions that are detected on a microtiter plate (Greenberg and Kapikian, 1978).

Another assay known as the blocking radio-immunoassay (RIA) rapidly replaced IAHA (Brandt et al., 1981; Nakata et al., 1983) because it requires less antigen and is more sensitive (Greenberg and Kapikian., 1978). Nakata et al., (1988), converted the RIA into the more widely applicable enzyme immunoassay (EIA). However, these techniques are limited in use by diagnostic and public health laboratories because required reagents can not be derived from non-human sources (Jiang et al., 2000).

A number of sero-epidemiological studies have been conducted worldwide using antibody-detecting EIAs because these assays, when designed to detect human calicivirus, are broadly reactive. Caul, 1996; Jiang et al., 2000) stated that serologically-based assays are not likely to be used as a diagnostic tool since it is difficult to discern if antibody presence is from a current or previous infection.

Jiang et al. (1992), reported that difficulties in sero-epidemiological studies involving human caliciviruses have largely been overcome by the advent of VLV baculovirus capsid protein expression systems. In 2000, they also reported EIAs had been designed to detect antibodies (Abs) to the prototype Norwalk virus as well as eight other human calicivirus strains (Jiang et al., 2000). These Ab assays, which are based on recombinant virus-like protein (VLP), are more widely reactive than antigen (Ag) tests (Gray et al., 1994; Jiang et al., 1992; Okhuysen et al., 1995; Treanor et al., 1988). Therefore, antibody (Ab) assays have been used in a number of studies which have concluded that human caliciviruses are extremely common worldwide (Black et al., 1982; Chiba et al., 1980; Cubitt et al., 1998; Greenberg et al., 1979; Jing et al., 2000; O’Ryan et al., 1998; Parker et al., 1994; Smit et al., 1999).

Molecular Biology of Caliciviruses

Typical caliciviruses (CVs) have a characteristic “Star of David” appearance by electron microscopy (M. Sugieda et al., 1998). The caliciviruses take their name from the characteristic cup-shape depressions on the virions when viewed by negative stain electron microscopy (Clarke et al., 1997). Lui et al. (1999),

described caliciviruses as being approximately 30 to 35 nm in diameter and having an amorphous structure with a ragged edge. Guo et al. (2001), described caliciviruses as small, nonenveloped viruses 27 to 38 nm in diameter and possessing a single-stranded, plus-sense RNA genome of 7.3 to 8.3 kb and a single capsid protein of 56 to 71 kDa. Research reported by Guo et al. (1999) detailed caliciviruses as small, nonenveloped viruses that possess a single-stranded, plus-sense genomic RNA that is 7 to 8 kb in length and that encode a single structural protein of 58 to 80 kDa and a polyprotein that contains motifs indicative of a putative 2C helicase, 3C-like protease, and RNA-dependent RNA polymerase 3D.

Members of the caliciviridae possess a 7.5 RNA genome with a characteristic arrangement of open reading frames (ORFs) which clearly differentiates them from members of other viridae (Clarke et al., 1997). The NV genome, a positive-sense, single-stranded RNA molecule approximately 7.7kb in length, is predicted to contain three open reading frames (ORFs). The first and third ORFs are in reading frame 2 of the cDNA, while ORF 2 is in reading frame 3 (Glass et al., 2000). In addition, the calicivirus nonstructural proteins apparently are encoded at the 5' end, while the structural proteins are at the 3' end of the genome. A polyprotein that undergoes post translational cleavage is predicted for the calicivirus nonstructural proteins. Several replication strategies may be used for the synthesis of the single calicivirus capsid protein (Jiang et al., 1993). Dunham et al.(1998) identified a primate calicivirus (Pan-1) VPg as a viral protein that is covalently attached to the 5' end of the genome of many positive- sense,

single-stranded RNA viruses, including picornaviruses, caliciviruses (CVs) and many plant RNA viruses.

Venkataram et al. (1999) reported the first x-ray structure of a calicivirus capsid, which consists of 180 copies of a single protein. The capsid protein has a protruding (P) domain connected by a flexible hinge to a shell (S) domain that has a classical eight-stranded β -sandwich motif. The structure of the (P) domain is unlike that of any other viral protein with a subdomain exhibiting a fold similar to that of the second domain in the eukaryotic translation elongation factor-Tu. This subdomain, positioned at the exterior of the capsid has the largest sequence variation among Norwalk-like human caliciviruses and is unlikely to contain the determinants of strain specificity and cell binding.

Norwalk virus and Norwalk antigen have been purified from fecal specimens obtained from infected volunteers (Fields Virology). These purified materials immunoprecipitate by convalescent-phase serum antibodies, after dissociation were examined by polyacrylamide gel electrophoresis. A single virion-associated protein and a single soluble protein have estimated molecular weights of 59,000 and 30,000, daltons respectively. Porcine enteric viruses have been characterized. Purwani et al., (1999), reported the molecular weight and antigenicity of proteins of a porcine enteric calicivirus (PEC). The PEC virions were purified from intestinal contents of infected pigs and from infected cell culture lysates. The average buoyant density of the purified virus was 1.37 gm/cm³ in cesium chloride. One major structural protein with a molecular weight of approximately 58 kD was found in the gut and cell culture-passaged PEC,

using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Purwani et al., 1999).

In spite of extensive research, the study of the molecular biology of NV has been hampered by a lack of cell culture and suitable small animal models. While chimpanzees inoculated with NV developed serologic responses, infection was usually asymptomatic (Tamura et al., 2000). Therefore, physicochemical studies of NV have only been carried out with virions obtained from clinical stool specimens of human patients (Tamura et al., 2000). Some physicochemical properties of the Norwalk virus include a buoyant density ranging from 1.36 to 1.41g/cm³. The virus also exhibits stability in that it can retain infectivity upon exposure to a pH of 2.7 and treatment with 20% ether. Chlorine concentrations of 3.75 to 6.25mg, which are similar to those present in drinking water distribution systems, do not inactivate the Norwalk virus. The virus is however not resistant to a chlorine concentration of 10mg/L, an amount used to treat water supply systems upon viral infection (Fields Virology).

Molecular Detection ***Diagnostics for Calicivirus Infection***

Electron microscopy

Since a cell culture system for human calicivirus has not been developed, electron microscopy (EM), has been a fundamental tool used by investigators. In the United Kingdom for example, a reporting network of the EM units of the diagnostic public health laboratories has been the basis of the national surveillance system (Caul, 1996). Highly skilled microscopists are required to reliably detect human caliciviruses from prepared stool samples (Kapikian, 1994).

Specimens are prepared for EM by a fairly simple and inexpensive negative staining technique (Doane, 1994), however direct detection of human caliciviruses by EM is only possible in samples with a high viral load. EM is too insensitive to detect human caliciviruses in samples with less than 10^6 particles per ml (Doane, 1994). Caul and Appleton (1982), reported that these enteric viruses can only be detected for approximately 48 hours after disease symptoms cease.

In immunoelectron microscopy (IEM), stool samples are visualized after reaction with antibody derived from convalescent phase sera from infected individuals with gastroenteritis (Atmar and Estes, 2001). Antigen and antibody form immune complexes, which can be negatively stained (Doane, 1994). IEM was used by Kapikian et al., (1972) in their discovery of the Norwalk agent. Although IEM's use as a diagnostic tool has been limited by the lack of defined antisera, it has been used to characterize other human caliciviruses (Dolin et al., 1982; Thornhill et al., 1977; Vial et al., 1990).

Immunoassays

The design of a baculovirus expression system for Norwalk viral capsid proteins by Jiang et al.(1992) provided a means of harvesting large amounts of virus particles. These particles were similar in antigenicity and morphology to native viruses (Green et al., 1993). In a discussion by Jiang et al.(1992) antibodies for EIA were generated by exposure of animals to the engineered Norwalk virus proteins (rNV). Recombinant antigens and EIAs have been designed for other NLV variants such as Southhampton, Snow Mountain, and

Lordsdale viruses (Atmar and Estes, 2001). Antigen-detecting EIAs using monoclonal antibodies or native baculovirus expressed proteins have also been prepared for a number of NLVs (Hardy et al., 1996; Herrman et al., 1995). While these assays are highly sensitive, they have a limited diagnostic laboratory use due to their narrow range of specificity. Antigen-detecting EIAs will only detect a narrow range of related human caliciviruses, not the majority of viruses that are genetically diverse.

RT-PCR

Amplification of genome sequences of the Norwalk virus by reverse transcription-polymerase chain reaction (RT-PCR) was first reported by Jiang et al., (1992) and De Leon et al. (1992). In the RT-PCR method, mRNA is converted to DNA using a reverse transcriptase; the mRNA is reverse transcribed to make a single-stranded DNA. Once this occurs, a primer is used to convert the single-stranded DNA to a nucleic acid that is double-stranded. Standard PCR is then used to amplify the cDNA made. RT-PCR has become a common diagnostic and research tool worldwide. Primers are small pieces of RNA that provide the free end needed for DNA replication to begin. The complete sequencing of a range of human caliciviruses has led to the development of many primer pairs for use in RT-PCR (Dingle et al., 1995; Jiang et al., 1993; Lamben et al., 1993). In a statement by Parashar et al. (1998) and Yamazaki et al. (1996), RT-PCR, compared to EM, is a far more sensitive diagnostic tool, able to detect virus 2 weeks after infection and possibly longer. Due to the genetic diversity among Caliciviridae, it has been difficult to find an

appropriately sensitive and specific primer pair to detect all NLVs. A number of primer pairs for the most conserved region of the genome, the RNA polymerase gene, have been designed and the ones described by Ando et al. (1995), Greene et al. (1995), Vinje and Koopmans (1996) and Le Guyader et al. (1996) are among those most commonly used (Atmar and Estes, 2001). The helicase, capsid and open reading frame regions of the virus genome have also been used as a target for RT-PCR (De Leon et al., 1992; Green et al., 2000; Vinje et al., 2000; Yamazaki et al., 1996), and analysis of more than one region may be important in the detection of unique or recombinant strains (Vinje et al., 2000). It is likely that sensitivity of detection of the RT-PCR assay varies substantially for different genotypes, though there are no reports in the literature of a full evaluation of this assay against the full range of genotypes that currently exist.

Nested RT-PCR

The polymerase chain reaction (PCR) is a simple and elegant technique that amplifies a region of DNA using primers that flank the region and repeated cycles of DNA polymerase action. Nested RT-PCR is a variation on PCR that has been used to further increase sensitivity. Green et al. (1998) demonstrated that by using two rounds of PCR, with the second set of primers 'nested' within the region captured by the first round, sensitivity can be increased 10 to 1000 times and the assay can also have a greater specificity. They also cautioned that though nested RT-PCR may have great application to environmental and foodstuff surveys where virus is found in smaller amounts, there lies a risk of contamination that may result in false positive data.

Electrophoresis

Electrophoresis involves applying voltage to charged molecules, inducing them to migrate. This technique is used to separate DNA fragments, RNAs, or proteins. It is also used to confirm and further analyse RT-PCR products.

Running products on electrophoresis gel is a simple means of visualizing whether DNA products of predicted length have been amplified (Green et al., 1998). A caution here is that non-specific DNA amplification will produce other bands on the gel, thus making results difficult to interpret.

Hybridization assays

Hybridization assays are also used to confirm RT-PCR analysis. There are a number of hybridization assays such as dot, slot, liquid, and Southern hybridization, where a labeled, virus-specific probe or a piece of nucleic acid is hybridized with the PCR product. NLVs have such genetic diversity that there is not a single broadly reactive probe, but a small panel can of probes usually capture the majority of circulating strains (Ando et al., 1995; Green et al., 1995; Le Guyader et al., 1996; Vinje and Koopmans 1996). The Southern hybridization assay is widely employed by molecular experimenters, discovered by Edward Southern, who pioneered this technique by transferring or blotting DNA fragments from an agarose gel to nitrocellulose by diffusion (Weaver).

Reverse line blot (RLB)

An enhanced application of the Southern hybridization principle is the reverse line blot or RLB. Vinje and Koopman. (2000) reported that the reverse line blot

technique makes use of a set of genotype-specific probes simultaneously, thus providing both confirmation of nucleic acid and genotyping information.

Heteroduplex mobility assay (HMA)

Another method of typing PCR amplicons is the heteroduplex mobility assay (HMA). Mattick et al. (2000) stated that HMA is a useful screening device that can serve to screen for commonly circulating viral strains as well as discern them from others, but in its current format it, runs the risk of not detecting virus if there is a change in the prevalent genotype.

Both HMA and RLB techniques are useful in processing multiple samples simultaneously, and can therefore be utilized in large-scale epidemiological studies.

DNA sequencing

The DNA sequencing method is used in order to determine the exact base sequence of a defined piece of DNA. DNA sequencing of amplicons, while expensive and labor-intensive, offers the most virus-specific information (Fankhauser et al., 1998; Kukkula et al., 1999; Vinje et al., 2000; Maunula et al., 2000; Yamazaki et al., 1996). For example, sequences can be compared with those from other samples as a means of providing information about the common source of an outbreak. However, since relatively few complete NLV genomes have been sequenced, and genetic recombination can occur here, a great deal remains to be learned about the use of genomic sequence information in regards to molecular epidemiology of the NLV's.

(Atmar and Estes, 2001; Vinje et al., 2000).

Molecular Detection

RT-PCR in Research

Jiang et al. (1992) reported that current diagnosis of Norwalk virus infection relies on immunologic tests, including immune electron microscopy, radioimmunoassay (RIAs), immune adherence hemagglutinin assays, and enzyme immunoassays. However, these tests use human convalescent-phase serum from patients, are low in sensitivity due to low affinity of antibodies for the virus, and sources of serum are limited. Moe et al. (1994) discussed investigations of SRSV outbreaks and the characterization of this group of viruses as having been limited by the lack of adequate laboratory techniques that detect these agents. Direct electron microscopy is often not sensitive enough to detect SRSVs, which are usually shed in low concentrations (Ando et al 1995). Radioimmunoassay, enzyme-linked immunosorbent assay (ELISA) and solid phase immune electron microscopy (SPIEM) to detect SRSVs in stool samples are not routinely performed because the reagents used in these methods are derived from nonreplenishable clinical samples (Ando et al 1995).

Recently, the entire genome of NV was cloned and characterized. The availability of their sequences led many researchers to develop methods to detect SRSVs by reverse transcription (RT)-PCR (Ando et al 1995). A year earlier, Moe et al. (1994) also discussed the recent cloning and sequencing of the NV genome that led to its classification in the family Caliciviridae and how the utilization of such techniques has made it possible to detect NV and Norwalk-related virus from fecal specimens by reverse transcription (RT)-PCR. For example, during winter 1998-1999, 406 fecal samples were received from

patients with suspected calicivirus infection. Of these, 76 (19%) were calicivirus positive by a nested RT-PCR (Vainio et al., 2001). Using RT-PCR, Daniels et al. (2000) proved that an outbreak was the result of human calicivirus when they detected the virus sequence in stool of university students who ate from a sandwich bar. The recent success in cloning Norwalk virus (NV) genes and the production of a recombinant NV protein have resulted in the development of new methods for the study of NV and other small round structured viruses (SRSV), including morphologically typical HuCV's (human caliciviruses) (Kogawa et al. 1996). Norwalk virus and most, if not all, SRSVs have been shown to be caliciviruses which can be divided into two genogroups. An RT-PCR technique for detection of the predicted RNA-dependent RNA polymerase (RDRP) has been introduced to detect and analyze SRSV's (Kogawa et al 1996).

Methods incorporating reverse transcription-PCR (RT-PCR) amplification that target short segments of the viral genome have been developed for NLV detection from clinical and environmental samples, including water concentrates, shellfish, and stools (Schwab et al 2001). Molecular methods like reverse RT-PCR provide hope for rapid and sensitive detection of pathogenic enteric viruses in water at levels that could predict water safety (Huang et al 2000). Outbreaks have occurred due to municipal sewage and septic tank leaks as well as run off contamination into springs and streams (Hedburg and Osterholm., 1993). A Swiss investigation detected NLVs by RT-PCR in 21 of 63 bottles of commercially available mineral water (Beuret et al., 2000). In addition, RT-PCR

methods have been used to detect NLVs in bathing and recreational waters (Schvoerer et al., 2000).

RT-PCR is very specific and sensitive, enabling the detection of as few as 10 to 40 genomic copies of viral nucleic acid (Schwab et al 2001). Schwab et al. 1997 identified RT-PCR as extremely sensitive and specific, thus enabling epidemiological studies to identify NV and related viruses as the causative agents in outbreaks of gastroenteritis. Chung et al. (1996) hailed the nucleic amplification method for increasing the detection of noncytopathic human enteric viruses in oysters. Oyster outbreaks are frequently reported, though people who eat oysters often accept the illness that comes with them (Ang, 1998; Chalmers and McMillian, 1995; Dowell et al., 1995; Godoy et al., 2000; Gunn et al., 1982; Stafford et al., 1997). Alternative virus detection methods such as nucleic acid hybridization are not capable of detecting the low levels of virus contamination anticipated in shellfish (Chung et al 1996). However, virus detection methods based on in vitro enzymatic amplification of target nucleic acid sequences, such as PCR, provide great sensitivity as well as specificity (Chung et al 1996).

RT-PCR has been utilized to detect Norwalk-like virus genes in the cecum of pigs. Sugieda et al. (1998) reported positive PCR products from four out of 11 samples by nested PCR using human SRSV primers. Ouardani et al. (1999) reported PCR for detection and typing of Porcine coronaviruses. When tested with clinical samples from pigs, the results of the single PCR method showed nearly 93% (13 of 14 samples) correlation with histopathological and immunohistochemical findings. Kirkwood et al. (2001) identified caliciviruses in

32 of 60 stool specimens negative for other enteric pathogens from children hospitalized in Melbourne, Australia with acute gastroenteritis by using RT-PCR, demonstrating the sensitivity of this technique.

Pathogenesis

The media and some politicians would often have society believe that viruses and infectious diseases are unremitting evils. However, in a quote from Sportin' Life in Gerswin's *Porgy and Bess*, this "ain't necessarily so." A significant part of understanding mechanisms of biological processes can be credited in whole or in part, to the impact of infectious disease and research carried out on viruses. In essence, viruses are collections of genetic information directed toward one purpose: their own replication. They are the ultimate and prototypical example of "**selfish genes.**" The viral genome contains the blueprints for virus replication encrypted in the genetic code, and must be decoded by the molecular machinery of the cell that it infects to gain this end. Viruses are therefore, obligate intracellular parasites dependent on the metabolic and genetic functions of living cells (Wagner and Hewlett).

Virus replication in the cell involves a production-infection cycle. Critical events characterizing this cycle take place when the virus specifically interacts with the host cell surface, resulting in the introduction of the viral genome into the cell. Viral genes are then expressed using host cell processes. Viral proteins modify the host and allow the viral genome to replicate using host and viral enzymes. New viral coat proteins will then assemble themselves into capsids in

which the viral genomes are included. Marking the end of this infectious cycle, virus is released so it can infect new cells, and the events are repeated.

Norwalk virus is a positive sense, single-stranded RNA virus. By definition viruses with RNA genomes whose genome is the same sense as mRNA are called positive (+) –sense RNA viruses. This RNA viruses use RNA also serves as genetic material, thus necessitating novel strategies to facilitate replication in a eukaryotic cell whose genetic material is DNA. Single-stranded RNA virus genome replication requires two strategies. In the first input strand must be transcribed using Watson-Crick base-pairing rules into a strand with a complementary sequence and opposite polarity (Wagner and Hewlett).

Replication occurs as a multibranched structure containing molecules of viral transcriptase or replicase, partially synthesized RNA “nascent” strands, and genome-sense template strand. This is termed a type-1 ribonucleoprotein complex or replicative intermediate 1 (RI-1). Virion RNA serves as the template in RI-1 which produces template RNA of opposite sense to virion RNA.

RNA that is complementary to virion RNA is the template in a second replicative intermediate known as RI-2. RI-2 is the intermediate for expression of RNA that is of the same sense as the virion.

Piglets have been widely utilized as models to describe the effects of viral infection on a host. Flynn et al., (1988) reported statistically significant villus atrophy in the small bowel of four-day-old gnotobiotic piglets infected with porcine enteric calicivirus-like virus. In this study, infected enterocytes were observed in the duodenum and histopathologic findings revealed short, stump-like villi, long

crypts and rounded, bulging epithelial cells. This morphology is typically associated with diarrhea because of loss of absorptive cells on the villus, and proliferation of secretory cells within the crypts. Guo et al., (2001), intravenously inoculated the gnotobiotic pig with wild-type porcine enteric calicivirus and reported induction of diarrhea and intestinal lesions. Nabuurs (2000), found that after rotavirus infection in piglets, mortality, due to diarrhea, was associated with severe villus shortening and crypt deepening in the small intestine.

Gnotobiotic pigs orally inoculated with human rotavirus developed diarrhea that correlated with the presence of viral antigen in epithelial cells and villous atrophy that correlated with peak fecal viral titres (Ward et al., 1996).

Human caliciviruses or Norwalk-like virus (NLV) are acid-stable viruses, and can therefore pass through the stomach. Replication is thought to occur in the small intestine (Caul, 1996) however an animal model of NLV has not been produced, making it difficult to study molecular mechanisms of NLV-induced disease.

Much of the knowledge concerning the pathogenesis of human caliciviruses comes from human volunteer studies performed in the U.S., whereby individuals agreed to intestinal biopsy before and after exposure to the viral agents (Agus et al., 1973; Dolin et al., 1975; Shreiber et al., 1973, 1974). Light and electron microscopy revealed that individuals with clinical illness exhibited lesions on the small intestinal mucosa. The mucosa presented with an inflamed lining and absorptive epithelial cells showed abnormal morphology.

Also apparent were blunt villi, short microvilli, dilated endoplasmic reticulum, swollen mitochondria, and intracellular edema.

Clinical Features and Treatment

Norwalk viral infections usually cause nausea, vomiting, diarrhea, weakness, abdominal pain, loss of appetite, headache, and fever (Wardlaw and Kessel). Chills, myalgia and sore throat have also been reported. Vomiting occurs more frequently than diarrhea in children, whereas diarrhea was observed more frequently in adults. In a report on 28 outbreaks of the illness, the onset lasted anywhere from 2 hours to several days, with a mean and median of 12 and 60 hours respectively in 26 of the 28 outbreaks (Fields Virology). The illnesses ranged in severity from mild vomiting and diarrhea to severe disease with dehydration that could be fatal (Glass et al., 2000).

Treatment of Norwalk gastroenteritis includes oral fluid and electrolyte replacement therapy. Although new, potentially useful drugs such as acetorphan, an enkephalinase inhibitor shown to be effective in reducing by half the amount of stool output of 135 young children with acute diarrhea, the cornerstone of treatment remains a proper oral rehydration (Guandalini 2002). Oral rehydration solutions (ORS) are formulated to correct dehydration and acidosis (Nappert et al., 2000). Blikslager et al. (2001) discussed the use of oral glucose-containing electrolyte solutions capable of stimulating NaCl and water absorption. These could counteract the enterotoxigenic second messenger-mediated events of increased intestinal epithelial cAMP or cGMP that inhibit an electrically neutral NaCl absorptive mechanism and elicit anion secretion,

resulting in diarrhea. Although the glucose-linked Na^+ absorption on the small intestinal villus remains intact in enterotoxigenic diarrheal diseases such as enterotoxigenic *Escherichia coli*, there may be damage to villous absorptive cells expressing the Na^+ -glucose transporters during Norwalk virus infection. This could limit the efficacy of glucose-containing oral rehydration solutions in this disease. Nonetheless, these solutions continue to provide a mechanism to induce salt and water uptake during diarrheal disease.

In Hungary, Arato et al. (2001) reported the great importance of rapid rehydration over 3-4 hours with oral rehydration solution containing 60mmol/l sodium. In a study presented by Szajewska et al. (2000), 66% of European primary care physicians and hospital-based pediatricians reported following The European Society for Pediatric Gastroenterology, Hepatology and Nutrition recommendation to use oral rehydration solution containing 60mmol/mol, whereas 16% followed the guidelines and used rapid oral rehydration over 3 to 4 hours. Forty-five percent would rehydrate infants in a 3- to 6- hour period, and 17% would extend the rehydration period to 12 to 24 hours.

Oral rehydration therapy is used to treat dehydration caused by diarrhea, however the rehydration solution does not reduce stool loss or length of illness (Fontaine et al., 2000). This study set out to assess the effects of rice-based oral rehydration salts solution compared with glucose-based oral rehydration salts on reduction of stool output and duration of diarrhea in patients with acute watery diarrhea. In people with cholera, randomized trials were updated comparing standard World Health Organization oral rehydration solution with an

experimental oral rehydration salts solutions in which 20 grams of glucose per liter was replaced by 50-80 grams per liter of rice powder, with electrolytes remaining unchanged. Results showed mean stool outputs to be lower by 67 milliliters/kg of body weight in the first 24 hours for children given rice oral rehydration salts, and by 51 millilitres/kg of body weight in adults, suggesting that rice-base oral rehydration appears to be effective for treating diarrhea. Lima et al., identified glutamine as a potential candidate to supplement or replace glucose in oral rehydration and nutrition therapy because it is the principle source of energy for enterocytes and plays a role in nucleotide and protein synthesis.

With severe diarrhea or vomiting, parenteral administration of fluids may be necessary, though oral (ideally) or enteral (directly into the GI tract) nutrition is always preferable to parenteral (intravenous) nutrition because of its ability to preserve the GI mucosal integrity (Shulman and Philips). Recently, the application of molecular engineering techniques involving the expression of NLV proteins in baculovirus has provided a powerful approach to the diagnosis of human calicivirus infection and illness (Jiang et al., 2000).

Control and prevention

Specific methods are not available for the prevention or control of human enteric calicivirus or NLV illness. Because Norwalk agent is highly infectious, sanitary handling of food is an essential barrier to infectious transmission. The potential of foodborne contamination from vomitus was proven by the investigation of an outbreak at a hotel in North Yorkshire, England where a kitchen employee suddenly vomited in a sink (Patterson et al., 1997). The sink

was cleaned with a chlorine disinfectant, but still had sufficient NLV load to contaminate a potato salad prepared in the sink on the following day.

Kuritsky et al. (1984) reported a baker having had multiple episodes of vomiting and diarrhea on his way to work and during his shift. As part of the preparation of some 10,000 frosted food items, he submerged his arms in 76L of cream frosting, thus contaminating the batch. The bakery employee infected over 3000 people.

Aside from effective handwashing and sanitary handling of food, clean water supplies, and proper sewage disposal may also decrease transmission.

Concluding Remarks

Norwalk-like virus (NLV) agents are the most common cause of non-bacterial gastroenteritis and have become increasingly recognized as a force to be reckoned with in the public health arena. The NLV agent is an enteric pathogen that is frequently responsible for food borne viral infections, and second only to rotavirus as a cause of severe diarrhea in children and infants.

Key factors underlying the high burden of infection associated with NLVs include their low infectious dose and stability in the environment, the wide diversity of strains and a lack of long-term immunity to infection or illness.

Recent advances in development of sensitive molecular techniques have provided assays for diagnosis quantification, and characterization of NLV that have contributed to its significance in infection. Future applications involving these techniques may lead to approaches toward an understanding of the routes of transmission, and effectiveness of control measures as they relate to the

epidemiology of human caliciviral disease as well as providing insight into mucosal immunity.

We propose a piglet model to study the infectivity of Norwalk-like virus.

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Chapter 2

DEVELOPMENT OF A PORCINE MODEL TO STUDY CALICIVIRUS PATHOGENESIS.

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ABSTRACT

MCPHATTER, LISA. The Piglet as a Model of Norwalk Gastroenteritis. (Under the direction of Dr. Jack Odle)

Norwalk-like viruses (NLV) are the most important cause of acute gastroenteritis in humans. However, little is known about their pathogenesis since no cell culture or animal model is available yet. Therefore, we have investigated if young piglets would be a suitable model to study NLV pathogenesis. Sixteen ~21-d-old piglets were orally gavaged with 3 doses of the NLV prototype strain (Norwalk virus; strain 8FIIb) previously confirmed to be infective for human volunteers. Stool samples were collected daily to assess viral shedding. Exposure to Norwalk virus had no detectable effect on pig growth. Using RT-PCR, viral NV-RNA was detected in two (high dose) of the twelve pigs gavaged with virus. In a second study, colostrum-deprived newborn piglets were orally gavaged with either the human strain (8FIIb, n=4), swine calicivirus (n=6) or a saline control (n=5). Neither swine calicivirus nor Norwalk virus could be detected by RT-PCR in any stool samples. At day 4 post infection intestinal samples were collected for histological and biochemical evaluation. Differences in diarrhea score and intestinal lactase activity were not detected, but weight gain of pigs infected with the swine calicivirus (158 g/d) was lower ($P<0.05$) than control pigs (280 g/d). In addition, there was a 49% reduction in ileal villus height in the swine calicivirus group ($P<0.05$). These results are consistent with swine calicivirus-induced villous atrophy, and attendant reduction in nutrient absorption.

INTRODUCTION

Norwalk Virus (NV) is the prototype strain of a group of human enteric pathogens recently classified as caliciviruses (Jiang et al., 1993; Lamden et al., 1993) and was named in 1972 as the result of a discovery of the virus in fecal specimens during a gastroenteric outbreak at an elementary school in Norwalk, Ohio (Adler et al., 1969; Kapikian et al., 1972). The group has emerged as the single most common cause of outbreaks of acute non-bacterial gastroenteritis in the United States and many other countries, striking people of all age groups (Djuretic et al., 1996; Green et al., 1997; Vinje et al., 1997) and humans do not appear to possess the ability to develop long-term immunity. It is now estimated that as many as 95% of non-bacterial gastroenteritis outbreaks are caused by Norwalk-like human caliciviruses (Green, 1997). Epidemics of diarrheal disease caused by human calicivirus infection occur in schools, communities, recreational facilities, day care centers, and nursing homes and among travelers and members of the military (Cukor et al., 1984; Sharpe et al., 1995; Kapikian et al., 1996). During Operation Desert Storm, an outbreak of NLV infection had an adverse impact on military operations (Sharpe et al., 1995). Furthermore, these viruses may be responsible for gastroenteritis episodes that number in the millions per year in individual countries (Green, 2000) and can be clinically debilitating and result in economic losses because of reduced ability to work. They are spread by ingestion of contaminated food or water and by secondary person-to-person transmission (Estes et al., 2000). Norwalk-like calicivirus has a small amorphous structure (27 to 38 nm in diameter) that is round, nonenveloped

and with a ragged edge (Lui et al., 1999). The virion possesses a single-stranded, plus-sense RNA genome of 7.3 to 8.3 kb and a single capsid protein of 56 to 71 kDa (Guo et al., 2001). The NLV group branches into two distinctive genogroups (GG1 and GG11) based on genetic divergence in the polymerase and capsid regions. The Norwalk virus is a reference strain included in GG1 (Ando et al., 1995).

Norwalk-like viruses replicate in the intestinal tract of the infected host and are excreted in the feces. Epidemic non-bacterial gastroenteritis was described for the first time in 1929, by Zahorsky, who proposed the descriptive name, winter vomiting disease, since the illness was characterized by violent forcible vomiting, diarrhea, and prostration and occurred most often in the winter (Andler et al., 1969). In the early 1990s, cloning and sequencing of the Norwalk virus revolutionized the study of the human calicivirus (Monroe et al., 2000). Although Norwalk virus was visualized more than twenty years ago, progress in understanding the molecular biology and replication strategies of NLV has been hampered by the lack of a cell culture system or an animal model (White et al., 1996).

Animal models offer valuable means to examine the infectivity and transmission routes of human pathogens and the piglet presents a number of advantages over other animal models because it closely resembles humans in physiology (Yuan et al., 1996). The piglet neonate and human infant have many similarities in their gastrointestinal physiology, milk diets, and mucosal immune development (Yuan et al., 1996). Furthermore, piglets have widely been utilized

as models to describe the effects of viral infection in the host. Flynn et al. (1988) reported statistically significant villus atrophy in the small bowel of four-day-old gnotobiotic piglets infected with a porcine enteric calicivirus-like virus.

Similarity of the porcine calicivirus-like virus and human caliciviruses in terms of infectivity or environmental stability is not known, but having an animal model to study the infectivity of human strains would be significant. Additionally, the piglet model could potentially be used as a tool for the development of a bioassay to evaluate the infectivity of NLV under food conditions and food treatment processes as well as investigating strategies in vaccine development.

We hypothesized that the piglet would develop acute gastroenteritis when exposed to caliciviral inoculants.

Materials and Methods

Subjects and experimental procedures

Trial 1. Sixteen 21-day-old Yorkshire cross-breed pigs of both sexes were randomly assigned to 4 treatment groups (n=4 pigs/trt) and orally gavaged with human calicivirus strain (Norwalk 8FIIb) prepared as three dosages: low (3×10^7 PCR amplifiable units), medium (9×10^7 PCR amplifiable units), high (3×10^8 PCR amplifiable units), and a non-inoculated sodium bicarbonate control. Animals were fasted overnight immediately prior to inoculation and were otherwise fed standard nursery diets. Stool was collected daily from all piglets to assess viral shedding via RT-PCR. Diarrhea was scored on a range of 1-5 (a score of five being the most severe).

Trial 2. In a second study, colostrum-deprived newborn piglets of both sexes and of the PIC genotype were delivered by C-section and adjusted to milk

formula for 2 days. Piglets were subsequently fasted overnight and orally gavaged with either a human caliciviral strain (Norwalk 8FIIb, n=4 pigs); a purported swine caliciviral preparation (n=6 pigs); or an uninfected saline control (n= 5 pigs). Following gavage, animal feed intake and growth was monitored daily. Feces were collected daily for assessment of viral shedding (via RT-PCR) and scored for fluidity on a scale of 1-5 (with 5 being most fluid). At day four post-gavage, duodenal, jejunal, and ileal intestinal samples were collected for measurement of histological and biochemical parameters.

VIRUS

The viral inoculum (8FIIb) was prepared from stool obtained from a Norwalk-infected human volunteer. A 2% stool suspension was prepared in an infusion broth containing 0.5% BSA then was trichloro-trifluoromethane (Freon)-extracted three times and filtered through a 1.2µm filter, and tested to ensure that it was free of known microbial agents. Porcine calicivirus was provided as a gift from J. Vinje at the University of North Carolina at Chapel Hill, Chapel Hill NC (van der Poel et al., 2000).

RT-PCR

The G1 & G2 primer system and protocol were used in this study. Location of the primer sets are shown in Fig. 1. Two sets of 21nt primer pairs were designed on the basis of conserved sequences in viral RNA polymerase region of 22 different strains of SRSVs (Ando et al., 1995). The G1 group includes Norwalk, Southhampton and UK2 viruses with pooled 5' primers, while the G2 group includes UK3 and UK4 viruses with one 5' primer. This primer

system can broadly react with many antigenically distinct SRSVs. RNA was isolated by Ultraspec-3 RNA reagent. Total RNA was extracted using a kit (Biotex Laboratories, Inc. Houston TX) according to Manufacturer's instructions. A single step heat shock RT-PCR method was used (K. J. Schwab et al., 1997) with G1/G2 primers. RT and PCR reactions were carried out sequentially in one tube with reverse transcription at 42°C for 1 hour, 40 PCR cycles of 94°C for 60", 50°C for 90" and 60°C for 120".

The expected PCR product is 123 bp.

Known Norwalk virus or SRSVs positive stools as well as ddH₂O were included as positive and negative controls in the RNA extraction and RT-PCR assay.

Information on RT-PCR Inhibitors

RT-PCR is now widely used for the detection of human enteric viruses in clinical specimens (De Leon et al., 1992; Ando et al., 1994; Moe et al., 1994). Although these methods have revolutionized detection, two major problems continue to hinder the further development of reliable, generic molecular detection methods for clinical, fecal diagnosis including: (1) the presence of matrix-associated RT-PCR amplification inhibitors; and (2) the genetic heterogeneity among HuCVs which limits the development of broadly reactive reagents. In this study, only the former is applicable. A range of techniques also have been employed in attempts to remove inhibitory substances from fecal specimens prior to RT-PCR amplification. Many of these protocols require multiple steps and involve the use of reagents such as guanidinium thiocyanate (GTC), polyethylene glycol (PEG), cetyltrimethylammonium bromide (CTAB),

phenol-chloroform, and Sephadex (Schwab et al., 1997). Other methods that have been tested include the combination of GTC and silica to further purify RNA (Boom and others 1990) and gel chromatography using spin columns (De Leon and others 1992). Hale and others (1996) compared four methods for RNA extraction from fecal specimens for detection of SRSVs using RT-PCR and found the GTC/silica method to be the most efficient in removing inhibitory substances. This has since been confirmed by Svensson (2000). Given even the best purification schemes, it is likely that residual matrix-associated inhibitors will remain in the final extract destined for nucleic acid amplification. These inhibitors are known to either prevent amplification, resulting in the potential for false-negative results, or else reduce its efficiency, resulting in poor detection limits. In fact, the effect of these inhibitors is sometimes more pronounced when target template levels are particularly low, which is precisely when one would need highly efficient amplification. The list of potential matrix-associated inhibitors is nearly endless; most have been poorly characterized and there are no doubt additional yet unidentified inhibitors. Careful design of more efficient and robust enzymes has improved the inhibition problem to a certain degree. Many investigators also add enhancement agents to increase amplification efficiency in the presence of matrix-associated inhibitors. For instance, bovine serum albumin (BSA) is particularly effective in enhancing the efficiency of DNA amplification from extracts with iron-containing molecules such as hemoglobin or humic acids. It has been hypothesized that BSA serves as a scavenger of these inhibitory compounds, preventing their binding to and subsequent inactivation of *Taq* DNA

polymerase. Dimethylsulfoxide (DMSO), dithiothreitol (DTT), and betaine are some other commonly used enhancement agents, among a list of many others.

The Ecl 3'- Oligolabeling and Detection System

Oligonucleotide labeling and hybridization was carried out according to the ECL protocol by Amersham International plc which utilized the enhanced chemiluminescence associated with a horse radish peroxidase (HRP) catalyzed oxidation of luminal, to detect the presence of oligonucleotides tailed at the 3'-end with fluorescein-11-dUTP (F1-dUTP), hybridized to target sequences on membranes (Fig. 2).

Interpretation of PCR Results

PCR products (10ul from 100ul reaction) were analyzed by 3% agarose gel electrophoresis stained with 0.5ug/ml ethidium bromide (EtBr) and visualized with UV light. Results were interpreted by the following criteria for both EtBr gel and probe hybridization:

“+” were samples with a clearly visible band that migrated at the same position as positive controls.

“+/-“ were samples with a faint band or multiple bands comigrating with the positive controls.

“-“ were samples with no DNA visible bands of the appropriate size.

Only the PCR results in which positive controls produced a visible PCR band of the right size and negative controls were not contaminated were included in the final results.

Intestinal Morphology

On day 4 post-gavage, samples of the small intestine composed of the duodenum, jejunum, and the ileum, a third and terminal portion of the small intestine extending from the jejunum to the cecum, were fixed in formalin-buffered saline. After 24h fixation, each section was dehydrated in 70% ethanol and then embedded in a paraffin wax block. Sections from each block were stained with hematoxylin and eosin (H&E) according to conventional methods (Luna, 1968) and measurements were performed using light microscopy with a computer assisted morphometric system (BioScan Optimetric, BioScan Inc., Edmonds WA). Villus width and height and crypt depth were measured. The morphological surface area was computed as follows: $SA = d(3.14)[2 h + r]$ where d =villus width, h =villus height, r =half width (radius) = $d/2$.

Lactase Specific Activity

Appropriately diluted mucosal homogenates were subjected to lactose-hydrolysis rate assays (performed at 37°C for 2 min) and glucose release was subsequently determined colorimetrically via a glucose-oxidase assay (The Sigma-Aldrich subclinical kit for glucose determination, 510-8). Blank corrected rates were then used to express activity as μmol lactose hydrolyzed/min/g of protein (Dahlqvist, 1964).

Statistics

Data from both trials were analyzed using the General Linear Model procedure of SAS (1989), as appropriate for completely randomized designs. All data were expressed as mean +/- standard error. Differences were considered statistically significant when $P < 0.05$.

Results and Discussion

In trial one, no pigs showed clinical signs of viral gastroenteritis. Pig body weights increased from 2kg initially to 12kg at the end of the trial. Norwalk had no detectable effect on pig growth (Fig. 3). Using RT-PCR (confirmed with Southern hybridization, fig. 4b), viral NvRNA was detected in two (high dose) of the twelve pigs gavaged with virus (Fig. 4). In the second study, diarrhea scores did not distinguish between the treatment groups (data not shown) nor was viral shedding detected via RT-PCR, but average daily gain of pigs infected with the swine calicivirus (158g/d) was lower ($P < .05$) than the control pigs (280g/d) (Fig. 5). However, milk intake was similar among treatments (Fig. 6). Likewise, differences in small-intestinal lactase specific activity (Fig. 7) were not detected ($P > 0.1$). However, there was a 42% reduction in villus height in the swine calicivirus group (Fig. 8). Significant differences also were observed in villus width and surface area, which were diminished in both human and swine infected animal groups in the duodenum, as compared to the uninfected group (Figs. 9, 10). In the jejunal samples there was a reduced villus surface area (mm^2) in the swine calicivirus treatment group compared with the untreated control group (Fig. 10). Furthermore, the villus/crypt ratio was reduced in the infected groups (Fig.

11) as a result of longer hyperplastic crypts and shortened villi in the duodenum, jejunum, and ileum. These changes are consistent with the histological abnormalities seen in Norwalk viral illness. For instance, Dolin et al. (1975) observed mucosal changes in the jejunum consisting of blunted villi, shortened microvilli, and cellular edema as a result of experimentally induced as well as naturally occurring illness with the Norwalk viral agent. In a later experiment by Flynn et al. (1988) statistically significant ($P < 0.01$) villus atrophy in the duodenum and/or jejunum was found and confirmed by scanning electron microscopy, which revealed shortening, blunting, and fusion or absence of villi in the duodenum and jejunum of inoculated pigs. The gain to feed ratio (g/g) showed a pattern identical to the average daily gain of the animals when the control and swine calicivirus treatment groups were compared (Fig. 12). These results are consistent with swine calicivirus-induced villus atrophy, and concurrent reduced nutrient absorption (detected as reduced average daily gain and feed efficiency). However, this pathology did not explicitly induce diarrhea, perhaps because of continued absorption by the large colon. An additional reason for a lack of diarrhea despite villous atrophy could be increased absorption by cells on the lower villus and crypt. For example, in a recent study, Blikslager et al. (2001) illustrated that the combined use of a PG synthesis inhibitor and glutamine can fully stimulate Na^+ and Cl^- absorption despite the severe villus atrophy, an effect associated with increased Na^+ -dependent amino acid transporter in infected crypts.

Caliciviruses are formidable enteric pathogens in animals as well as humans. Nonetheless, several unsuccessful attempts to infect animals (aside from chimpanzees) (Wyatt et al., 1978) as well as cell lines with human caliciviruses suggests that Norwalk-like viruses are highly species specific pathogens (Clarke and Lamden, 1997). If interspecies transmission does occur, it is likely to be a subclinical event.

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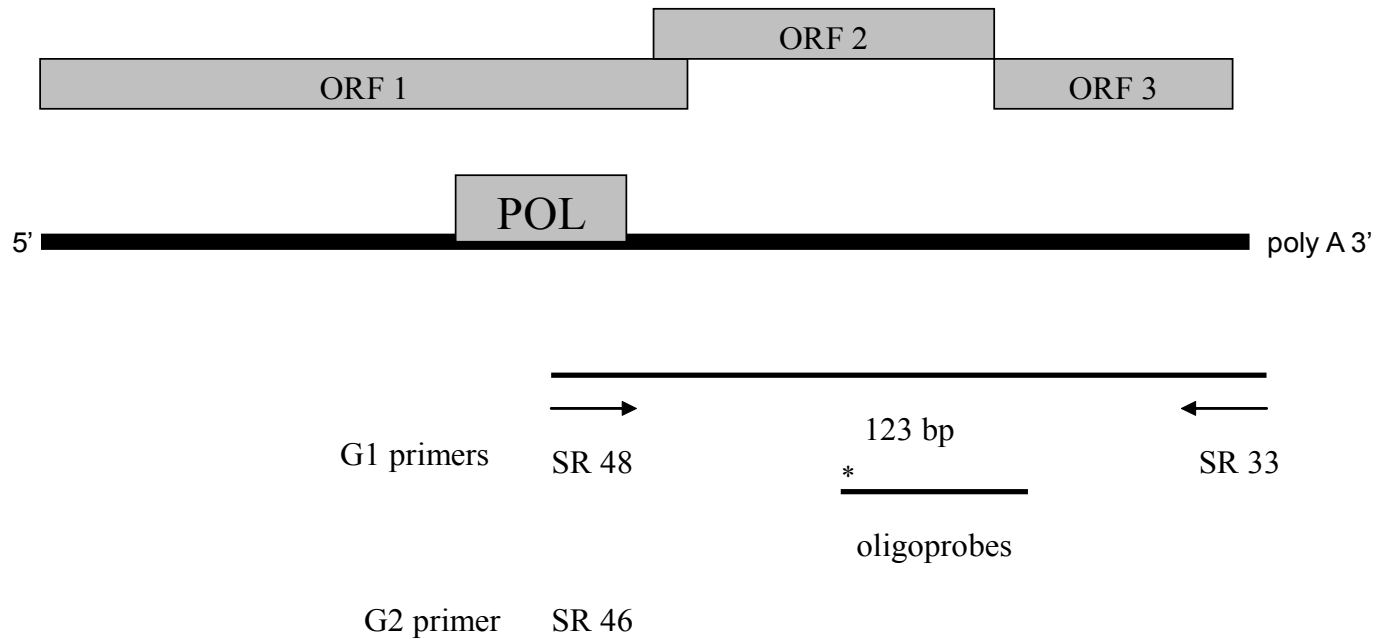
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Fig. 1 Location of the primers, oligoprobes and PCR products for G1 & G2 primer systems in Norwalk virus genome.



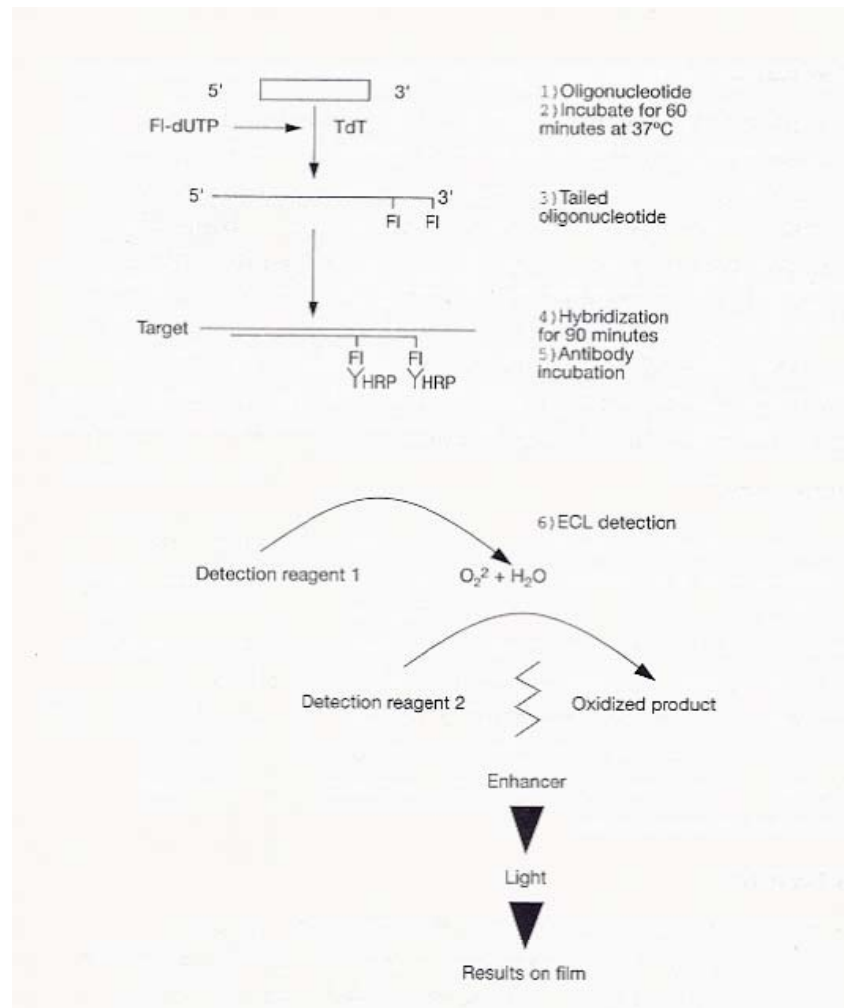


Figure 2. Amersham ECL 3'-oligolabeling and detection system for Southern analysis of Norwalk virus.

Effects of Norwalk Exposure on Growth

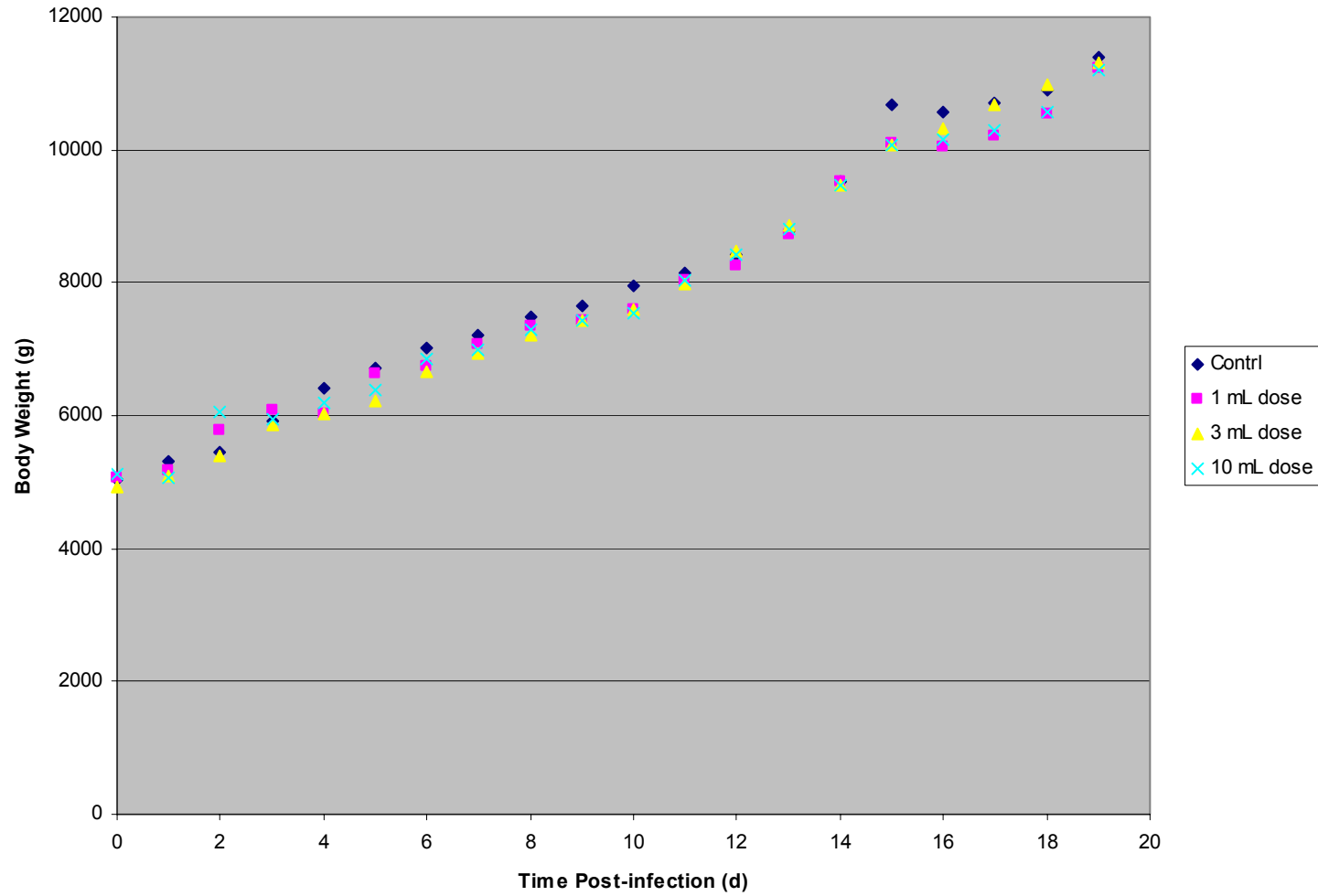


Figure 3. Trial one; Effects of Norwalk virus exposure at three levels (i.e., 3×10^7 , 9×10^7 , and 3×10^8 PCR amplifiable units) on growth of weaning pigs. Each point represents the average of 4 pigs

Cntrl & High Dose

Ultraspec

RT-PCR

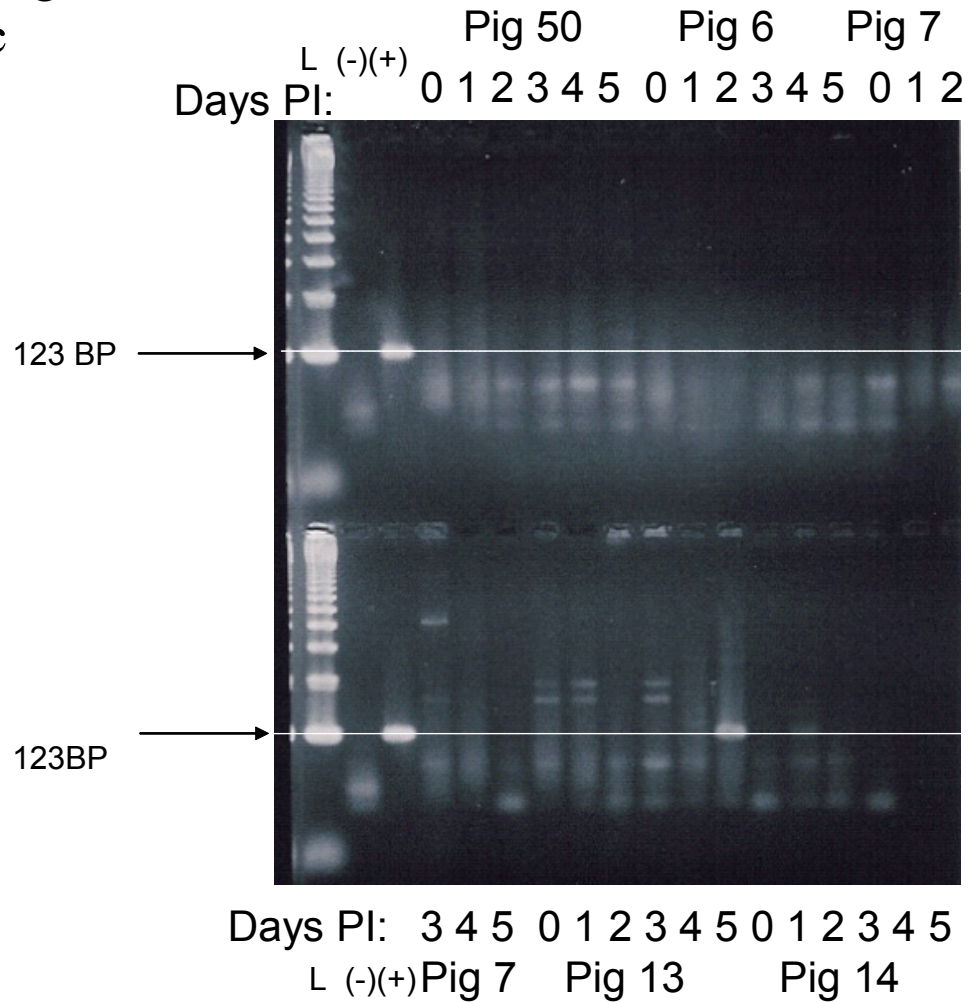


Figure 4. Agarose gel of PCR-amplified Norwalk viral cDNA. RNA was extracted from the stool of pigs at various time points following oral gavage with Norwalk virus, and was reverse transcribed to cDNA and subsequently amplified using primers shown in figure 1. High dose pig 13, day 5 and pig 14, day 1 showed positive stool.

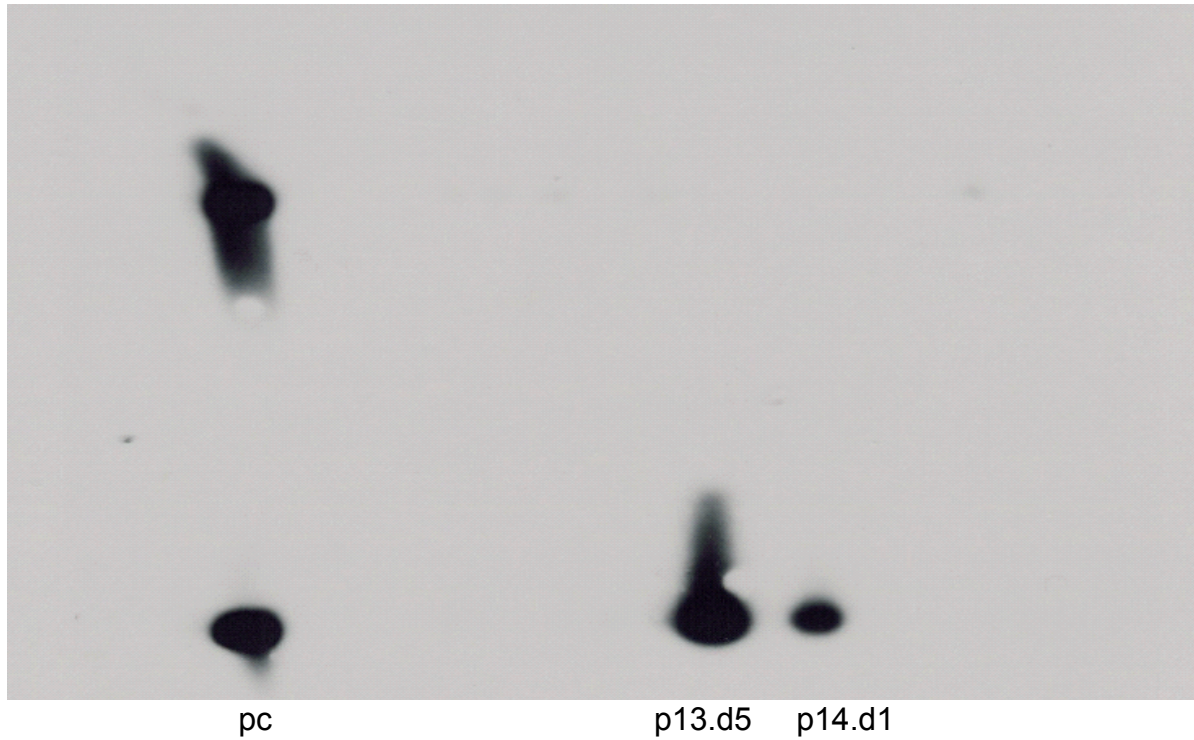


Figure 4b. High dose Southern Hybridization confirming RT-PCR from figure 4.
pc= positive control; p13.d5= high dose pig 13, day 5; p14.d1= high dose pig 14, day 1

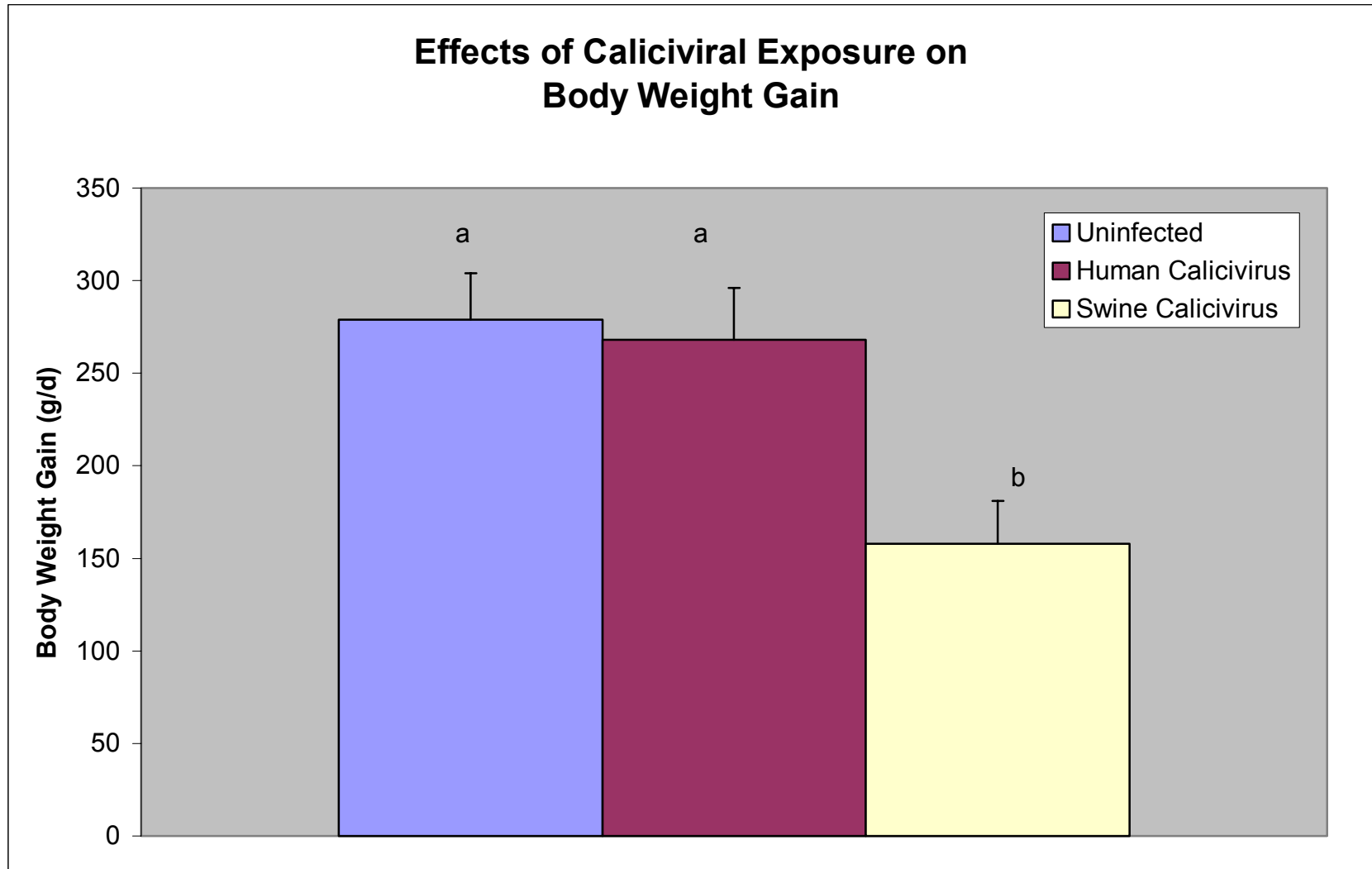


Figure 5. Trial two; effect of human and swine calicivirus exposure on growth of neonatal piglets. Bars represent means +/- standard error, n=4-6 pigs/trt. Bars lacking a common letter are different (P < 0.05).

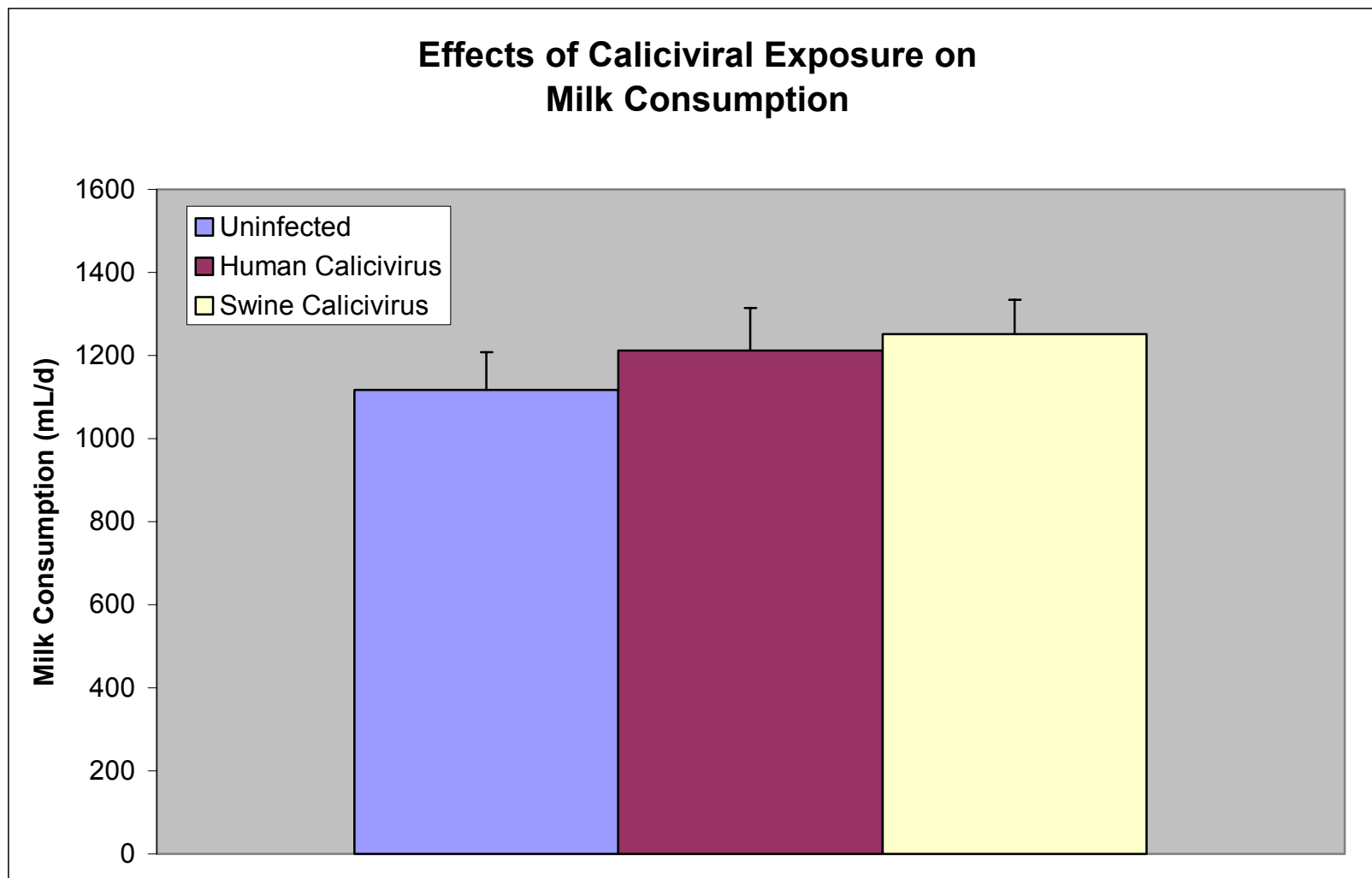


Figure 6. Trial two; effect of human and swine calicivirus exposure on milk intake of neonatal piglets. Bars represent means +/- standard error, n=4-6 pigs/trt.

Effects of Caliciviral Exposure on Intestinal Lactase Activity

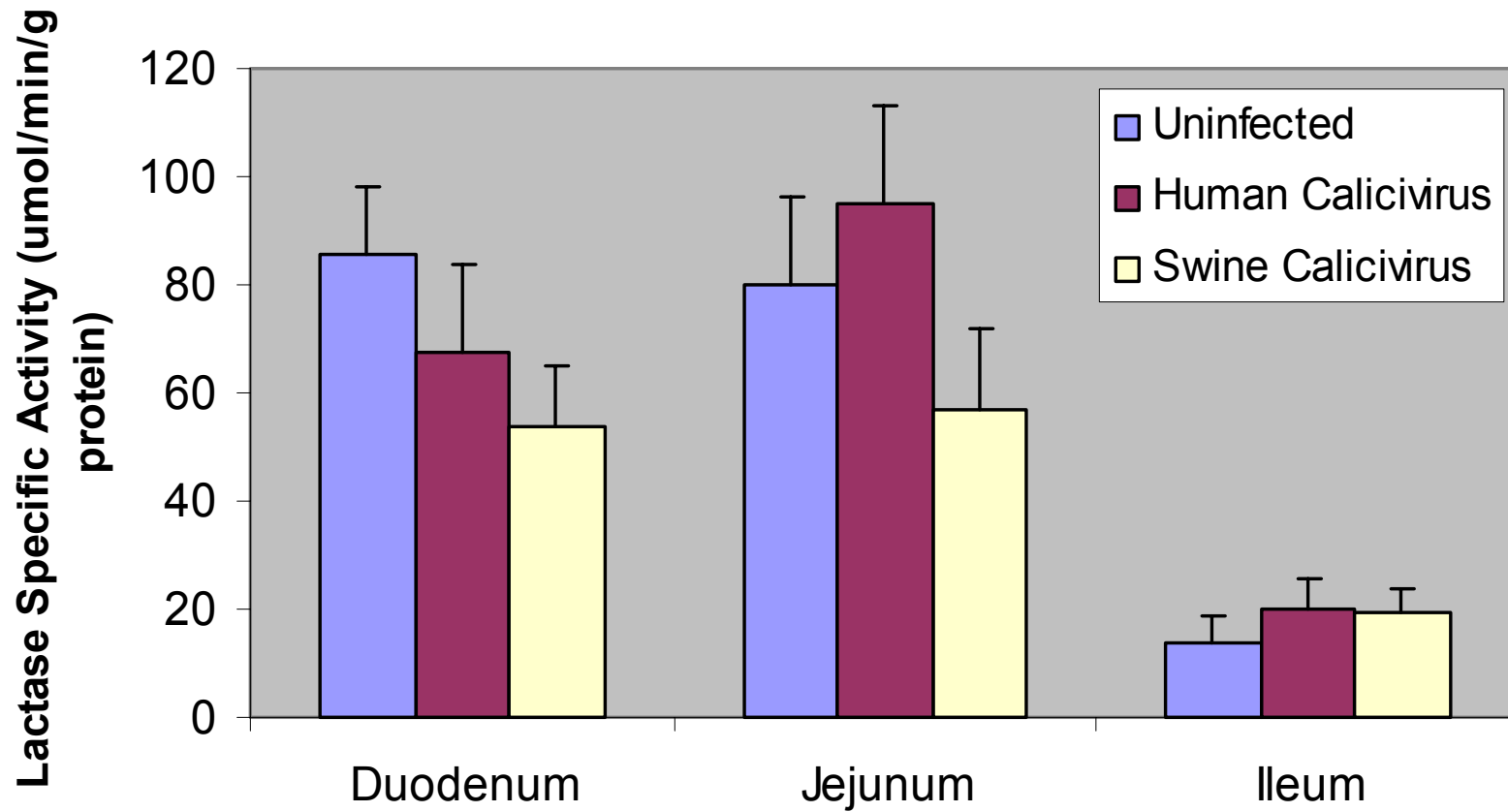


Figure 7. Trial two; effect of human and swine calicivirus exposure on intestinal lactase of neonatal piglets. Bars represent means \pm standard error, n=4-6 pigs/trt.

Effects of Caliciviral Exposure on Villus Height & Crypt Depth

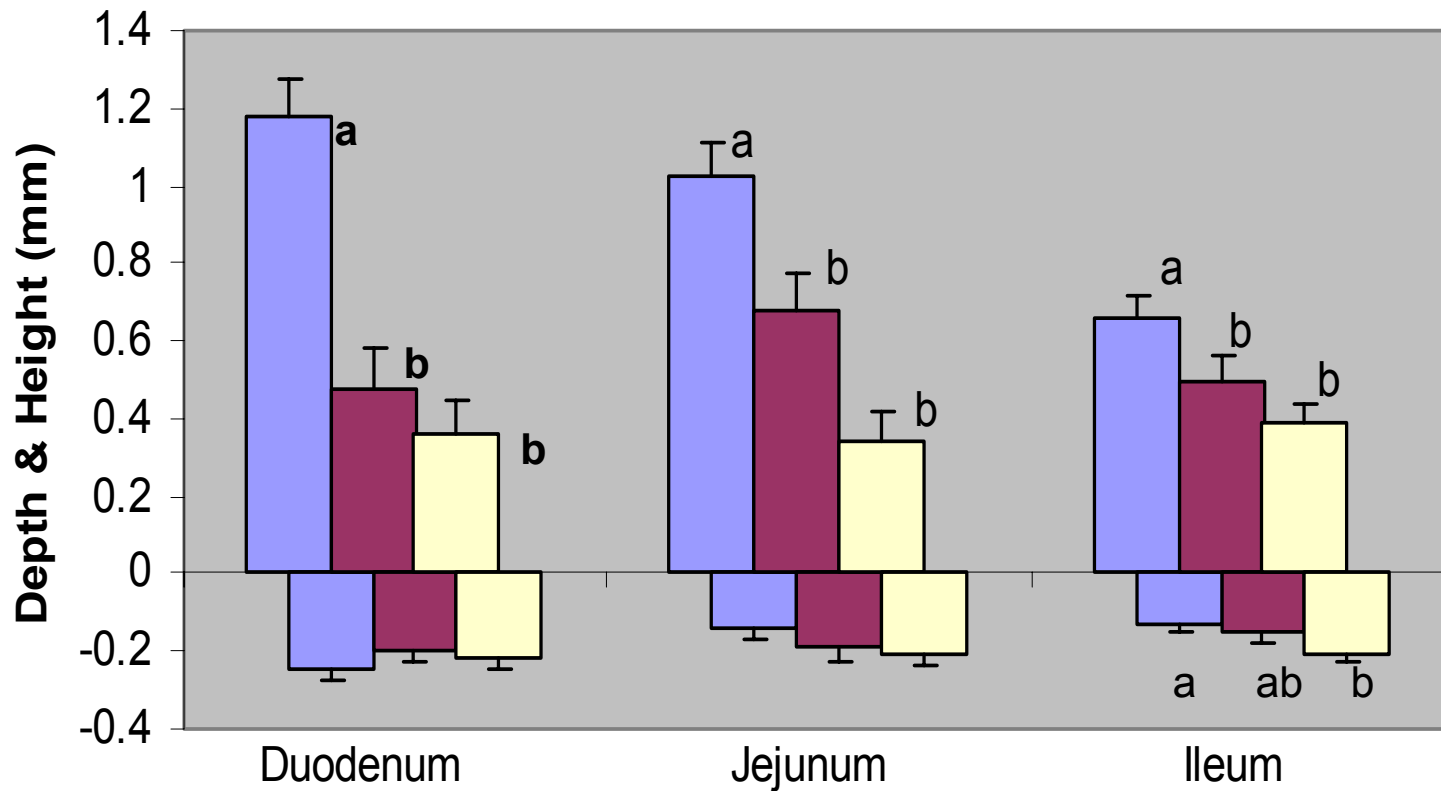


Figure 8. Trial two; effect of human and swine calicivirus exposure on intestinal morphology of neonatal piglets. Bars represent means \pm standard error, n=4-6 pigs/trt. Bars lacking a common letter are different (P < 0.05).

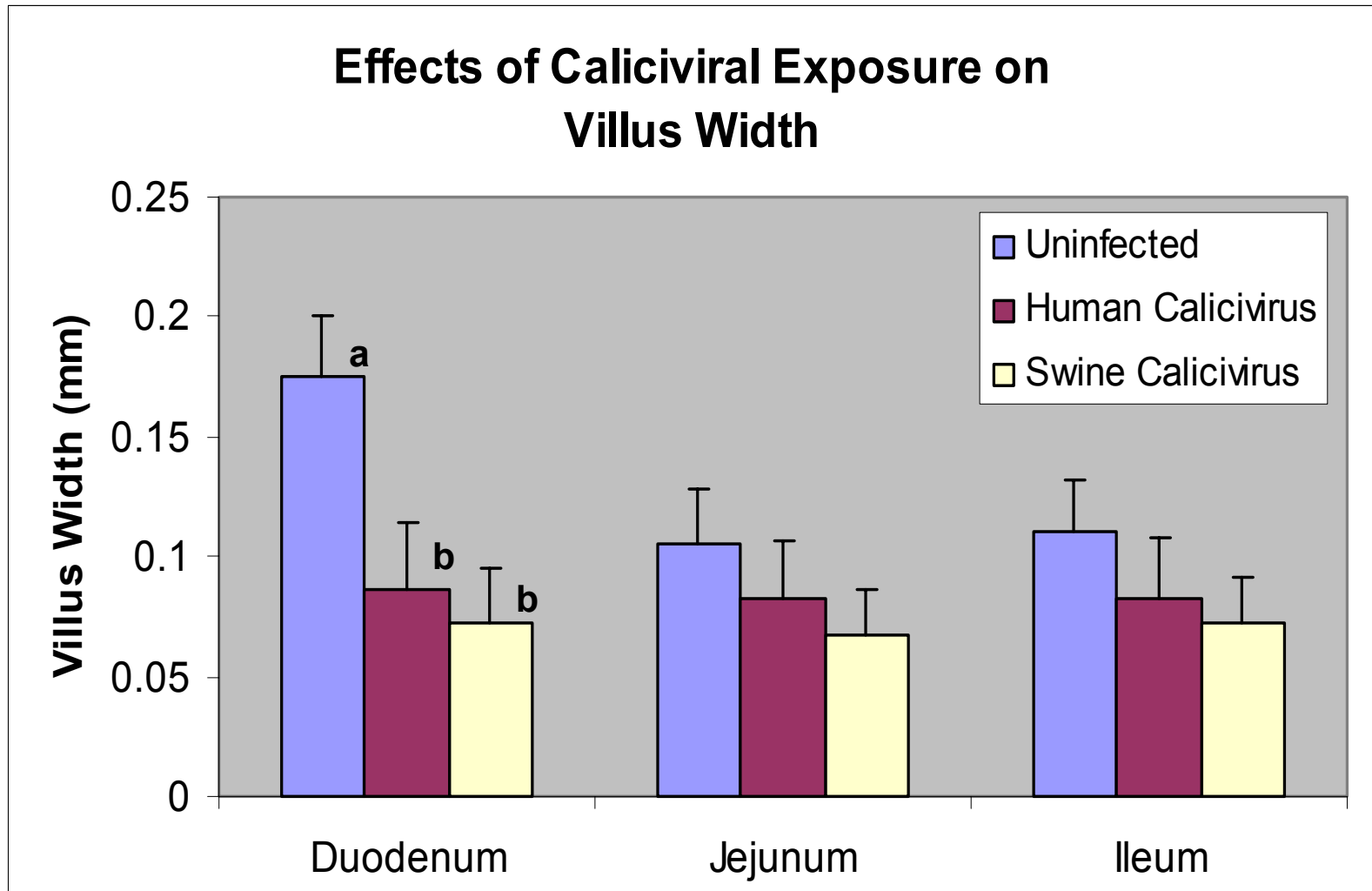


Figure 9. Trial two; effect of human and swine calicivirus exposure on intestinal morphology of neonatal piglets. Bars represent means +/- standard error, n=4-6 pigs/trt. Bars lacking a common letter are different (P < 0.05).

Effects of Caliciviral Exposure on Villus Surface Area

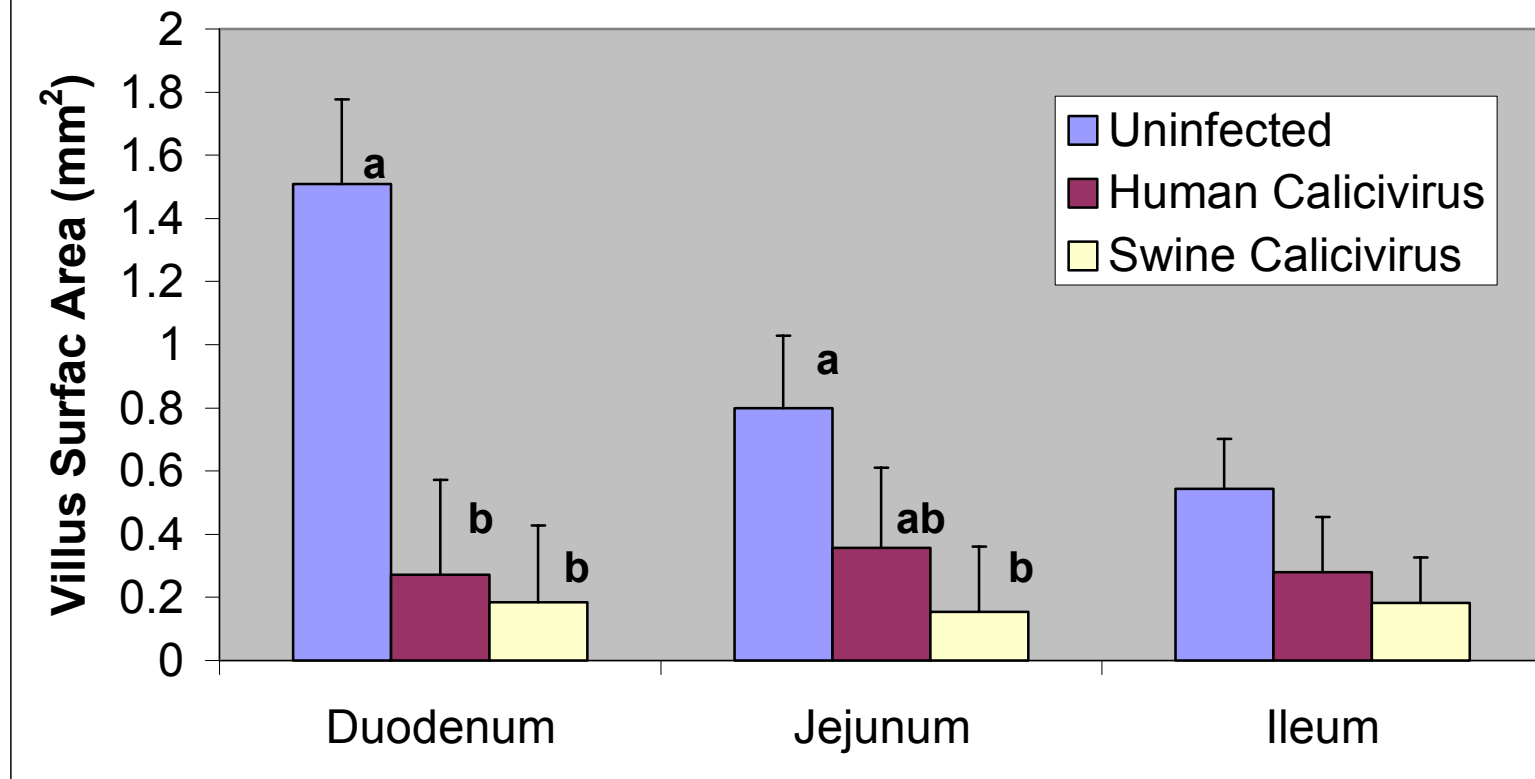


Figure 10. Trial two; effect of human and swine calicivirus exposure on intestinal morphology of neonatal piglets. Bars represent means \pm standard error, n=4-6 pigs/trt. Bars lacking a common letter are different ($P < 0.05$).

Effects of Caliciviral Exposure on Villus/Crypt Ratio

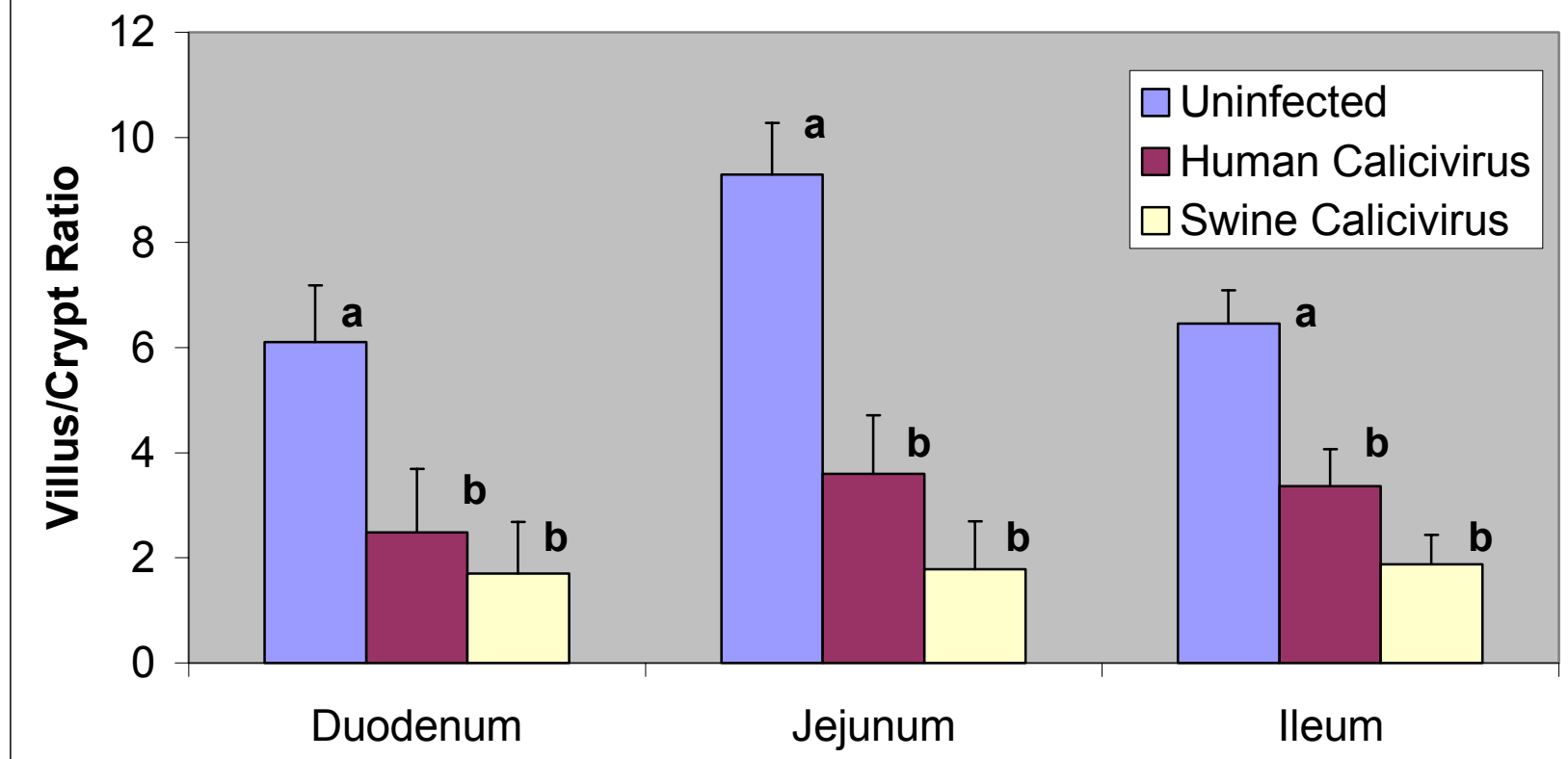


Figure 11. Trial two; effect of human and swine calicivirus exposure on intestinal morphology of neonatal piglets. Bars represent means \pm standard error, n=4-6 pigs/trt. Bars lacking a common letter are different ($P < 0.05$).

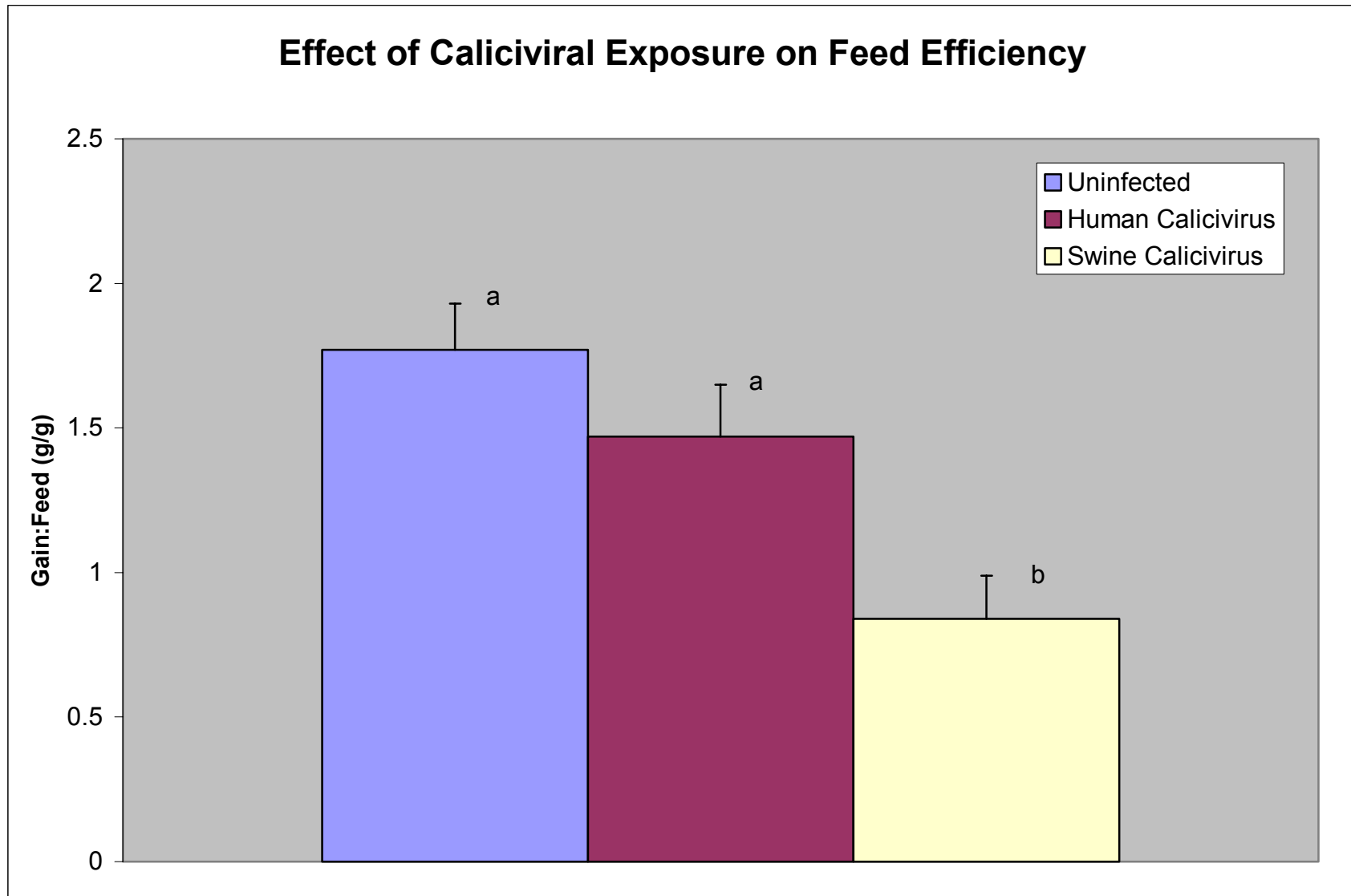


Figure 12. Trial two; effect of human and swine calicivirus exposure on feed efficiency (gain:feed). Bars represent means +/- standard error, n=4-6 pigs/trt. Bars lacking a common letter are different ($P < 0.05$).