1995

Salmonella choleraesuis in swine: carrier state studies

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Salmonella choleraesuis in swine: Carrier state studies

by

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A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Microbiology, Immunobiology and Preventive Medicine

Major: Veterinary Microbiology

Approved:

Signature was redacted for privacy.

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1995

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GENERAL INTRODUCTION

Introduction

Salmonella spp. are gram-negative, non-spore forming, facultative intracellular bacteria which belong to the family *Enterobacteriaceae*. Members of the genus *Salmonella* are extremely ubiquitous in nature, recovered from nearly all vertebrates as well as insects and are often referred to as universal pathogens.

Salmonella are differentiated by biochemical reactivity and agglutination of the somatic (O) and flagellar (H) antigens. There are nearly 2300 serotypes of *Salmonella* which have been identified and each serotype is named utilizing genus, species nomenclature.

Salmonella choleraesuis var. *kunzendorf* is the etiologic agent of swine paratyphoid and is the most common *Salmonella* serotype associated with swine. It has been estimated that swine salmonellosis is responsible for 28 million dollars in annual production losses in Iowa and 100 million in annual losses nationwide.

However, if one recognizes that *S. choleraesuis* is the most frequent porcine isolate, but is rarely isolated from swine feeds or non-porcine *Salmonella* reservoirs, the conclusion must be drawn that the infected shedding pig is the source of new infections. There is a paucity of information

regarding characterization and identification of subclinical *S. choleraesuis* carrier swine. In order to control and reduce the presence of *S. choleraesuis* in the swine environment a better understanding of the carrier state is needed.

The objectives of these investigations were to 1) determine if a *S. choleraesuis* carrier state model can be experimentally developed to study the affect of route of inoculation, the tissues of predilection and the immune response associated with the carrier state, 2) determine the affect of challenge dose on the immune response and persistence of *S. choleraesuis* and 3) study the natural transmission of *S. choleraesuis* to swine and assess the development of the carrier state and immune response.

Dissertation organization

The following dissertation consists of a general introduction, a literature review of salmonellosis with an emphasis on swine (to be submitted for publication), three manuscripts (submitted for publication), a general summary and acknowledgements. The doctor of philosophy candidate, Jeffrey T. Gray, is the senior author and principal investigator for these manuscripts.

CHAPTER 1: SALMONELLOSIS IN SWINE; A REVIEW OF SIGNIFICANT AREAS AFFECTING THE CARRIER STATE.

A manuscript in preparation for publication in
the Compendium om Continuing Education for
the Practicing Veterinarian

Jeffrey T. Gray and Paula J. Fedorka-Cray

THE ORGANISM

Introduction

Salmonella species (*spp.*) are facultatively anaerobic, non-spore forming, Gram-negative, facultative intracellular bacteria which belong to the family *Enterobacteriaceae*. The majority of *Salmonella* are motile, however nonmotile mutants may occur and one serotype, *S. pullorum* is always nonmotile (LeMinor 1984). Infection of animals with various species of *Salmonella* may or may not result in serious disease. It does, however, serve as a reservoir for potential transmission to humans. The interplay of *Salmonella spp.* with its host is varied and may include host specificity, inapparent infections, recovered carriers (subclinical carriers), enteritis, septicemia, abortion and combinations of disease syndromes. *Salmonella spp.* are zoonotic agents and are

readily transferred between animals, from animals to humans and between humans by direct or indirect means.

Classification

There is a variety of naming schemes associated with the genus *Salmonella* none of which are completely accepted by scientists in the field. DNA-DNA hybridization between salmonellas has indicated that there is not enough genetic variation to warrant species differentiation within the *Salmonella* genus (Hook 1990). Another study has divided the genus into three species, *S. typhi*, *S. choleraesuis*, and *S. enteritidis* (LeMinor 1984). In this schema *S. typhi* and *S. choleraesuis* each consist of a single serotype, while all other *Salmonella* are grouped under *S. enteritidis*.

Currently, it is recognized that the genus *Salmonella* is divided into two species, *S. enterica* and *S. bongori*. *Salmonella enterica* is further subdivided into six subspecies: *enterica*, *salmae*, *arizonae*, *diarzonae*, *indica* and *houtenae* (Leminor and Popoff 1987; Reeves et al. 1989). Most *Salmonella* belong to *S. enterica* subsp. *enterica*. Members of this subspecies are given a name which is usually based on the geographic location where the serovar was first isolated.

At the present time, there are approximately 2300 serotypes of *Salmonella* which differ in antigenic structure, host adaptation and biochemical reactions. The most widely

used, and probably the most useful method of differentiating between *Salmonella spp.*, is the Kauffman-White scheme (Hook 1990). Serotypes are differentiated by exhaustive cross-absorbsion and cross-reaction with antisera from the existing serotypes. The antigens responsible for differentiation are the somatic O antigens, as well as the flagellar H antigens and the Vi antigen (Falkow and Mekalanos 1990). Using this system *Salmonella* are named using a genus species format such as *S. typhimurium*. However, the species nomenclature clearly does not define a species but rather a serotype of *Salmonella*.

PATHOGENESIS

Infectious dose

While ingestion of large numbers of *Salmonella spp.* may be required to initiate disease, disease is facilitated often by factors such as peristaltic impairment, interference with intestinal flora and elevation of gastric pH (Clarke and Gyles 1993). The LD_{50} for *S. enteritidis* in germ free mice has been shown to be only 3-5 organisms. However, the comparable value in conventional mice is 10^6 CFU (Collins and Carter 1978). These data have implicated normal intestinal flora as one protective factor from development of clinical salmonellosis. They may also serve to explain the greater susceptibility of the very young whose intestinal flora is not fully developed.

Most studies suggest *Salmonella spp.* gain access to the host by an oral route, pass through the stomach (during which time populations are greatly reduced), then colonize the intestine (Hale 1988). Intraluminal replication varies between serotypes. In swine, *S. typhimurium* replicates to much higher numbers intraluminally than *S. choleraesuis* which is inherently more invasive (Gianella et al. 1973; Reed et al 1986).

Adherence

Attachment of *Salmonella spp.* to epithelial cells has been shown to be influenced by a nonfimbrial, mannose-resistant adhesin which can mediate attachment of *Salmonella spp.* to mammalian tissue culture cells *in vitro* (Tavendale et al. 1983). Interestingly, many of the serotypes which are less invasive in humans have the mannose-resistant adhesin, whereas the more invasive ones, such as *S. choleraesuis* and *S. typhi* lack the adhesin (Clarke and Gyles 1993). This indicates that other virulence factors mediate disease outcome. Most serotypes of *Salmonella* posses mannose-sensitive hemagglutinating pili (type 1) that bind to mannose derivatives on eukaryotic cells. However, the type 1 pili do not appear to play a significant role in adherence of the bacterium to the ileal mucosa (Finlay and Falkow 1988).

Invasion

Much of the knowledge regarding the penetration of the intestine by *Salmonella spp.* is based on work by Takeuchi (1967). The bacteria enter enterocytes through the microvilli or via the tight junctions in between enterocytes then migrate via membrane-bound vesicles to the basal region of the cell. The *Salmonella* pass through the enterocytes to the lamina propria where they stimulate an inflammatory response and are phagocytized by neutrophils and macrophages (Takeuchi and Sprinz 1967).

More recent studies have looked at the invasion process on a molecular basis and have divided this process into two stages. In the first stage, the bacteria adhere to an unidentified receptor on the epithelial cell surface and cause activation of a cascade of events on the cell surface which are mediated by the epidermal growth factor receptor (Galan et al. 1992b; Portnoy and Smith 1992). This series of events indicates that *Salmonella* invasion is dependant on both virulence factors of the pathogen and on the interaction of the host cell with the pathogen. In the second stage, invasion by *Salmonella spp.* induces a tyrosine phosphorylation of the epidermal growth factor receptor. It appears this phosphorylation induces increased intracellular calcium concentrations, microvilli depolarization, formation of extracellular blebs and internalization of the organism (Galan

et al. 1992a; Portnoy and Smith 1992). It has been shown that invasion is regulated by several global regulatory systems which are induced by environmental factors such as low oxygen concentration, temperature, and osmolarity (Galan and Curtiss 1990; Lee and Falkow 1990; Jones et al. 1992). Other factors influencing invasion include chemotaxis, motility and flagellar orientation (Jones et al. 1992). However, plasmids, which have been shown to be critical for virulence, have no effect on invasion (Gulig and Curtiss 1987).

Intracellular survival

Once inside the cell, *Salmonella spp.* multiply in membrane-limited vacuoles with a generation time of about 50 minutes. *Salmonella spp.* can survive within many cells; however, the most significant cell is probably the macrophage. Mutations which destroy this capacity include loss of LPS and certain auxotrophic and regulatory events. Cell wall composition has been shown to influence intracellular survival of *S. choleraesuis* (Griffith 1982). In the intracellular compartment the bacteria are protected from nonspecific defenses such as complement as well as antibody and some antibiotics (Falkow and Mekalanos 1990).

Salmonella spp. synthesize over 30 proteins which are selectively induced during infection of macrophages. Two of these are heat shock proteins, GroEL and DnaK. Avirulent,

macrophage sensitive mutants have been shown to produce heat shock proteins but fail to synthesize different subsets of proteins normally induced within the macrophage. This indicates that these proteins are important for survival (Buchmeier and Heffron 1990).

Intestinal inflammation, fluid production

Salmonella spp. are known to produce cholera-like and shiga-like enterotoxins and these toxins may induce diarrhea independent of mucosal damage (Kinsey et al. 1976; Clarke and Gyles 1987). The *Salmonella* enterotoxin gene has been shown to be coded on the chromosome (Chopra et al. 1987). It must be noted however, that the production of toxins *in vitro* is somewhat obscure and not easily studied in the case of *Salmonella*. Therefore the overall importance of toxin production is not understood. In general, diarrhea observed with salmonellosis is believed to be primarily associated with the inflammatory response induced by *Salmonella spp.* (Gianella 1979). This response stimulates local prostaglandin synthesis resulting in activation of the adenylate cyclase system increasing the secretion of fluid and electrolytes into the lumen (Falkow and Mekalanos 1990). The systemic signs and lesions relating to the septic form of this disease are commonly attributed to endotoxemia as a result of bacterial dissemination.

Cell free extracts of *Salmonella* have been shown to be cytotoxic and inhibit protein synthesis in eukaryotic cells (Koo and Peterson 1982, Koo et al. 1984). These studies provide a molecular basis for the cellular damage caused by *Salmonella* cytotoxin during experimental salmonellosis (Koo et al. 1984).

Extraintestinal infection

The struggle between *Salmonella spp.* and the host is usually not localized in the intestine. This is especially true with *S. choleraesuis* infection (Cherubin et al. 1974; Wilcock 1979; Reed et al. 1986). Bacteria which may be intracellular or free in the mucosa and submucosa are transported by the lymphatics to the regional lymph nodes which contribute to the inflammatory response. From the lymph nodes, *Salmonella spp.* may travel via the efferent lymph vessels and drain into the circulatory system. They are filtered out of circulation via the reticuloendothelial system, usually by the spleen and liver. Release of endotoxin into the circulation may account for many of the systemic effects of disease including fever and vascular damage. Thrombosis of the small vessels may lead to ischemic necrosis of the extremities and tips of the ears, particularly with *S. choleraesuis* infection in swine. Failure to contain the infection will result in septicemia resulting in pneumonia,

meningitis and septic arthritis (Wray and Sojka 1977).

Upper respiratory infection

It has suggested that infection of the upper respiratory tract may influence the outcome of infection. Aerosol experiments in chickens and mice have shown that infections with *Salmonella spp.* can be achieved more regularly via the lungs than by oral inoculation (Clemmer et al. 1960; Darlow et al. 1961).

Pneumonia associated with *S. choleraesuis* infection has been previously described (Baskerville and Dow 1973) and a recent increase in *S. choleraesuis* associated pneumonia has been reported (Turk et al. 1992). It is unclear whether this predilection for the lung is due solely to the pathogen, poor ventilation in large confinement buildings or some combination of these and other factors. Experimental infection models have not provided good answers because positive lung samples have been regarded as an artifact of intranasal or per os inoculation. However, Fedorka-Cray et al. (1995) has demonstrated that the lung is a primary site of colonization following intranasal inoculation of esophagotomized pigs. Additionally, Gray et al. (1995c) has also demonstrated that the lung is colonized in swine that are naturally exposed to pigs infected with *S. choleraesuis*. These data illustrate that lung colonization is not an artifact of experimental

inoculation.

Swine have a large number of alveolar macrophages in the lung (Winkler and Cheville 1987). Fedorka-Cray et al. (1995) hypothesized that swine alveolar macrophages may have an impaired ability to contain *Salmonella spp.* within the early hours after infection. However, once uptake has occurred, the alveolar macrophages may act as a vehicle for dissemination of *Salmonella spp.* (Fedorka-Cray et al. 1995).

Virulence factors

Many potential virulence factors have been identified for *Salmonella spp.* but few have been tested critically for their contribution to virulence. It has been estimated that *Salmonella spp.* posses over 200 virulence factors, only a fraction of which have been characterized (Curtiss III 1994). Many studies have relied on *in vitro* data to draw their conclusions. This makes it difficult to develop meaningful extrapolations for human and animal disease. In addition many studies utilize mice as a model for disease and these results often cannot be repeated in other hosts.

Several serovars have been shown to produce enterotoxins specifically cholera-like toxin (Prasad et al. 1990, 1992). Very little is known about this toxin as it relates to the pathogenesis of *Salmonella spp.* If it acts similarly to cholera toxin, the B subunit of the protein will bind to the

G_{M1} ganglioside on the membrane of intestinal epithelial cells after which the A subunit is internalized causing activation of cAMP and prostaglandin synthesis. These changes would result in fluid and electrolyte secretion (Falkow and Mekalanos 1990).

A common feature of *Salmonella spp.* induced enteritis is severe damage to intestinal epithelial cells likely the result of a cytotoxin. At least three cytotoxins have been identified. A wide variety of serovars posses a heat-labile cytotoxin described by Ashkenazi et al. (1988). Another cytotoxin is a low molecular weight, membrane associated toxin which has not been characterized (Reitmeyer et al. 1986). A third toxin, described by Libby et al. (1990), appears to be present in nearly all *Salmonella spp.*, *Shigella* and enteroinvasive *E. coli*. This cloned protein is a 26 kDa cell-associated hemolysin and its role in virulence is under study.

The LPS of *Salmonella spp.* is a major determinant of host specificity and virulence. The intact LPS affords resistance to phagocytosis and killing by macrophages and complement-mediated killing (Saxen et al. 1987; Robbins et al. 1992). In addition it has been shown that LPS is a major contributor to survival of *Salmonella spp.* in the intestinal tract (Nnalue and Lindberg 1990). The LPS component of *Salmonella spp.* also contributes to vascular damage and thrombosis. Endotoxic properties result in fever, disseminated intravascular

coagulation, circulatory collapse and endotoxic shock associated with salmonellosis (Takeuchi and Sprinz 1967; Clarke 1985).

Motility provided by flagella appears to be important for invasion for some, but not all, serotypes of *Salmonella* Regardless of the other contributions the flagella may make, their presence increases the probability that the organism will come in contact with an epithelial cell. It has been shown that strains with polar rather than peritrichous flagella have increased ability to come in contact with, and potentially invade, epithelial cells (Jones et al. 1992).

A siderophore has been identified in *S. typhimurium* called enterobactin (Benjamin et al. 1985). This protein does not appear to be necessary for full virulence and the importance of the protein may be relative to the amount of extracellular growth which occurs. Interestingly, pigs infected with *S. choleraesuis* have a reduction in serum iron, total-iron binding capacity and transferrin. The intracellular environment is low in iron and it has been suggesting that *S. choleraesuis* has a nonsiderophore mechanism for scavenging iron (Clarke and Gyles 1993).

Finally, heat shock proteins have been shown to be produced by *S. typhimurium* inside murine macrophages. Mutants which are defective in this ability to produce these proteins are less virulent in mice and do not survive well in

macrophages (Falkow and Mekalanos 1990).

DISEASE IN SWINE

Associated serotypes

Clinical swine salmonellosis can be separated into two syndromes. *Salmonella typhimurium* is associated with enterocolitis, while *S. choleraesuis* is usually associated with septicemia. In the United States clinical swine salmonellosis is almost solely due to infection with *S. typhimurium* or *S. choleraesuis*. Clinical disease has also been associated with *S. typhisuis*. This serotype is difficult to isolate and because of this difficulty may be responsible for more outbreaks than it is directly associated with by culture (Wilcock and Schwartz 1992; Glock 1994). In addition, their have been reports of both *S. dublin* (Lawson and Dow 1966) and S. *enteritidis* (Reynolds et al. 1967) causing disease in swine. In contrast, other countries see clinical disease from many serotypes and *S. choleraesuis* may or may not be one of them (Nielsen 1995).

The vast majority of *S. choleraesuis* outbreaks in swine are due to the H_2S producing variant *kunzendorf* (Wilcock and Schwartz 1992). However, the non-H_2S producing *S. choleraesuis* has been as high as number 2 in the top 10 most common *Salmonella* isolates from swine in a given year (Ferris

and Thomas 1993).

Populations affected

Intensely reared weaned pigs are most often affected by *Salmonella* infection. In general, *S. typhimurium* tends to cause disease in young pigs from six to twelve weeks of age. Disease from this serotype is rare in adult animals; however, infection is not. *Salmonella choleraesuis* causes disease among a wider range of ages. Mortality tends to be higher in younger rather than older pigs, while morbidity is often equal regardless of age. Disease from *S. choleraesuis* in the adult is not a common occurrence. However, if a susceptible population is exposed, the animals will be affected significantly (Wilcock and Schwartz 1992). It is not known how common subclinical infection is in the adult. Normally only moribund, suspect cases are cultured for *S. choleraesuis*. In suckling pigs disease is distinctly uncommon but infection is not (Gooch and Haddock 1969; Wilcock et al. 1976). The occurrence of salmonellosis in suckling pigs is rare, presumably because of lactogenic immunity, while neonatal swine are susceptible to oral challenge with salmonellae and develop disease similar to that observed in weaned pigs (Wilcock and Olander 1978).

Septicemia

The septic form of porcine salmonellosis is usually caused by *S. choleraesuis*. Affected pigs are inappetent, lethargic and febrile with temperatures up to 107°F. Respiratory signs may consist of a shallow moist cough and diaphragmatic breathing. Clinical signs first appear after 24-36 hours of infection (Reed et al. 1986). Often, producers will find the first evidence of disease as dead pigs with cyanotic extremities and abdomens. In most outbreaks, mortality is high and morbidity is variable but generally less than 10% (Reed et al. 1986; Wilcock and Schwartz 1992). Diarrhea is normally not a feature of *S. choleraesuis* infection until at least the fourth or fifth day of infection. It may last from five to seven days after onset if chronic reinfection is not occurring.

Gross lesions include colitis, infarction of gastric mucosa, swollen mesenteric lymph nodes, splenomegaly, hepatomegaly and lung congestion. Random white foci of necrosis are often observed on the liver (Reed et al. 1986; Wilcock and Schwartz 1992).

The microscopic lesion which is most often associated with *S. choleraesuis* in swine is the paratyphoid nodule. This lesion can be viewed in the liver as clusters of histiocytes amid foci of acute coagulative hepatocellular necrosis and corresponds to the white foci seen grossly (Lawson and Dow

1966). Other lesions may include fibrinoid thrombi in venules of gastric mucosa, cyanotic skin and glomerular capillaries. Swelling of histiocytes and epithelial cells typical of gram negative sepsis, as well as hyperplasia of reticular cells of the spleen and lymph nodes are often observed (Wilcock et al. 1976).

Enterocolitis

Salmonella spp. enterocolitis in pigs is typically associated with *S. typhimurium* infection and occasionally with *S. choleraesuis* infection. In contrast to the septicemic disease, the initial sign of infection is often a watery yellow diarrhea. Infected pigs are inappetent, febrile and lethargic. Mortality is usually very low however morbidity can be very high within a few days after infection (Wilcock and Schwartz 1992).

The major gross lesion at necropsy is focal or diffuse necrotic colitis and typhlitis. Mesenteric lymph nodes are greatly enlarged. Intestinal lesions develop as red, rough mucosal surfaces that may also have gray-yellow debris. Colon and cecal contents are bile stained and scant, often with black or sand-like gritty material on the surface. Intestinal necrosis may be seen as sharply delineated button ulcers often associated with resolving lesions (Wood and Rose 1992, Wilcock and Olander 1978; Wilcock and Schwartz 1992).

In cases of *S. typhimurium* enterocolitis, the liver and spleen are not enlarged except by terminal congestion (Wilcock and Schwartz 1992).

Histopathologic examination reveals necrosis of cryptic and surface enterocytes which may be local or diffuse. The lamina propria and submucosa contain macrophages and lymphocytes with neutrophils observed only in the very early stages of disease. It is not uncommon to see lymphoid atrophy or regenerative hyperplasia associated with this disease (Wilcock et al. 1976, Jubb et al. 1985, Reed 1986).

EPIDEMIOLOGY

General introduction

Members of the genus *Salmonella* are extremely ubiquitous in nature, recovered from nearly all vertebrates as well as insects and are often referred to as universal pathogens (Taylor and McCoy 1969; Falkow and Mekalanos 1990). Taken as a whole, it is useful to group *Salmonella spp.* into three groups on the basis of host-adapted preference. The first group are *Salmonella* serotypes highly adapted to humans. The prototype of this group is the typhoid bacillus, *S. typhi*. The second group are *Salmonella* serotypes highly adapted to specific hosts other than humans. Some examples of this group are *S. pullorum* or *S. gallinarum* which are adapted to avian

hosts or *S. typhisuis* and *S. choleraesuis* which are adapted to swine. In addition, *S. abortusovis* is a serotype highly adapted to sheep and is a major cause of abortion in ewes. Their are also serotypes such as *S. dublin* which is viewed primarily as a pathogen of cattle but is often found in other hosts (LeMinor 1984). However, some serotypes in this second group can cause severe disease in humans which may result in high mortality. This has been observed following infection by *S. choleraesuis* (Cherubin 1980). The third group of salmonellae would be those with a broad host range. Most *Salmonella spp.* belong to this category and *S. typhimurium* is the best known serotype of this group. It is the serotype most frequently associated with gastroenteritis worldwide (Falkow and Mekalanos 1990).

Salmonella in pork products

Wilcock and Schwartz (1992) consider the epidemiology of *Salmonella* in swine as two relatively separate problems: 1) The contamination of pork carcasses and retail products with *Salmonella spp.* and 2) salmonellosis as a disease of pigs. They also point out that failure of prevalence surveys to distinguish the two conditions has led to considerable confusion about the etiology and epidemiology of clinical salmonellosis in swine. It should be noted that infection of swine and swine products by a wide variety of serotypes is

common, but clinical disease caused by serotypes other than *S. typhimurium* or *S. choleraesuis* is distinctly uncommon.

Due to the potential threat of foodborne illness in humans resulting from consumption of contaminated pork, it is appropriate that we briefly consider this subject. The results of these studies will not be discussed in depth here. It is accepted that the infected pig leaving the farm is most often considered the original source of abattoir infections. Also of importance is that *S. choleraesuis* is rarely associated with contamination of carcasses and pork products. As mentioned earlier, *S. choleraesuis* is a host adapted serotype that rarely infects humans; however, in cases where it has infected humans, it presents a disease of grave consequence that is difficult to diagnose and treat (Cherubin 1980).

In contrast, the top 10 serotypes isolated from swine in 1994 (Ferris and Thomas 1994) include at least 3 (*S.typhimurium, S. heidelberg, S. agona*) of the top 10 serotypes commonly associated with human disease (Bean and Griffin 1992). This indicates that serotypes which commonly cause disease in humans may be closely associated with pork and pork products.

Distribution and prevalence

Salmonellosis as a disease in swine occurs worldwide but varies markedly in estimated prevalence and mortality as there seems to be variation in prevalence between serotypes. Some of this variation can likely be explained by the virulence of the specific strains endemic to an area or from the genetic variation of breeding stock. Investigators have added to the confusion in this area. Often reviews report epidemiologic data from slaughterhouse or federal surveillance studies which are unsupported by clinical or pathologic criteria for salmonellosis (Wilcock and Schwartz 1990). There is also marked variation in the prevalence of *Salmonella spp.* responsible for disease production in data reported from diagnostic laboratories. There could be many explanations for this amount of variation. Of particular concern are variations in bacteriologic culture methods utilized to isolate the organism. *Salmonella typhimurium* is much less difficult to isolate than *S. choleraesuis* because it grows readily in all of the standard selective media used, whereas host adapted strains often require more specialized media (Ewing 1986).

Overall, regardless of animal species, the number one *Salmonella* isolate is *S. enteritidis*. Ferris and Frerichs (1990) found that *S. choleraesuis* has been, and is currently, the second most frequently isolated *Salmonella spp.* from all

animal sources in the United States since 1979. The isolation rate was greater than 99% from swine when compared to all other animals.

In the late eighties and early nineties, the reported isolations of salmonellosis due to *S. choleraesuis* were increasing. One laboratory reported 256 isolations in 1981 with gradual increases to 788 in 1989. In 1989 *S. choleraesuis* was isolated from >95% of swine salmonellosis cases while *S. typhimurium* represented 4% of cases (Schwartz 1990). Causes for the increase are unknown. Recent reports indicate the trend may be declining (Ferris and Thomas 1994).

Cost

Owen (1990) estimated that the cost of salmonellosis as a disease to Iowa swine producers ranks second to swine dysentery caused by *Serpulina hyodysenteriae*. The National Animal Health Monitoring Survey estimated that swine salmonellosis is responsible for 28 million dollars in annual production losses in Iowa and 100 million in losses nationwide (Schwartz 1990). There are no estimates of costs associated with subclinical infections of *Salmonella* in swine.

Source of infection

As previously noted, *Salmonella* is ubiquitous in nature. However, if one recognizes that *S. choleraesuis* is the most

frequent porcine isolate, but is rarely isolated from swine feeds or non-porcine salmonella reservoirs, the conclusion must be drawn that the infected shedding pig is the source of new infections (Wilcock and Schwartz 1992). It has been shown that experimentally challenged pigs can shed up to 10^6/g of *S. choleraesuis* (Smith and Jones 1967) and 10^7/g of *S. typhimurium* (Gutzmann et al. 1976) in the feces. The challenge inocula used in these studies were as high as 10^{11} cfu which seems to be an inoculum unlikely found in the environment. The minimum infective dose for either *S. choleraesuis* or *S. typhimurium* has not been established. Often investigators infect swine with doses of 10^8-10^{11} cfu. Fedorka-Cray et al. (1994) demonstrated that pigs infected with 10^4 CFU of *S. typhimurium* will develop a short term carrier state. Gray et al. (1995b) demonstrated that experimental infection of pigs with 10^3 CFU of *S. choleraesuis* will be cleared with no apparent shedding or clinical signs. In contrast, a dose of 10^6 CFU results in persistent infection for at least 9 weeks.

Natural transmission studies with *S. typhimurium* in swine have indicated that subclinical carriers develop when naive swine are exposed to a population of swine shedding $\leq 10^3$ CFU (Fedorka-Cray et al. 1994). Gray et al. (1995c) demonstrated that natural exposure of *Salmonella*-free swine to a population shedding 10^3 CFU *S. choleraesuis*/g of feces will result in a

severe clinical outbreak with some of the population carrying the organism for at least 12 weeks. In comparison to experimental models, the naturally exposed swine would have needed to ingest between 250 g (10^6 CFU dose) and 25000 g (10^8 CFU dose) of feces each to manifest the severe clinical signs observed in this experiment. This amount of coprophagia is unlikely. These data suggest that during natural transmission, the infectious dose of *S. choleraesuis* is much lower than experimental models have previously described. Swine may also be exposed to a large dose of *S. choleraesuis* by a mechanism other than fecal oral transmission.

CARRIER STATE AND SHEDDING

Species other than swine

Although *Salmonella spp.* may survive for long periods in the environment, it is widely believed that the carrier animal is the major source of infections for both animals and humans (Wray and Sojka 1977).

The carrier state is defined as the absence of evidence of disease in animals that are able to transmit infection to susceptible individuals (Thrusfeild 1986). Carrier animals develop as a result of the interaction of several factors including the serotype of *Salmonella*, age of the animal, and number of bacteria ingested. Young cattle often shed

Salmonella only during convalescence, whereas adults are more likely to become chronic shedders. In addition a low dose which is insufficient to cause disease may result in a carrier state (Wray and Sojka 1977).

In cattle various types of carrier states have been identified (Wray and Sojka 1977). The active carrier state may follow recovery from clinical disease and cattle may excrete *Salmonella spp.* for months or years in the milk and/ or the feces. The active carrier state often persists in the presence of high serum antibody titers to *Salmonella* O and H antigens.

Passive carriers are described as cattle which ingest *Salmonella spp.* and pass the organisms through the intestine into the feces with little or no invasion of the mesenteric lymph nodes. These animals cease shedding *Salmonella spp.* shortly after they have been removed from the contaminated environment (Wray and Sojka 1977). Latent carriers are cattle which have deep tissue infection with *Salmonella spp.* but do not excrete the organism in their feces (Wray and Sojka 1977). Excretion may be reactivated by unknown mechanisms.

Treatment of adult animals with antibiotics during the course of disease is ineffective in eliminating the carrier state in cattle infected with *S. dublin* (Wray and Sojka 1977). It is widely accepted that the antibiotic treatment of humans following infection with *S. typhi* (typhoid) is contraindicated

as it prolongs the carrier state (Askerkoff and Bennett 1969).

Certain stress factors have been shown to promote activation or reactivation of clinical signs and shedding in *Salmonella* carrier cattle. These factors include, but are not limited to, transportation of animals, overcrowding, cortecosteroids, parturition and concurrent infection.

Tannock and Smith (1971b) described the carriage of *S. typhimurium* in sheep for up to 6 weeks after intranasal inoculation. When the same inoculum was given orally by use of a gelatin capsule a prolonged carrier state was not observed. Tannock and Smith (1971a) also compared the effect of route of inoculation on the carrier state in mice. They concluded the upper respiratory tract provides a focus of infection. When the inoculation route is intranasal, a carrier state results for at least 6 weeks. As observed in sheep, the gastric route of inoculation did not result in development of a carrier state.

Carrier state in swine

After experimental challenge of swine with *S. typhimurium*, *Salmonella* could be isolated from the feces daily for the first 10 days and frequently over 4-5 months. Four to seven months post challenge carrier animals were necropsied and greater than 90% of the pigs were positive for *S. typhimurium* in the mesenteric lymph node, tonsil, cecum or

feces (Wilcock and Olander 1978; Wood et al. 1989). In an unrelated experiment it has been shown that a sub-clinical, undetectable infection can progress to a high level of shedding after the occurrence of stressful events such as farrowing or transport to slaughter (Wilcock and Schwartz 1992).

In contrast to this minimal understanding of the carrier state of *S. typhimurium* in swine, the duration of shedding and the locations of organisms for *S. choleraesuis* in carrier swine has not been studied until recently. Gray et al. (1995a) has recently described the carrier state of *S. choleraesuis* in swine. These data demonstrate that a subclinical carrier state exists for at least 12 weeks after experimental challenge. The tissues in which *S. choleraesuis* can most commonly be found in carrier swine are the ileocolic junction, ileocolic lymph node, cecal contents, tonsil, lung and colon, regardless of route of inoculation. Swine can also shed *S. choleraesuis* in the feces sporadically throughout the 12 week period (Gray et al. 1995a) A dose dependant affect on persistence of *S. choleraesuis* in swine has also been observed (Gray et al. 1995b). Lower challenge doses such as 10^3 CFU may be cleared by pigs. In contrast, a moderate dose (10^6 CFU) may result in persistent infection for at least 9 weeks. High challenge doses (10^9 CFU) have been shown to result in long term carriers which may be related to an observed

lymphocyte immunosuppression.

Recent experiments have indicated that *S. choleraesuis* which have been shed from infected swine can survive for at least 3 months in a wet fecal slurry and at least 6 months in dry, desiccated feces (Gray et al. 1995d). This indicates the importance of decontamination of the environment when reduction of *Salmonella spp.* is a goal.

The influence of antibiotics on the frequency and duration of shedding of *Salmonella* in swine is not well understood. However, it is known that antibiotics do not affect the magnitude or intensity of shedding of *S. typhimurium* in swine (DeGeeter et al. 1976; Jacks et al. 1988). Conversely, it has been shown that antibiotics may reduce the magnitude and duration of shedding of *S. choleraesuis* (Jacks et al. 1981).

IMMUNITY AND VACCINATION

Introduction

There is continual debate over the importance of the humoral versus the cell-mediated immune response following *Salmonella* infections. This controversy stems from the pathogen's ability to reside successfully in both an intracellular and extracellular environment. Taken as a whole, these data suggest that both humoral and the cell-

mediated response are important in resistance to *Salmonella spp.* infection. At the present time, there is a lack of information regarding the immune response in swine following *Salmonella spp.* infection.

Humoral immunity

Antibodies against *Salmonella* are common in sera following exposure to the pathogen. Passive transfer of antibodies against *Salmonella spp.* to offspring is observed (Royal 1968). Antibody can provide protection through opsonization of the pathogen, neutralization of toxins and initiation antibody dependent cell-mediated cytotoxicity. Many antigens from *Salmonella* have been shown to induce antibodies including LPS (Jimenez-Lucho and Leive 1990), proteins (Foulaki et al. 1989; Saxen et al. 1986; Udhayakumar and Muthukkaruppan 1987) and ribosomal fractions (Eisenstein 1975).

The importance of humoral immunity in protection from *Salmonella* infection is discussed by Eisenstein and Sultzer (1983). The experiments conducted in mice show increased host survival following challenge, increased duration of survival and passive protection against homologous strain challenge. Evidence suggests that specific antibodies in the colostrum of cows vaccinated with *Salmonella* may interact with organisms in the lumen of the gut of calves and influence the outcome of

infection (Royal 1968).

Natural and antibody-dependent antibacterial mechanisms may be important in defense against *Salmonella spp.*, particularly in the gastrointestinal tract. Secretory IgA is also found in the intestine of animals that have recovered from disease or that have been vaccinated orally (Clarke and Gyles 1994; Stabel 1993). Intestinal antibodies constitute the first line of specific immune defense to organisms which enter the gastrointestinal system.

The amount of specific antibody in serum has not been shown to correlate with protection against experimental challenge of calves vaccinated with either a live attenuated mutant or heat-killed preparations of *Salmonella spp.* (Habasha 1981; Lindberg and Robertsson 1983). In addition, not all animals which are infected or immunized with *Salmonella* develop titers to the organism even though they show increased survival from subsequent challenge (Clarke and Gyles 1994). Recently, antibody titers to nontyphoidal *Salmonella* O-antigen have been suggested to confer protection against challenge (Robbins et al. 1992).

Cell-mediated immunity

Salmonella is a facultative intracellular pathogen which can evade antibody in the intracellular environment. This suggests that a strong cellular response is needed for

pathogen elimination (Sell 1987). Infection with *Salmonella* has been shown to effectively induce cell-mediated immunity (Hanna et al. 1979a; Hanna et al. 1979b; Hassan and Curtiss III 1990; Jones et al. 1991). Transfer of sensitized T cells confers protection, whereas transfer of macrophages and B cells are not protective in the absence of T cells. Delayed-type hypersensitivity develops in animals with natural and experimental salmonellosis (Robertsson et al. 1982a,b; Lindberg and Robertsson 1983).

Generally, the cell-mediated immune response correlates well with protection in calves and mice immunized with live attenuated vaccines and subsequently challenged with large numbers of *Salmonella spp.* (Habasha 1981; Lindberg and Robertsson 1983; Eisenstein and Sultzer 1983). Cross-protection has been demonstrated for *S. typhimurium* and *S. dublin* and may be attributed to shared O-antigenic components and porin antigens (Habasha 1981; Lindberg and Robertsson 1983).

Interestingly, investigators have demonstrated that live vaccines were superior to killed bacteria in providing increased host survival which was facilitated by elimination of the organism from the spleen and liver (Collins 1974; Eisenstein and Sultzer 1983). Using a mouse model of typhoid fever the immunity induced by an *aroA* mutant of *S. typhimurium* was attributed to natural killer cell activity that was

observed in the early stages of disease. It was suggested that these cells may also contribute to host defense in the later stages of disease (Schafer and Eisenstein 1992).

Mucosal immunity

Mucosal surfaces are the major sites in the body in which antigens are encountered. Throughout life, they are continuously bombarded by antigens, whether they are ingested food particles, microbes such as *Salmonella*, toxins, parasites, or allergens. Most infectious diseases develop on mucosal surfaces and in many the organism is limited to these sites. To combat this constant threat, vertebrates have developed a complex mucosal immune system that undertakes the task of limiting infections without interfering with the function of the fragile mucosal tissue (Klein 1989).

The major humoral immune factor at these sites is locally produced secretory IgA antibody (Mestecky 1987). It has been estimated that 65 to 90% of immunoglobulin-producing cells produce IgA and 75% of the total immunoglobulin produced in humans is IgA (Michalek et al. 1995).

An antigen that has come in contact with the mucosal surface must cross the epithelium to reach the lymphoid tissue in the lamina propria. On some mucosal surfaces, this crossing is affected by specialized cells overlying the lymphoid follicles (Michalek et al. 1995). In the intestine,

and probably also in the respiratory system, crossing the epithelium is regulated by M cells. On the side opposite the luminal surface, lymphocytes attach themselves closely to the M cells. The M cells bind antigen on the luminal side, endocytose it and then exocytose the antigen to the lymphocytes on the opposite side. The M cells differentially bind antigens excluding commensal organisms and food antigens. Some invasive pathogens such as *Salmonella spp.* can use this transport mechanism to their advantage which results in access to macrophages and lymphocytes in which they can survive (Klein 1989).

The M cells transport material in both directions. Lymphocytes, for example, have been seen moving through M cells by diapedesis and then entering the lumen of the gut. It has been hypothesized that most of the lymphocytes found on the luminal surface of the gut mucosa may have reached their destination by this manner (Klein 1989). This may be the mechanism by which swine challenged with *S. typhimurium* by mechanisms completely excluding oral gastric exposure are observed to have *S. typhimurium* positive intestinal tissues after only a few hours (Fedorka-Cray et al. 1995). It should be noted that the M cells take up antigens, but are unable to carry out the crucial presentation step which is required to initiate an immune response because they lack class II Mhc molecules and are unable to process the antigen for

presentation (Klein 1989). Therefore, the M cells must release the antigen to the mucosal associated lymphoid tissue (MALT) where presentation can take place.

The collaboration of B lymphocytes with T helper lymphocytes (Th) may occur in the MALT or the draining lymph nodes. The B cells that encounter antigen and receive the necessary stimulation from Th cells leave the MALT without secreting antibody. As they mature and begin secreting antibody, they home back to the various MALTs where they settle back into the lamina propria (Bergmann 1986, Ogra and Karzon 1969). If the B cells encounter the same antigen again they begin to produce antibody resulting in a mucosal antibody response. What determines the B cells specificity to the MALT sites remains unclear. However, the homing of the B cells for the various MALTs is one characteristic of the mucosal immune system; another characteristic is the dominance of IgA-producing B-cells over any other B lymphocytes although IgM, IgG and IgE antibodies are also produced but in lesser amounts (Klein 1989).

The cells found in the MALT include lymphocytes, natural killer cells, macrophages, mast cells and eosinophils. At least some of the lymphocytes, the intraepithelial lymphocytes, seem to be unique in the mucosae. Among this population about 15% are T lymphocytes. There is a dispute regarding the remaining 85% of intraepithelial lymphocytes.

The lamina propria contains true lymphocytes, both T and B cells, providing the necessary components for the initiation of an immune response in this area (Klein 1989).

Mucosal antibody responses to *Salmonella* antigens have been shown in swine after challenge (Gray et al 1995 a,c) and vaccination (Stabel et al. 1993). However, there have not been any studies providing a correlation of mucosal antibody response and increased resistance to salmonellosis in swine despite the critical importance of mucosal immunity.

Vaccination

It is generally accepted that live attenuated, orally-administered *Salmonella* vaccines provide the best protection against *Salmonella* infection. The superior protection achieved in comparison to killed *Salmonella* bacterins and subunit vaccines is generally attributed to the ability of live vaccines to stimulate a more effective cell-mediated immune response. Oral administration allows the attenuated mutant to utilize natural routes of infection which facilitates the crucial step of antigen presentation to lymphocytes in the gut-associate lymphoid tissue. These events induce the production of secretory IgA on mucosal surfaces (Clarke and Gyles 1994).

Recently the development of specific nonreverting mutations to construct both homologous and heterologous

vaccine vehicles with multiple attenuating mutations has been achieved (Chatfield et al. 1992).

A mutation in the *galE* region in *S. typhi* results in a deficiency in UDP-glucose-4-epimerase, the enzyme which converts UDP-glucose to UDP-galactose, an essential component of *Salmonella spp.* smooth LPS (Levine et al. 1989). In several large trials utilizing human subjects this mutant has appeared to be very efficacious. Because of this success, this mutation has been employed for many *Salmonella* serotypes including *S. typhimurium* (Nnalue and Lindberg 1990). However, the *galE* mutation was not successful when utilized in *S. choleraesuis*. The O antigen of *Salmonella* serogroups are the main component of host specificity and facilitate survival in the gastrointestinal tract and entry onto deeper tissues (Nnalue and Lindberg 1990). The *galE* mutation in *S. choleraesuis* does not reduce virulence in swine. This is due to the fact that galactose is missing from the oligosaccharide repeating unit of the O antigen side chain of *S. choleraesuis* (Nnalue and Stocker 1986).

Another common attenuation involves the creation of auxotrophic mutants that require metabolites not available in animal tissues. Aromatic mutants, which have a complete block in the aromatic biosynthetic pathway have a requirement for aromatic metabolites such as para-aminobenzoate and 2,3-dihydroxybenzoate. Oral vaccination with *aroA*, *aroD* mutants

in mice and calves has been effective in reducing disease and have been shown to be safe (Hook 1990; Robertson et al. 1983; Smith et al. 1984).

Mutations in global regulatory pathways have also been a popular means of attenuation. Several studies have utilized strains with deletions (Δ) in the genes for adenylate cyclase (*cya*) and for cAMP-receptor protein (*crp*). Cyclic AMP and cAMP-receptor protein regulate at least 200 genes, many of which are required for breakdown of catabolites. *Salmonella* with deletion mutations in the *cya,crp* genes have been shown to be safe and effective in eliciting protective immunity in mice, chickens and pigs (Coe and Wood 1992; Curtiss and Kelly 1987; Stabel et al. 1990; Stabel et al. 1991). A large study evaluating the safety and efficacy of a battery of *S. choleraesuis Δcya,Δcrp* isogenic mutants in mice indicates that several of these strains are protective and safe (Kelly et al. 1992).

Recently a *S. choleraesuis* strain which has been cured of the 50 kb virulence plasmid has been shown to safe and efficacious in swine (Kramer et al. 1991). The nonspecific mutation was obtained by repeated passage through porcine neutrophils. The plasmidless variant lacks the ability to invade Vero cell monolayers and porcine neutrophils as well as having increased resistance to killing by H_2O_2 and phagocytic killing by porcine neutrophils (Roof et al. 1992).

DETECTION OF *SALMONELLA*

Culture

A great interest has developed in the animal production and food processing industries to create and evaluate new methods to detect, either directly or indirectly, the presence of *Salmonella spp.*. Traditional culture methods are slow, cumbersome, expensive and require considerable manpower to complete. However, the culture of *Salmonella* is the standard by which all other methods are measured. Recovery of the organism is the only means by which definitive serotyping can be achieved. In addition, the isolation of the organism serves as an invaluable source of epidemiologic data which cannot be overlooked.

It is always advisable to employ enrichment culture in the examination of various kinds of specimens for *Salmonella spp.*. The first step in the culture process should include an assessment of the competing flora and the physical state of the *Salmonella* in the sample. For example, for a pelleted feed sample which has been heated and dried in the processing step there will likely be relatively few competing flora and the *Salmonella* may be in a desiccated state. This warrants use of a non-selective nutrient broth (Ewing 1986). In contrast, fecal samples contain large numbers of competing flora and the *Salmonella* may be in any stage of growth

indicating the use of selective media for enrichment (Ewing 1986).

The media suggested for enrichment of *Salmonella spp.* in fecal specimens from carriers or suspected carriers are tetrathionate broth and selenite F broth (Ewing 1986). Many modifications have been made to these media and the application should be considered when choosing a medium for a specific purpose. Another useful medium for the enrichment of *Salmonella spp.* is Rappaport medium (Vassiliadis 1983), which utilizes malachite green and magnesium chloride as selective agents. However, this medium is easily overloaded when used as an initial enrichment and care must be exercised when developing an inoculation plan (1986).

Smith (1952) found it absolutely necessary to utilize media other than tetrathionate broth and selenite F broth for the isolation of *S. choleraesuis*, both of which have been reported to be toxic for *S. choleraesuis*. The same caution is warranted when any host adapted serotype is suspected as many of the of the host adapted serotypes do not grow well in the highly selective *Salmonella* media, including *S. typhi* (LeMinor 1984). It has been suggested that this may explain the infrequent isolation of *S. choleraesuis* in swine associated epidemiologic surveys (Ewing 1986). When possible, a combination of enrichment media should be employed and may include GN-Hajna broth and tetrathionate broth for the

isolation of host adapted serotypes as well as broad host range *Salmonella spp.* (Ewing 1986).

Many plating media have been devised for the isolation and differentiation of *Salmonella spp.*. Plating media for use of isolation and differentiation of *Salmonella spp.* and other members of the genus Enterobacteriaceae may be divided into categories according to their selectivity.

Differential media, with little selectivity for enterobacteriaceae, are used with some frequency. This group includes MacConkey agar on which the lactose negative *Salmonella spp.* appear as white colonies. All *Salmonella spp.* grow well on MacConkey agar (Ewing 1986). Moderately selective differential media includes Shigella-Salmonella agar and Hektoen Enteric (HE) agar. Hektoen Enteric agar is often considered a standard by which other *Salmonella* isolation media are measured (Dusch and Altwegg 1995). Again, all serotypes of *Salmonella* grow well on moderately selective differential media (Ewing 1986).

The final category is the highly selective media which include brilliant green (BG) agar. The BG agars are very popular and can be considered as a one purpose medium for the isolation of *Salmonella spp.* *Salmonella* appear as smooth pink colonies on BG agar. Only a few other genus of bacteria can also appear as pink colonies similar to *Salmonella* and include pseudomonads, aeromonads, proteus and late lactose fermenting

E. coli. Although these agars are very useful in the isolation of *Salmonella spp*. some serotypes such as *S. typhi* do not grow well on these agars (Ewing 1986).

A recent study compared HE agar, Rambach agar, SM-ID medium, xylose-lysine-Tergitol 4 agar (XLT4), novobiocin-brilliant green-glycerol-lactose agar (NBGL) and modified semisolid Rappaport Vassiliadis medium (MSRV) for the isolation of *Salmonella spp*. The test of these relatively new media found MSRV to be the most sensitive and specific but it was also the most difficult to use. The XLT4 plates were found to be as sensitive as HE with improved specificity. The other media did not perform as well (Dusch and Altwegg 1995).

In all cases, pooled fecal samples are preferred over rectal swabs for the detection of *Salmonella*-carrier pigs (McCall et al. 1966).

Enzyme linked immunosorbent assays

Enzyme linked Immunosorbent Assays (ELISA) can be used to detect either the organism or a humoral immune response to the organism. Assays utilized to detect microorganisms in food and feed stuffs are gaining widespread use in the industry and are called antigen-capture ELISA. Whereas culture may take 3-7 days to identify the organism, ELISA can detect the organism in a much shorter period of time, usually 1 day or less. However the reliability of some of these assays is

questionable. In general, the cleaner the sample the better the assay will perform. Usually feces does not test as well as food and feedstuffs. Feng (1992) listed and described several commercial rapid screening assays. Several antigen capture immunoassays have been utilized to detect *Salmonella spp.* in swine feces (Araj and Chugh 1987; Lambiri et al. 1990; van Poucke 1990). They have the same disadvantage of many ELISA tests in that they require 10^4-10^5 CFU of *Salmonella* per ml to detect the organism (Dziezak 1987). In order to achieve these numbers, a time consuming and expensive concentration protocol or a lengthy pre-enrichment must be employed. Some investigators have had success utilizing rapid enrichment protocols to detect *Salmonella spp.* in swine feces (Cherrington and Huis in't Veld 1993a,b)

The second use of ELISA is to detect animals which have been, or are currently, infected with *Salmonella spp.* This procedure is not new, and was first described by Carlsson et al. (1972) to detect antibodies specific for *Salmonella* LPS. Several studies have focused on this approach and have found that LPS antibody titer can be an important diagnostic tool for detection of *Salmonella* infected cattle (Smith et al. 1989; Spier et al. 1990, 1991).

The detection of antibodies to the O antigen of *Salmonella* has also been utilized successfully in swine (Nielsen et al. 1994). The mixed ELISA utilizes LPS produced

by the method of Westphal (1965) from either *S. typhimurium* or *S. choleraesuis*. The majority of swine produce high titers to the O-antigen which are present whether or not shedding can be detected (Nielsen et al. 1994). The test can be utilized as a herd test but is not suited as an individual pig test.

Unfortunately, experimetally and naturally infected swine have been shown to have a titer to LPS for at least 12 weeks after exposure to *S. choleraesuis* even after clearing the bacteria (Gray et al 1995a,c). This may result in a number of ELISA positive pigs which are no longer infected. It is unclear what effect vaccination has on the outcome of this assay. However, data indicates swine vaccinated with a commercially available modified live, plasmidless *S. choleraesuis* vaccine do not initiate a humoral immune response to *S. choleraesuis* antigens (Gray 1995e). This would suggest that swine vaccinated with this strain would appear as noninfected pigs on a diagnostic test.

Another ELISA has been utilized to detect antibodies in *Salmonella* carrier swine employing a heat-extracted antigen (Kramer et al. 1994). The results from this study indicate that most pigs infected with *S. typhimurium* or *S. choleraesuis* have an antibody response to this antigen. This assay shows a correlation between higher magnitude of infectivity and a higher antibody response.

Polymerase chain reaction

The extraordinary ability of the polymerase chain reaction (PCR) to exponentially replicate a target DNA sequence has made it a very powerful tool in the armamentarium of the diagnostician, epidemiologist and molecular biologist. This assay is based on the ability of target (organism) specific primers which, through complimentary DNA base-pairing, anneal only to the target sequence. Thermostable DNA polymerase recognizes the template primer complex as a substrate which results in the simultaneous copying of both strands of the segment of DNA between the two annealed primers. The denaturation annealing and elongation steps take place in a cyclical fashion relying on the thermostability of the *Taq*-polymerase until the target sequence is amplified to detectable amounts (Ehrlich and Sirko 1994).

The PCR assay has been used to identify *Salmonella spp.* in food and clinical samples (Araj and Das Chugh 1987; Rahn et al. 1992; Cohen N.D. et al. 1993). However, obstacles in the detection of organisms include the presence of substances inhibitory to PCR (Rossen et al. 1992; Wilde et al. 1990) and the inability to detect $<10^3$ CFU per gram of sample without preenrichment (Ehrlich and Sirko 1994). Investigators have improved detection methods in PCR assays by combining it with immunomagnetic separation (Widjojoatmodjo et al 1991; Widjojoatmodjo et al. 1992) or by enrichment culture (Stone et

al. 1994).

FUTURE DIRECTION

Control of infection caused by serotypes other than *S. choleraesuis* is reliant on detecting the carrier pig, contaminated feed, or environmental sources of infection. Pigs are most likely to develop disease during periods of stress or when exposed to large numbers of salmonellae. The commingling and transport of weanling pigs from different sources to finishing farms enhances activation of latent carriers and assures exposure of stressed pigs to salmonellae (Allred 1972).

Because *S. choleraesuis* is rarely, if ever, isolated from feed or feed ingredients the source of new infections would seem to be limited to carrier pigs and facilities previously contaminated with this serotype. It is not uncommon for outbreaks to occur in facilities with good sanitation suggesting that stress is a likely contributor to disease.

Management practices which allow filling of grower and finisher rooms with single source and age pigs is beneficial. In addition, careful attention to good management practices such as proper animal density, dry comfortable pens, temperatures and adequate ventilation is critical (Wilcock and Schwartz 1992).

sporadic at best because of the unpredictable nature of fecal shedding. Even repeated negative cultures may not ensure that a herd or individual is not a potential source of infection. Use of *Salmonella* serology will determine if the animal has had previous exposure to salmonella but this has not been shown to have relevance to the carrier status or to the predictable probability of shedding of an individual animal (Nielsen et al. 1994; Kramer et al. 1994). However, serological testing of herds will provide valuable information regarding the ongoing prevalence of infection in the herd and allow a measurement regarding success of control strategies. Additionally, refusal to introduce animals which have a positive titer will eliminate the introduction of potential carriers, but may also eliminate a proportion of the population which is not infected (Wilcock and Schwartz 1992).

In the United States monitoring herds for *Salmonella spp.* is not commonly practiced. However, other countries have had success with a monitoring and reduction program (Nielsen 1995). Current interest suggests that monitoring herds for the presence of *Salmonella spp.* in the United States may become more common. In addition, there is a great deal of research being conducted regarding the analysis of hazards and the critical control points for reduction of *Salmonella spp.* in both the pre and post-harvest setting of swine production.

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CHAPTER 2: INFLUENCE OF INOCULATION ROUTE ON THE CARRIER STATE OF *SALMONELLA CHOLERAESUIS* IN SWINE

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Running Title: *Salmonella choleraesuis* carrier state in swine

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Abstract

This study was designed to investigate the carrier state of swine infected with *Salmonella choleraesuis*. Thirty-five pigs were divided into 3 groups. Groups 1 (n=15) and 2 (n=16) were challenged with 10^8 CFU of *S. choleraesuis* intranasally or by gastric route, respectively. Group 3 (n=4) served as uninoculated controls. Pigs were necropsied at 2, 4, 6, and 12 weeks post inoculation. Clinical signs and microscopic lesions were more severe for group 1. *Salmonella choleraesuis* was recovered from a greater percentage of tissue samples for group 1 versus group 2 at 2, 4, and 6 weeks post inoculation. No differences were observed between groups at 12 weeks post inoculation. Regardless of route of inoculation, *S. choleraesuis* was most often recovered from the ileocolic junction, ileocolic lymph node, cecal contents, tonsil, lung and colon. Both groups shed *S. choleraesuis* in the feces sporadically throughout the 12 week period indicating that a carrier state is maintained for at least 12 weeks. However, group 1 shed higher numbers of *S. choleraesuis* initially. Serum IgG, IgM, and IgA antibody responses to *S. choleraesuis* lipopolysaccharide and heat extract antigens were observed for both groups. Higher serum IgG antibody titers to *S. choleraesuis* lipopolysaccharide were observed for group 2. Intestinal antibody responses for both groups included IgG and IgM responses but not an IgA response. Both routes of

inoculation stimulated peripheral blood B-cells while the intranasal route (group 1) was more effective at stimulating peripheral blood T-cells. The reduction in numbers of tissues infected and shedding observed for both groups coincided with the development of the host immune response. These data indicate that route of inoculation affects the development of humoral and cellular immunity, influences the amount of *Salmonella* shed into the environment and the distribution of *Salmonella* within tissue.

Keywords:

Salmonella choleraesuis, carrier state, swine.

Introduction

Salmonella choleraesuis is a host adapted, facultative intracellular pathogen that causes swine paratyphoid (Wilcock and Schwartz, 1992). It is the most frequent *Salmonella* serotype recovered from swine (Ferris and Miller, 1990) and was isolated from >95% of swine salmonellosis outbreaks in Iowa in 1989 (Schwartz, 1990). The National Animal Health Monitoring Survey estimated that swine salmonellosis is responsible for 28 million dollars in annual production losses in Iowa and 100 million in losses nationwide (Schwartz, 1990).

The carrier state of *S. typhimurium* in swine has been described (Wood et al., 1989, Wilcock and Olander, 1978). It has also been reported that a build up of *Salmonella* in the intestinal tracts of asymptomatic carrier swine can occur when they are subjected to stress suggesting that the asymptomatic carrier may be important in dissemination of *Salmonella spp.* in the environment (Hansen et al., 1964, Morgan et al., 1987, Williams, 1970). Although *S. choleraesuis* is the most frequent porcine isolate, it is rarely isolated from swine feeds or non-porcine *Salmonella* reservoirs. The source of *S. choleraesuis* seems to be limited to carrier pigs and facilities previously contaminated with this serotype (Wilcock and Schwartz, 1992).

The carrier state of *S. choleraesuis* in swine, including the duration of shedding, location of organisms and immune status, has not been studied (Wilcock and Schwartz, 1992). Lack of information regarding the carrier state of *S. choleraesuis* in swine has hindered efforts to control the disease.

Pneumonia caused by *S. choleraesuis* has been induced by intranasal inoculation (Baskerville and Dow, 1973) while oral inoculation of *S. choleraesuis* typically results in enterocolitis and septicemia (Lawson and Dow, 1965). Interestingly, the occurrence of pneumonia associated with *S. choleraesuis* infection in swine has increased in recent years

(Turk et al., 1992). No information is available regarding the effect of route of inoculation on development of the carrier state.

The purpose of this study was to determine if the route of inoculation of swine with *S. choleraesuis* affects the induction of the carrier state and to evaluate the subsequent immune response.

Materials and Methods

Bacterial strains and challenge cultures. Wild type *S. choleraesuis* var. *kunzendorf* χ3246 (Kelly et al., 1992) was kindly provided by the laboratory of Roy Curtiss III, Washington University, St Louis, MO. A 12 week old pig was intranasally inoculated and the isolate was recovered from the ileocolic lymph node (ICLN), stored at -70°C in glycerol, and used as the challenge strain (3246pp). Challenge cultures were prepared by inoculating 10 ml of LB broth (Sambrook et al., 1989) with 100 µl of a -70 frozen stock culture, and incubating the culture overnight at 37°C on an orbital shaker at 150 rpm. A 1% inoculum was transferred into fresh LB broth and the culture was grown for 3.5 hours at 37°C and 220 rpm. The culture was centrifuged, the pellet was resuspended in 1/2 volume of 0.01 M phosphate buffered saline (PBS, pH 7.0) and adjusted to a concentration of $1x10^8$ CFU/ml (OD_{600}=.123) in PBS.

Swine. Thirty-five 10 to 14 day old pigs were weaned, transported to isolation facilities at the National Animal Disease Center and raised in isolation (Fedorka-Cray and Harris, 1995). Tonsil (T), nasal (N) and rectal (R) swabs and fecal samples of piglets were repeatedly cultured to determine if *Salmonella spp.* were present through 6 weeks of age. Fecal samples from maternal sows were cultured pre- and post-farrowing.

Experimental design. At 6 weeks of age pigs were randomly divided into three groups. Each group was housed in separate isolation facilities and allowed to acclimate to the new environment for 1 week. Pigs were challenged at 7 weeks of age (day 0). Group 1 (n=15) was challenged intranasally with 1 ml (0.5 ml in each nostril dropwise on inspiration, alternating nostrils) of strain 3246pp at $1x10^8$ CFU/ml in PBS. Group 2 (n=16) was challenged via gastric route using size 000 gelatin capsules loaded with approximately 0.75 g of pelleted feed and 1 ml of strain 3246pp at $1x10^8$ CFU/ml in PBS. The capsule was inserted past the tonsil into the proximal end of the esophagus by use of a Pet Piller^R (H-Bar-S, Boerne, TX). Group 3 (n=4) served as uninoculated controls.

Two mls of whole blood was collected in sodium heparin from all pigs on days 2,4,6 and 8 post-inoculation (PI) and cultured as described below.

Four pigs from group 1 and 2, and one pig from the control group were euthanized and necropsied at 2,4,6 and 12 weeks PI, except that only 3 pigs from group 1 were necropsied at 12 weeks. Tissues were collected aseptically (sterile gloves and instruments for each tissue) for bacteriologic and histologic examination and included the turbinate (1 g; approximate weight), tonsil (4 g), thymus (3 g), mandibular lymph nodes (mandib-LN; 3 g), trachea (1 g), esophagus (1 g), lungs (8 g), bronchial lymph nodes (bronch-LN; 2 g), stomach wall (stom wall; 2 g), spleen (5 g), liver (5 g), middle ileum (ileum-mid; 4 g), ileocolic junction (ICJ; 6 g), ICLN (3 g), cecum (4 g), and colon (4 g); stomach contents (stom cont; 20 g) and cecal contents (cec cont 25 g) were also collected and cultured.

Histopathology. Tissues for microscopic examination were placed in 10% neutral-buffered formalin and fixed for 24 hours before processing. Tissues were embedded in paraffin wax, sectioned, stained with hematoxylin and eosin, and examined under a light microscope.

Clinical signs. Rectal temperatures and clinical signs were taken 2 times daily until day 3 PI for pigs in all groups. Between days 4 and 14 PI, pigs were monitored once daily then once weekly after day 14 PI.

Rate of gain. Pigs were weighed on days -3, 4, 11, 21, 25, 32, 39 and 46 PI. The daily rate of gain was calculated for each group.

Bacteriologic examinations. The schedule for bacteriologic sampling is summarized in Table 1. Fecal pools consisted of fresh 1-2 g samples of feces randomly collected from 10 different areas in a pen. Tissues (collected at necropsy) were minced using a sterile scalpel then homogenized. Fecal pools and tissues were homogenized in a stomacher 80 lab blender (Tekmar, Cincinnati, OH). All T, N, R swabs, fecal pools and tissues were incubated at 37°C in GN-Hajna broth (GN; Difco, Detroit, MI) for 18 to 24 hours then streaked on brilliant green agar with sulfadiazine (80 μg/ml; BGS; Difco, Detroit, MI). Additionally, at 18 to 24 hours 100 μl of GN was transferred to Rappaport-Vassiliadis (RV) medium (Vassiliadis, 1983), incubated at 37°C for 18 hours, then streaked to BGS. All BGS plates were incubated 24 hours at 37°C. Colonies having the appearance typical of *Salmonella* were picked and inoculated into triple sugar iron and lysine iron agar slants. Isolates having biochemical reactions typical of *Salmonella* were confirmed as group C by agglutination with *Salmonella* antiserum group C_1O (Difco, Detroit, MI). Additionally, all isolates from the cecum, cec cont, ICJ, colon, and feces were confirmed to be urease and

indol negative. Representative isolates were serotyped at the National Veterinary Services Laboratory.

Quantitative bacteriology was conducted on the tonsil, lung, ICLN, ICJ, and cec cont using the 5 tube most probable number (MPN) method (Wood and Rose, 1992) with GN, BGS and RV media as described above and are reported as the mean of the respective group for each necropsy day.

Antigen preparation. Antigens used in ELISA assays were prepared as follows. *Salmonella choleraesuis* 3246pp lipopolysaccharide (LPS) antigen was prepared by the method of Westphal (1965) and lyophilized. Heat extract antigen (HE) from *S. choleraesuis* 3246pp was prepared by growing 1 liter of culture in super broth (Greenstein and Besmond, 1993) for 5 hours at 37°C and 220 rpm. The culture was centrifuged (12000 x g for 10 min) and resuspended in 20 ml sterile distilled H_2O. The resuspended pellet was placed in an Omni Mixer® (Omni International, Waterbury, CT) and homogenized on setting 6 for 20 minutes. The homogenate was removed, placed in an Erlenmeyer flask and heated in a 65°C water bath for one hour. The cells were removed by centrifugation at 19000 x g for 6 minutes and the supernatant was removed and filtered through a Millex-GV© 0.22 μm filter and stored at -20°C until use.

Antigens used in lymphocyte blastogenesis assays were prepared as follows. Endotoxin from *S. choleraesuis* 3246pp

was prepared by the method of Morrison and Leive (1975). *Salmonella choleraesuis* 3246pp HE was prepared as described above, except that the homogenization step was omitted.

Protein determination. Total protein (μg/ml) was determined by the method of Bradford (1968) using bovine serum albumin as the standard.

Intestinal antibody. Local intestinal antibody secretion was measured in intestinal wash collected as previously described (Dean, 1990).

ELISA. Serum and intestinal antibody titers were determined by ELISA in 96-well Immulon 1 (Dynatech, Chantilly, VA.) plates incubated overnight at 4°C with 6 μg/well of 3246pp LPS antigen diluted in 0.01 M PBS (pH 7.2). Immulon 2 plates (Dynatech, Chantilly, Va.) were coated with 6 μg/well of 3246pp HE antigen diluted in 0.01 M PBS (pH 7.2).

Serum samples were serially diluted two-fold in diluent buffer (PBS, pH 7.2, 0.04% Tween 20 (Bio-Rad, Richmond, CA), 0.5% BSA). Samples were added to coated plates (50 μl/well) and incubated 1h at 37°C then washed 3x with wash buffer (WB; PBS, pH 7.2, 0.04% Tween 20). Immunoglobulin titers were determined by the addition of 50 μl/well peroxidase conjugated goat anti-swine IgM (mu specific) antibody diluted 1:1000 or

goat anti-swine IgG (gamma specific), peroxidase conjugated antibody diluted 1:1500 (Kirkegaard and Perry, Gaithersburg, MD). Plates were incubated 1h 37°C and washed 3x with WB. Enzyme-substrate reactions were initiated by the addition of 25 µl of TMB microwell substrate (1 component, Kirkegaard and Perry), as per manufacturers instructions.

Serum IgA titers were determined by the method of Stabel et al. (1990) using the Immulon plates and antigens described above. For all assays, absorbance of all wells was measured at 410nm. Titers were determined by taking the reciprocal of the highest dilution having an optical density greater than the highest optical density of the day 0 serum sample. Titers are reported as the mean of the experimental value minus the mean of the control pig values.

Intestinal antibody responses were determined by adding 50 µl of undiluted intestinal wash to antigen coated plates in triplicate. Plates were incubated overnight at 4°C. The assay was completed as described above. Optical densities are reported as the mean of the experimental value minus the mean of the uninoculated control value.

Lymphocyte Blastogenesis Assay. Blastogenesis assays were performed at weeks 2, 4, 6 and 12 PI on necropsy pigs. Peripheral blood lymphocytes (PBL) were separated from heparinized blood by centrifugation over Ficoll-Paque

(Pharmacia, Upsala, Sweden) according to manufacturer's instructions. Cells were resuspended in 10 ml of Hank's balanced salt solution without calcium or magnesium (HBSS) and 10 ml of lysing solution (0.013 M Na_2HPO_4, pH 7.2), incubated for 40 seconds at room temperature and returned to isotonicity with 20 ml of restoring solution (0.1 M PBS, pH 7.4). Cells were centrifuged (450 x g), washed twice in HBSS and resuspended to 2.5 x 10^6 cells/ml in RPMI (RPMI 1640 with L-Glutamine; Gibco, Gaithersburg, MD) containing 10% fetal bovine serum, 25 mM HEPES (Sigma) and 50 mg/ml gentamicin (Sigma). Microtiter plate wells contained cells (100 µl/well in triplicate) and 100 µl of the either 3246pp endotoxin or 3246pp HE antigen diluted to 50 µg/ml in RPMI media. Plates were incubated at 37°C with 5% CO_2 for 48 hours for B-cell proliferation assays or 5 days for T-cell proliferation assays. After incubation plates were pulsed with 0.5 µCi of ^{3}H-thymidine per well, incubated for 18 hours and harvested. Values are reported as the mean stimulation index (SI; defined as experimental values divided by negative control values) plus or minus the standard error of the mean for the necropsied pigs in each group. Wells containing media and cells alone served as negative controls and Concanavalin A (Sigma) at 10.0 µg/ml served as a positive control.

Statistical analysis. Postmortem results of *S. choleraesuis* recovery between groups, between tissues and ELISA titers between groups were evaluated by the X^2 test.

Results

Clinical signs. A febrile response occurred in group 1 which peaked at 41.3°C, 48 hours PI; group 2 peaked at 41°C, 72 hours PI (data not shown). On days 2 through 7, all group 1 and 2 pigs exhibited signs of depression and coughing, diaphragmatic breathing and chills were observed. However, clinical signs were more severe in group 1. Diarrhea (mild to severe) was also observed in 94% of group 1 pigs between days 5 through 10 and in 25% of group 2 pigs. One group 1 pig developed a self-limiting rectal prolapse. No mortality occurred in either group. Clinical signs resolved for both groups by day 14 PI.

Microscopic lesions. Group 1 pigs had more numerous and more severe microscopic lesions than group 2 pigs. Three group 1 pigs necropsied at 2 weeks PI had lesions in the cecum, liver, or lung. One of these pigs had marked focally-extensive erosive typhlitis and mild multifocal acute necrotizing hepatitis; the second pig had moderate multifocal necrotizing hepatitis; the third pig had marked focally

extensive suppurative bronchopneumonia. At four weeks PI, 2 pigs from group 1 had mild erosive typhlitis. Conversely, at 2 weeks PI, only 1 pig in group 2 had microscopic lesions which consisted of marked erosive typhlitis and marked multifocal necrotizing hepatitis. Remaining pigs in the study lacked microscopic lesions.

Weight gain. The average rate of gain for group 1 was negative for the first 11 days PI, whereas the control group and group 2 each gained at approximately 227 g/day. After day 11 the average daily gain for all three groups was similar (approximately 386 g/day; data not shown).

Antemortem bacteriologic results. All source sows and pigs were culture negative for *Salmonella spp* prior to challenge. *Salmonella choleraesuis* was recovered from antemortem blood culture from one group 1 pig on day 4 PI. The frequency of recovery of *S. choleraesuis* from fecal and T, N and R swab cultures is presented in Table 2.

Excluding day 0, *S. choleraesuis* was recovered from 8 of 15 (53%) fecal pools from group 1 versus 9 of 15 (60%) from group 2 (Table 2). *Salmonella* was not recovered from swabs or feces of group 3.

The magnitude of fecal shedding was markedly higher for group 1 than group 2 (Fig. 1). Both groups shed low numbers

sporadically after 2 weeks PI.

Postmortem bacteriologic examination. The frequency of recovery of *S. choleraesuis* is presented in Tables 3 and 4 for groups 1 and 2, respectively. *Salmonella choleraesuis* was recovered from more tissues in group 1 than group 2 at 2, 4 and 6 weeks PI. The frequency of isolation was equal for the two groups at 12 weeks.

Qualitative bacteriological data was combined for both groups by tissue for statistical analysis. These data indicate that *S. choleraesuis* is significantly more likely ($p<.01$) to be found in the ICJ, ICLN, cec cont, tonsil, lung, colon, liver and cecum than any other tissue (higher to lower order of likelihood, except that colon, liver and cecum are equal). Significant differences between group total positives (by necropsy) were not observed.

The mean population ($\log_{10}$/g tissue) of *S. choleraesuis* was determined for the tonsil, lung, ICLN, ICJ and cec cont and is presented in Table 5. *Salmonella choleraesuis* was not detected in the tonsils of either group at 2 weeks however, tonsils were positive for both groups at 4 weeks. Tonsil populations increased markedly at 6 weeks PI (4 $\log_{10}$ CFU/g). Group 2 populations remained at this concentration until 12 weeks PI while group 1 populations declined. Group 1 had higher populations of *S. choleraesuis* in the lung at 2 weeks PI, the ICJ at 2,4, and 6 weeks PI, the ICLN at 4 and 6 weeks

PI and the cec cont at 6 weeks PI. At 12 weeks PI a reversal was observed in that the cec cont population was higher in group 2 (Table 5.).

Serum and intestinal antibody responses. The mean serum IgG titers to 3246pp LPS antigen are shown in Fig. 2a. The peak response for group 1 was 262 at 10 weeks PI. In contrast, the peak serum IgG titer to LPS for group 2 was 548, 12 weeks PI.

The IgM titers to 3246pp LPS were similar for both groups. The titers peaked at 112 and 85 within 3 weeks PI for groups 1 and 2, respectively then declined steadily (Fig. 2b). The serum IgA response to LPS was similar for both groups through 5 weeks PI (Fig. 2b). After 7 weeks PI the group 1 IgA titer increased to 69 then declined steadily to 25 at 12 weeks while the group 2 titer decreased to 0 at 7 weeks PI (Fig. 2b).

Challenge with 3246pp resulted in relatively low titers to 3246pp HE antigen (Fig. 3). Serum IgG titers for groups 1 and 2 peaked at 10 weeks PI at 58 and 83, respectively. The IgM titers to the HE antigen peaked 3 weeks PI for group 1 (127) and 4 weeks PI for group 2 (73) then decreased sharply. No serum IgA titers were detected to the HE antigen. The serum antibody titers varied considerably between animals within a group. No statistically significant differences in

antibody titers were observed between groups.

Intestinal antibody response to 3246pp LPS occurred in both groups within 2 weeks PI (Fig. 4a). The IgG response of group 1 increased throughout the 12 weeks experiment. In contrast, the group 2 IgG intestinal response to LPS decreased until 6 weeks then rose sharply at 12 weeks (0.48 OD). The intestinal IgM responses were higher for group 1 versus group 2 through 6 weeks PI. However, at 12 weeks the group 2 intestinal IgM response to LPS increased to 0.11 OD (Fig. 4a). No IgA response to 3246pp LPS was detected for either group.

Intestinal antibody titers to *S. choleraesuis* HE antigen were not as high as intestinal responses to LPS antigen (Fig. 4b). However, IgG and IgM HE antigen trends for both groups were similar to the LPS response. No IgA response to 3246pp HE antigen was detected for either group.

Blastogenic responses. Peripheral blood mononuclear cells from both group 1 and 2 developed an antigen-specific proliferative response to *S. choleraesuis* endotoxin and HE antigens. Uptake of ^{3}H-thymidine after 2 days *in vitro* incubation is indicative of B-lymphocyte activation (Fig. 5), whereas ^{3}H-thymidine incorporation after 5 days is more indicative of T-lymphocyte activation (Fig. 6). Results indicated no significant difference in the proliferative response of lymphocytes group 1 and 2 swine following a 2 day

incubation with endotoxin or HE antigens. However, after a 5 day incubation of lymphocytes with the same antigens, an increase in ^{3}H-thymidine incorporation occurred at 2 weeks PI for group 2 and 4 weeks for group 1.

Discussion

Results of this study indicate that regardless of route of infection, *S. choleraesuis* can persist in the tonsil, ICLN, ICJ and colon and can be excreted in feces of experimentally infected pigs for at least 12 weeks PI. Therefore, upon infection with 10^{8} organisms and after resolution of initial clinical signs, the bacteria persist at relatively low numbers in clinically normal pigs establishing a carrier state.

Nnalue (1991) demonstrated that route of inoculation affects *S. choleraesuis* virulence in mice. In our study the intranasal (group 1) route of inoculation resulted in more lesions and more severe clinical disease suggesting that *S. choleraesuis* is more virulent in swine following exposure by this route. The pathology that was observed is consistent with acute disease caused by *S. choleraesuis* (Wilcock et al., 1976). Studies by Fedorka-Cray and Stabel (1993) with *S. typhimurium* indicate that the intranasal route of inoculation results in a wider tissue distribution in swine. This result was attributed to the involvement of lymphatogenous (which

includes the nasopharyngeal lymphoid tissues and tonsil) and hematogenous exposure.

Baskerville and Dow (1973) suggested that intensive rearing of pigs may contribute to pneumonia caused by *S. choleraesuis* and that feeding habits of swine ensure that pathogens present on food and in the environment enter the nasal cavity. In this study group 1 had a greater magnitude and duration of fecal shedding when compared to group 2, resulting in more environmental contamination. In addition, a larger number of *S. choleraesuis* positive tissues were observed for group 1 versus group 2 for the first 6 weeks PI. These results suggest that respiratory exposure affects the pathogenesis and maintenance of *S. choleraesuis* in swine.

There is a paucity of information regarding the magnitude and duration of fecal shedding of *S. choleraesuis*. One study indicated that 10^6 CFU of *S. choleraesuis*/g of feces is shed by pigs during acute infection following challenge with 10^{10} CFU of *S. choleraesuis* in sodium bicarbonate (Smith and Jones, 1967). The challenge in this study was 10^8 CFU in PBS and marked clinical illness was observed. The magnitude of shedding was 10^3 CFU/g which peaked between 72 hours and 3 weeks PI. Beyond 3 weeks PI *S. choleraesuis* was only detected in the feces in low numbers. However, shedding was detected in both groups throughout the 12 week period. The T, N and R swab data and fecal cultures indicate that although these

samples may be sufficient to detect *S. choleraesuis* within a herd, they are not effective in detection of individual carrier animals. However, since *S. choleraesuis* is mainly found in swine and swine operations (Wilcock and Schwartz, 1992) isolation from any sample suggests that an asymptomatic carrier is present, thereby increasing the likelihood for an outbreak within the herd. These data indicate that carrier animals shed low numbers of *S. choleraesuis* in feces and suggest that suspected shedders should be isolated from naive animals to control outbreaks involving *S. choleraesuis* in confinement units.

Under experimental conditions, there appears to be no significant loss in rate of gain for the subclinical carrier when compared to the control. These data suggest that following recovery from acute illness the length of time to market weight should not be affected. However, chronic *S. choleraesuis* infection, concurrent disease and/or stress may compromise these results.

Infection of swine with *S. choleraesuis* in the present study showed that the tissue distribution was similar to that reported for *S. typhimurium* (Wood et al., 1989) except that there was no predilection for *S. choleraesuis* to colonize the tonsil during acute disease. As the subclinical infection progresses the numbers of *S. choleraesuis* in the tonsil increase markedly. The organism may persist in the tonsil of

intranasally infected pigs until 19 weeks post challenge (data not shown). This suggests that the tonsils are a critical site for maintenance of *S. choleraesuis* in a herd. Although *S. choleraesuis* is often associated with pneumonia in swine, we only recovered the pathogen from the lung up to 6 weeks PI. This suggests that recovery from the lung, but not the tonsil, is indicative of acute disease, while recovery from the tonsil is more likely to be indicative of a carrier animal. Additionally the data indicate that the tissues in which *S. choleraesuis* is most likely to be found at necropsy are the ICJ (68% recovery; groups combined) and ICLN (65% recovery; groups combined) suggesting that they should be included in slaughterhouse surveys and necropsies where *S. choleraesuis* is a potential isolate.

The intestinal antibody responses observed were similar for both routes. Interestingly, no intestinal IgA response could be detected in contrast to the measurable serum IgA responses. However, a response may have occurred in the interval between necropsies or may have been too weak to detect in our assays.

The serum IgG titers to 3246pp LPS continued to increase through 10 and 12 weeks for the group 1 and 2, respectively. The IgM titers peaked between 2 and 3 weeks PI, then dropped as the IgG titers increased, indicating the point when isotype switch likely occurred. The trends in isotype responses

observed in the serum were also evident in intestinal immunoglobulin responses. This sequence of antibody isotype titers is indicative of a classic humoral response to bacterial pathogens.

It is also of interest that the increase in serum IgM titers at 2 weeks PI correlate with the marked increase in B-cell activity at 2 weeks PI. The times associated with increased T-cell activity may be correlated with the isotype switch observed for the humoral response.

Both routes of inoculation were equally effective at stimulating peripheral blood B-cells. However the intranasal route was more effective, although slower, at activating peripheral blood T-cells.

Eisenstein and Sultzer (1983) proposed that adequate immunity to *Salmonella spp.* requires both humoral and cellular responses in the mouse model. This study indicates that swine can harbor *S. choleraesuis* for at least 12 weeks in the presence of relatively high IgG response to *S. choleraesuis* antigens. In general, we observed a combined humoral, mucosal and cellular immune response to *S. choleraesuis* antigens which coincided with the reduction in overall of *S. choleraesuis* infection. This suggests that adequate immunity to *S. choleraesuis* in swine requires both humoral and cellular immunity.

In conclusion we have demonstrated that route of

inoculation impacts on the carrier animal. However, regardless of route of inoculation, a carrier state persists for at least 12 weeks following challenge with 10^8 CFU *S. choleraesuis*.

Acknowledgements

The authors thank Dr. Greg Mahairas, Dr. Roy Curtiss III and Sandra Kelly of Washington University, St. Louis, MO. for providing the original bacterial culture. The authors also thank Nels Nord, Ruth Willson, Kathy Foss, Brad Chriswell, Jeannie Frey, Nathan Adair and Jenny Emerson for excellent technical assistance.

The authors also thank Dr. Ted Kramer for sharing his expertise regarding *S. choleraesuis* in swine and his critical review of this manuscript and Dr. Evelyn Nystrom for her consultation on mucosal antibody response.

This research was completed as partial fulfillment of requirements for the degree Doctor of Philosophy at Iowa State University, Department of Microbiology, Immunology and Preventative Medicine.

All programs and services of the U.S. Department of Agriculture are offered on a nondiscriminatory basis without regard to race, color, national origin, religion, sex, age, marital status, or handicap.

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Figure Legends

Figure 1. Quantitative recovery of *S. choleraesuis* from fecal pools. Reported values are the MPN/g of feces for each group.

Figure 2. Serum antibody response of swine following challenge with *S. choleraesuis* by either intranasal (group 1) or gastric (group 2) route to *S. choleraesuis* LPS antigen. Reported values are the mean ELISA titer minus the mean control titer of either IgG (panel a) or IgM and IgA (panel b) isotype specific assays.

Figure 3. Serum antibody response of swine following challenge with *S. choleraesuis* by either intranasal (group 1) or gastric (group 2) route to *S. choleraesuis* HE antigen. Reported values are the mean ELISA titer minus the mean control titer of either IgG or IgM isotype specific assays.

Figure 4. Intestinal antibody response of swine following challenge with *S. choleraesuis* by either intranasal (group 1) or gastric (group 2) route, to *S. choleraesuis* LPS (panel a)

or HE antigen (panel b). Reported values are the mean ELISA IgG or IgM specific OD minus the mean control OD collected from intestinal wash samples (undiluted).

Figure 5. Two-day *in vitro* blastogenic response to *S. choleraesuis* endotoxin (shaded bars) and HE (hatched bars) antigen of peripheral blood lymphocytes from swine challenged with *S. choleraesuis* by either intranasal (group 1) or gastric (group 2) route and uninoculated control (group 3) swine.

Figure 6. Five-day *in vitro* blastogenic response to *S. choleraesuis* endotoxin (shaded bars) and HE (hatched bars) antigen of peripheral blood lymphocytes from swine challenged with *S. choleraesuis* by either intranasal (group 1) or gastric (group 2) route and uninoculated control (group 3) swine.

Table 1 - Bacteriologic sampling schedule

	Day			Week											
Sample	D1	D2	D3	1	2	3	4	5	6	7	8	9	10	11	12
T,N,R[a] Swabs	x[b]	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Fecal Pool[c]	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Necropsy[d]					x		x		x						x

a - T = tonsil, N = nasal, R = rectal; qualitative bacteriology.
b - x designates sample taken.
c - quantitative bacteriology.
d - qualitative and quantitative bacteriology

Table 2 - Number of samples positive for *S. choleraesuis* from tonsil, nasal, rectal swabs and fecal pools

		Day			Week												
		D1	D2	D3	1	2	3	4	5	6	7	8	9	10	11	12	
Group 1	Sample	15[a]	15	15	15	15	11	11	7	7	3	3	3	3	3	3	Total Pos.
	Tonsil	2	1	0	0	0	0	0	1	0	1	1	1	1	1	2	11
	Nasal	0	1	1	0	0	0	0	0	0	0	1	1	1	1	0	6
	Rectal	5	1	6	9	0	0	0	0	0	0	0	1	1	1	0	24
	Fecal	+[b]	+	+	+	-	-	+	-	-	-	+	+	-	+	-	8
Group 2		16[a]	16	16	16	16	12	12	8	8	4	4	4	4	4	4	
	Tonsil	8	2	3	0	0	0	0	1	2	0	0	0	0	1	1	18
	Nasal	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	3
	Rectal	8	1	0	0	0	0	0	0	0	0	1	0	0	0	0	10
	Fecal	+[b]	+	+	-	-	-	+	-	+	+	+	-	+	+	-	9

a- Number of pigs swabbed at each time point.
b- Results reported as *S. choleraesuis* present (+) or absent (-).

Table 3 - Recovery of *S. choleraesuis* from tissue of 15 intranasally challenged pigs.

	Necropsy (week)				
Tissue	2	4	6	12	Percent Pos
Turbinate	0	0	1	0	7%
Tonsil	1	1	1	2	33%
Thymus	0	0	0	0	0%
Mandib-LN	1	0	0	0	7%
Trachea	1	0	0	0	7%
Esophagus	0	0	0	0	0%
Lung	3	3	1	0	47%
Bronch-LN	2	1	1	0	27%
Stom wall	0	0	0	0	0%
Stom cont	0	0	0	0	0%
Spleen	2	2	1	0	33%
Liver	3	2	1	0	40%
Ileum-mid	1	0	2	0	20%
ICJ	3	4	4	1	80%
ICLN	4	4	2	0	68%
Cecum	1	3	1	0	33%
Cec Cont	2	3	2	2	60%
Colon	1	3	2	1	47%
Number Pos	25	26	19	6	
Collected	72	72	72	54	
Percent Pos	35%	36%	26%	11%	

Table 4 - Recovery of *S. choleraesuis* from tissue of 16 gastric (group 2) challenged pigs.

	Necropsy (week)				
Tissue	2	4	6	12	Percent Pos
Turbinate	0	0	0	0	0%
Tonsil	0	2	2	2	38%
Thymus	0	0	0	0	0%
Mandib-LN	0	0	1	1	13%
Trachea	0	0	0	0	0%
Esophagus	0	0	0	0	0%
Lung	2	1	0	0	19%
Bronch-LN	1	0	0	0	6%
Stom wall	0	0	0	0	0%
Stom cont	0	0	0	0	0%
Spleen	1	0	0	0	6%
Liver	2	1	0	0	19%
Ileum-mid	1	3	0	0	25%
ICJ	2	4	1	2	56%
ICLN	4	3	2	1	63%
Cecum	1	1	2	0	25%
Cec Cont	1	3	1	2	44%
Colon	0	0	2	0	13%
Number Pos	15	18	11	8	
Collected	72	72	72	72	
Percent Pos	21%	25%	15%	11%	

Table 5 - Mean populations of *S. choleraesuis* recovered from tissues of pigs challenged by intranasal (group 1) or gastric (group 2) inoculation from 12 weeks PI.

Log_{10}CFU/g tissue *S. choleraesuis* (n=4)

Week	Tonsil	Lung	ICJ	ICLN	Cec Cont
Group 1					
2	P[a]	3.6	5.8	4.4	<0.1
4	0.1	<0.1[b]	5.2	3.3	<0.1
6	4.1	<0.1	3.8	3.4	3.2
12	1.1	N[c]	<0.1	N	0.1
Group 2					
2	N	0.6	4.5	4.1	P
4	0.7	P	4.2	0.4	0.1
6	4.1	N	0.1	1.4	0.6
12[d]	4.2	N	0.7	0.7	1.1

a- positive by qualitative bacteriology only
b- <1/g designation
c- negative
d- 12wk group 1 n=3

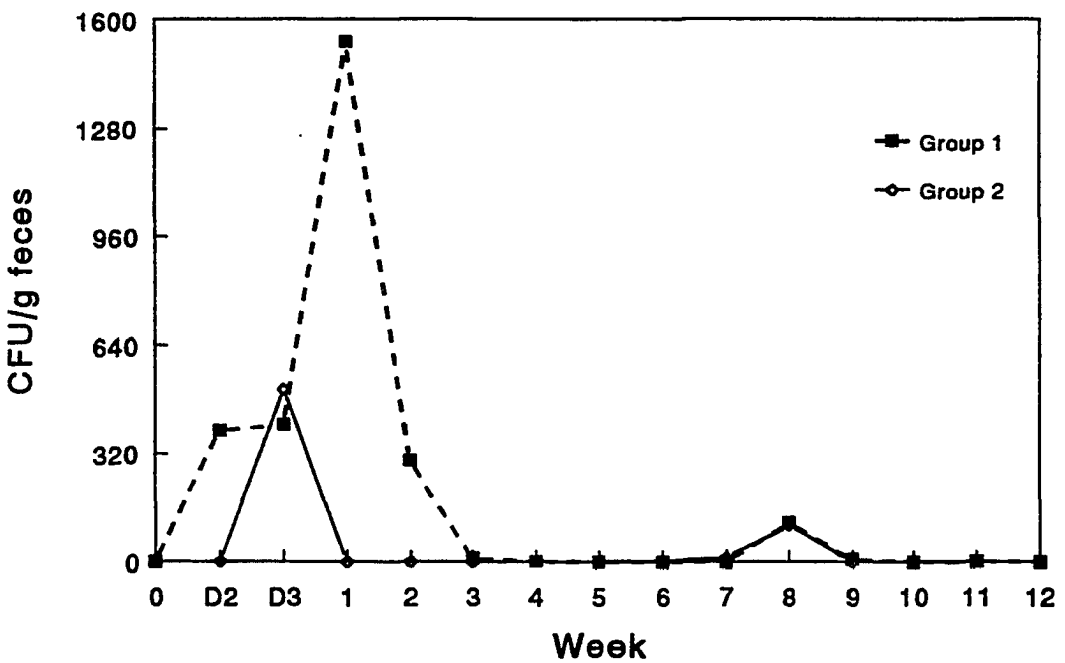
1600
1280
960
640
320
0
CFU/g feces
Group 1
Group 2
0
D2
D3
1
2
3
4
5
6
7
8
9
10
11
12
Week

Figure 1.

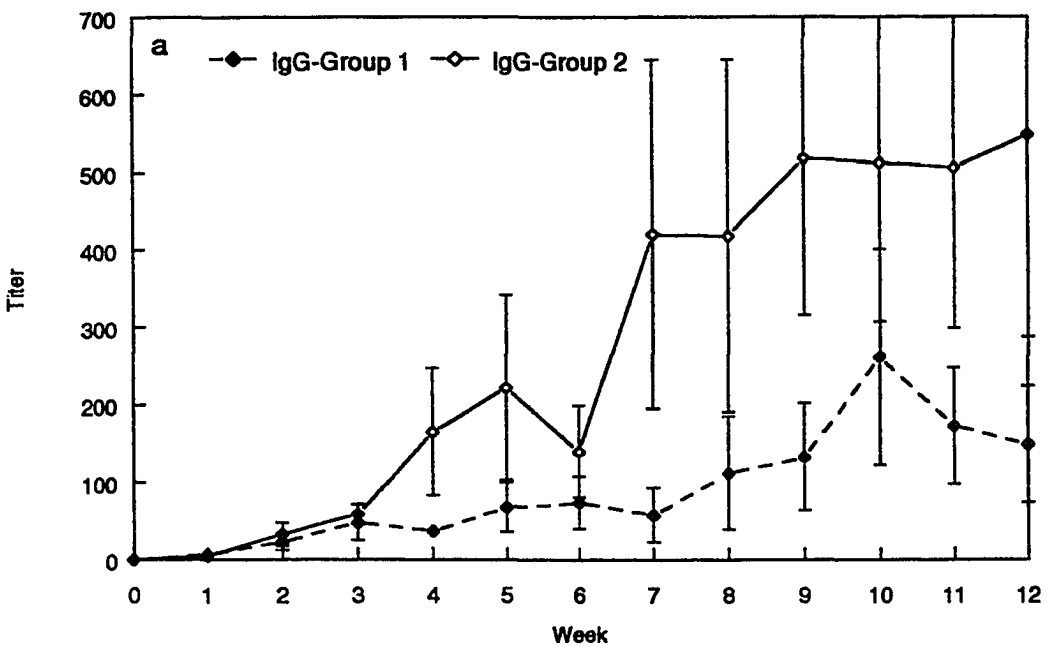
a
IgG-Group 1
IgG-Group 2
Titer
700
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
9
10
11
12
Week

Figure 2a.

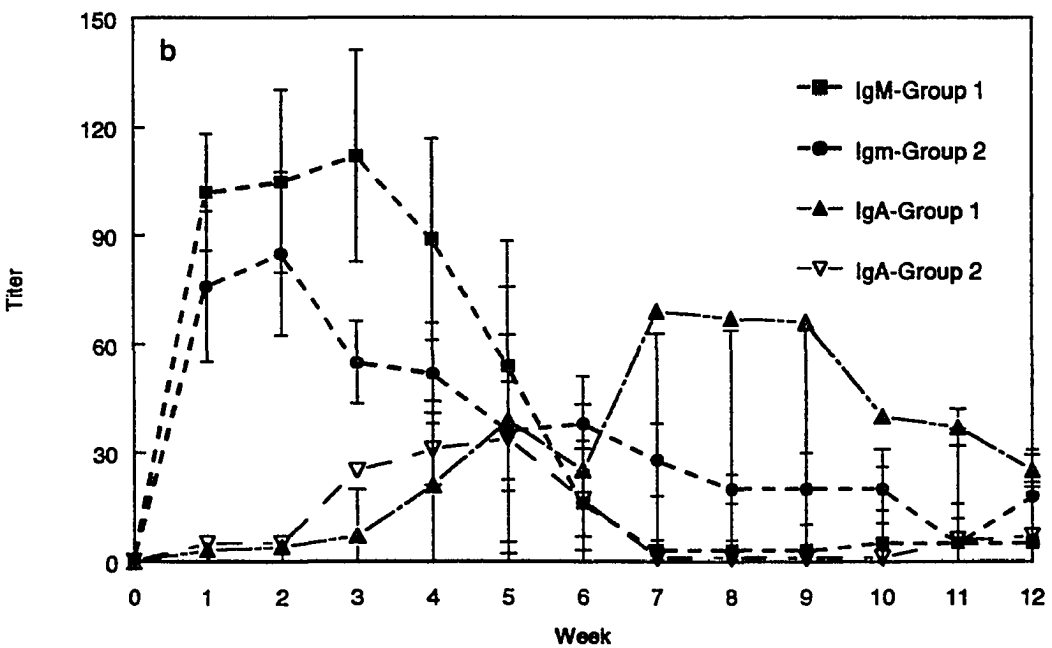
b
IgM-Group 1
Igm-Group 2
IgA-Group 1
IgA-Group 2
Titer
Week
150
120
90
60
30
0
0
1
2
3
4
5
6
7
8
9
10
11
12

Figure 2b.

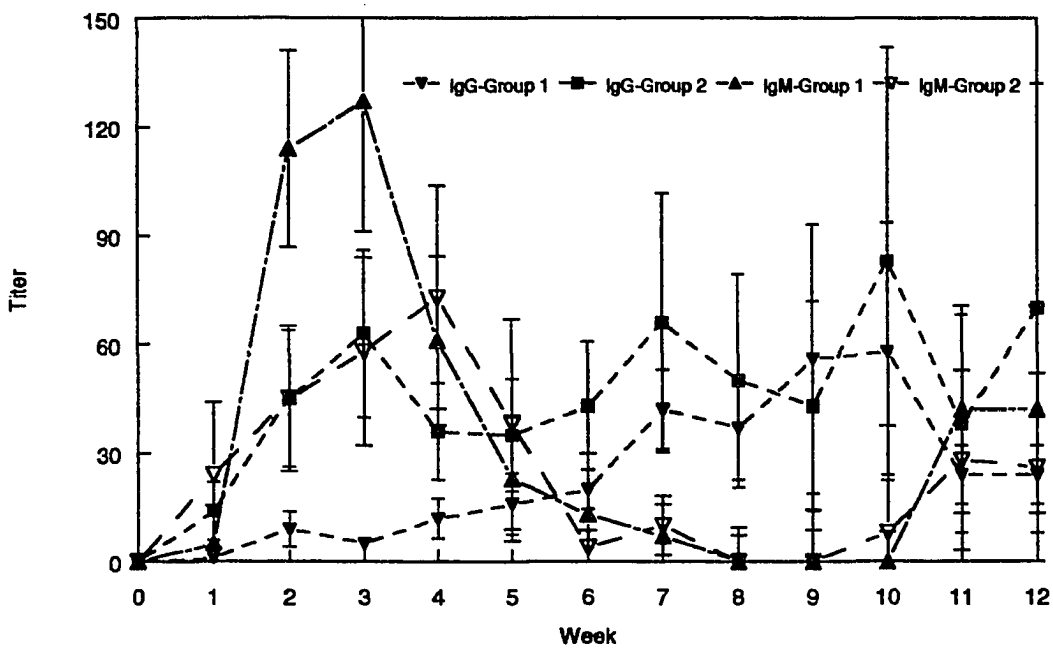
IgG-Group 1
IgG-Group 2
IgM-Group 1
IgM-Group 2
Titer
Week
150
120
90
60
30
0
0
1
2
3
4
5
6
7
8
9
10
11
12

Figure 3.

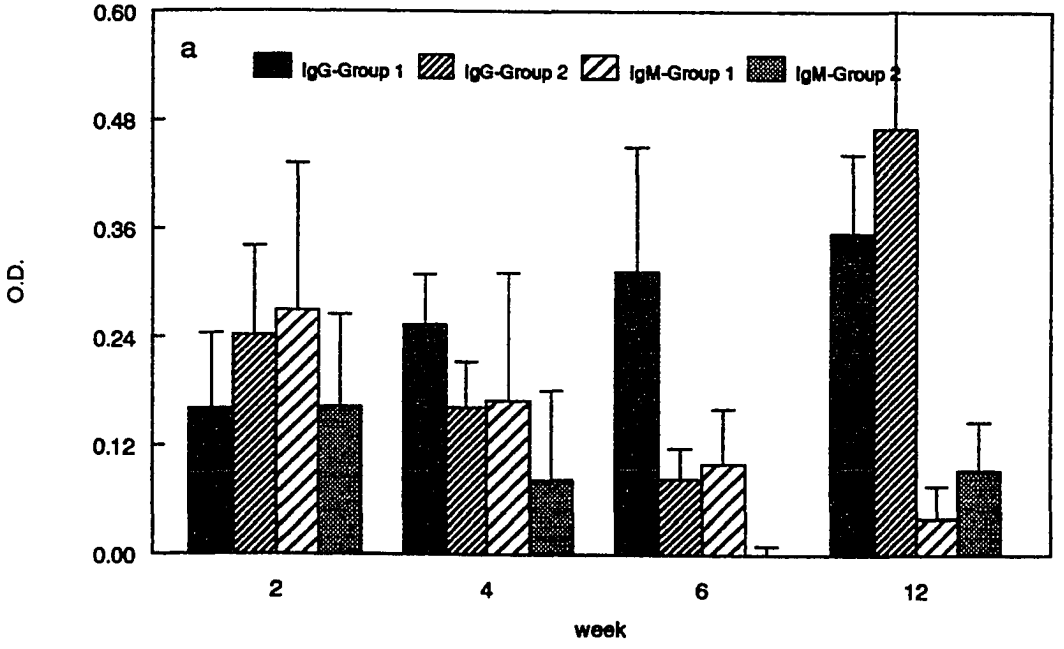
a
IgG-Group 1
IgG-Group 2
IgM-Group 1
IgM-Group 2
0.60
0.48
0.36
0.24
0.12
0.00
O.D.
2
4
6
12
week

Figure 4a.

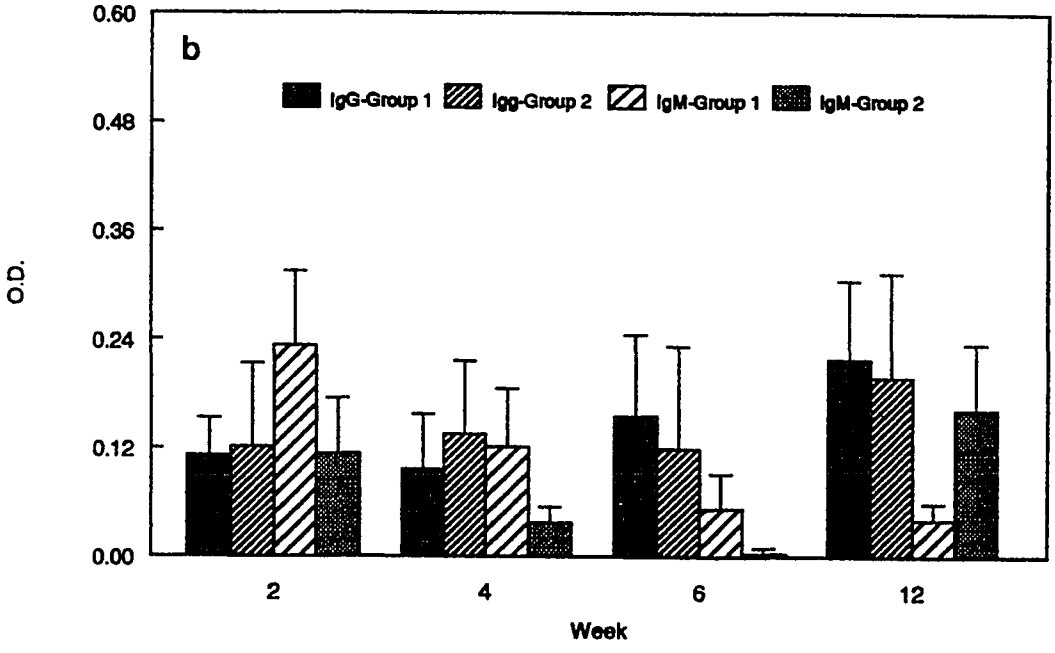
b
IgG-Group 1
Igg-Group 2
IgM-Group 1
IgM-Group 2
0.60
0.48
0.36
0.24
0.12
0.00
O.D.
2
4
6
12
Week

Figure 4b.

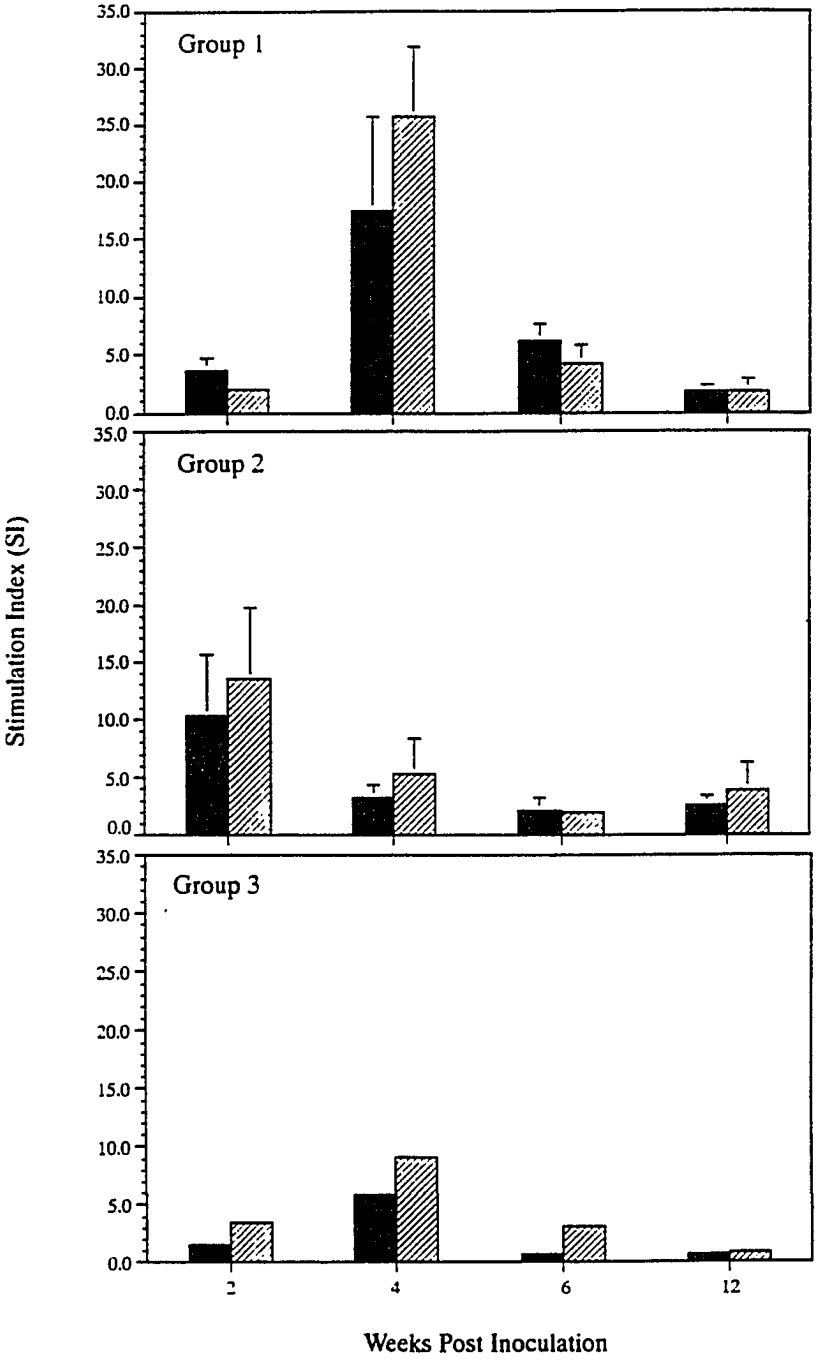
Group 1
Group 2
Group 3
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
2
4
6
12
Stimulation Index (SI)
Weeks Post Inoculation

Figure 5.

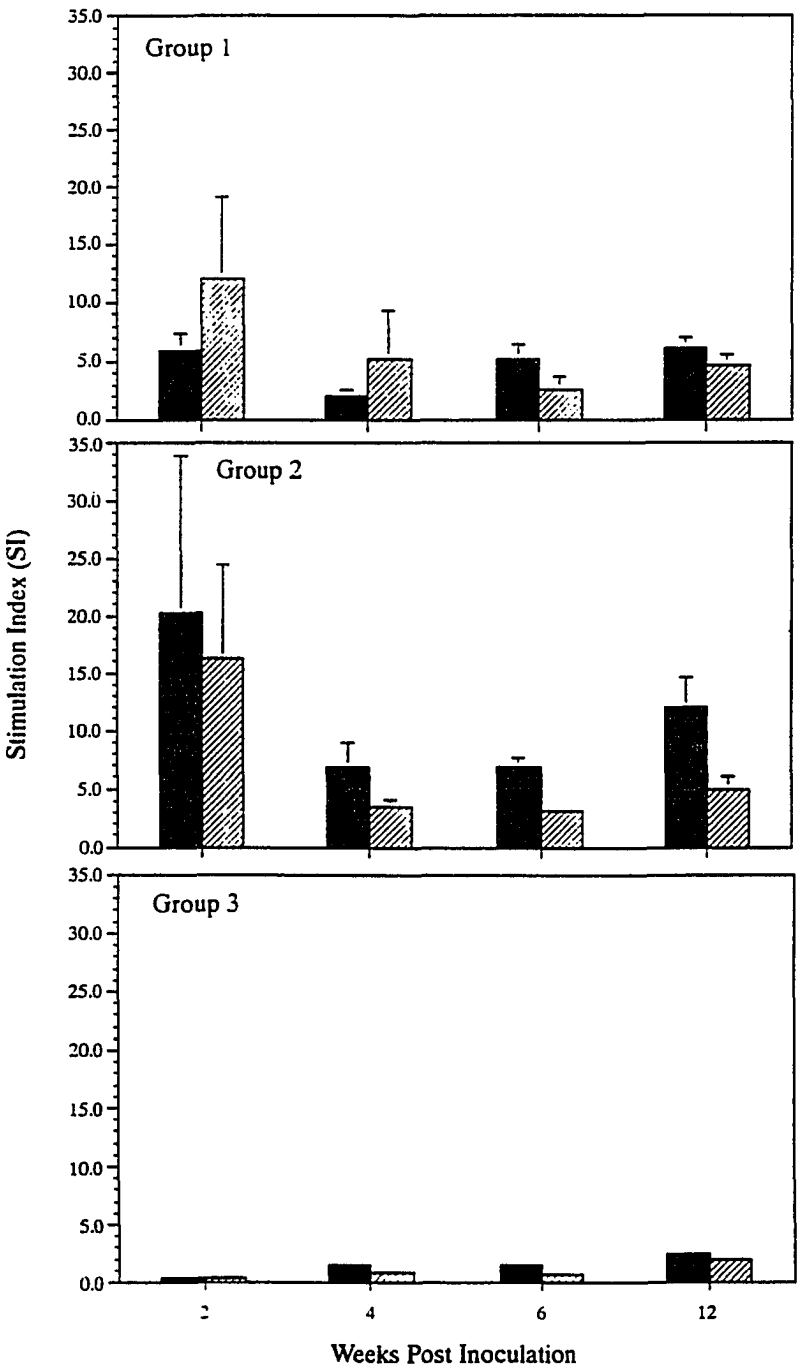
Group 1
Group 2
Group 3
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Stimulation Index (SI)
2
4
6
12
Weeks Post Inoculation

Figure 6.

CHAPTER 3: EFFECT OF DOSE ON THE IMMUNE RESPONSE AND PERSISTENCE OF *SALMONELLA CHOLERAESUIS* IN SWINE.

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Submitted to The

American Journal of Veterinary Research: (1995 accepted)

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¤ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

§ All programs and services of the U.S. Department of Agriculture are offered on a nondiscriminatory basis without regard to race, color, national origin, religion, sex, age, marital status, or handicap.

Footnotes

[a] Difco, Detriot, MI

[b] Omni International, Waterbury, CT

[c] Millipore, Bedford, MA

[d] Dynatech, Chantilly, VA

[e] Sigma, St. Louis, MO

[f] Bio-Rad, Richmond, CA

[g] Kirkegaard and Perry, Gaitherburg, MD

[h] Pharmacia, Upsala, Sweden

[i] Gibco, Gaithersburg, MD

Summary

Experiments were conducted to establish the effect of dose on the persistence of *S. choleraesuis* infection in swine and to elucidate the immune response. Nineteen pigs were divided into three groups which were housed in separate isolation facilities. Groups 1 (n=5), 2 (n=5) and 3 (n=5) were inoculated intranasally with 10^9,10^6 and 10^3 CFU of *S. choleraesuis*, respectively. Group 4 (n=4) served as uninoculated control pigs. Pigs were monitored for clinical signs, shedding patterns were determined, and serum was collected. Lymphocyte blastogenesis assays were conducted at 0, 2, 4 and 6 weeks post inoculation (PI). Three pigs from groups 1, 2 and 3 and 1 pig from group 4, were necropsied 6

weeks PI. Two pigs from group 3 and 1 pig from group 4 were necropsied 10 weeks PI. All remaining pigs were necropsied 15 weeks PI. Pigs in group 1 shed *S. choleraesuis* throughout the 15 week experiment. A high percentage of the tissues sampled for were positive for *S. choleraesuis* at both 6 (89%) and 15 (67%) weeks PI for group 1. The highest concentration of infection was found in the tonsils of group 1 at both necropsies. For group 2 pigs, *S. choleraesuis* was recovered from ileocolic lymph nodes at 6 weeks PI and shedding was observed through 9 weeks PI. *Salmonella choleraesuis* was not recovered after nine weeks PI from pigs in group 2. In contrast, *S. choleraesuis* was not recovered at any time point from tonsil, nasal and rectal swabs, fecal samples or necropsy tissues of group 3 or group 4 pigs. High serum IgG and IgM titers to *S. choleraesuis* lipopolysaccharide and soluble antigens were observed for group 1. Group 2 had moderate IgG and IgM titers to LPS and low titers to soluble antigens. Group 3 had mild serum IgG and IgM titers to *S. choleraesuis* antigens. Increased lymphocyte proliferation was observed for groups 1 and 2 in response to *S. choleraesuis* endotoxin. All four groups demonstrated a lymphocyte response to concanavalin A. However, a lower stimulation index was observed in group 1 in response to *S. choleraesuis* endotoxin and concanavalin A which indicated that some form of lymphocyte immunosuppression occurred after the high dose challenge. These results

indicate that persistence of *S. choleraesuis* in host tissues is dose dependent. Short term persistence can occur after a dose as low as 10^6 CFU of *S. choleraesuis* (9 weeks). However with doses less than or equal to 10^6 the bacteria are eventually cleared. Higher doses of *S. choleraesuis* result in development of a long term carrier animal (at least 15 weeks) which may be related to observed lymphocyte immunosuppression.

Introduction

Salmonella choleraesuis is a host adapted, facultative intracellular pathogen that causes swine paratyphoid[1]. *Salmonella choleraesuis* is the most frequent *Salmonella* serotype recovered from swine[2] and was isolated from >95% of swine salmonellosis outbreaks in Iowa in 1989[3]. Although *S. choleraesuis* is the most frequent porcine isolate, it is rarely isolated from swine feeds or non-porcine *Salmonella* reservoirs. The source of *S. choleraesuis* appears to be limited to carrier pigs and facilities previously contaminated with this serotype[1]. It has also been reported that an increase in amounts of *Salmonella* in the intestinal tract of asymptomatic carrier swine can occur when they are subjected to stress[4,5,6]. Together these studies indicate that the persistently infected or asymptomatic carrier pig is critical in the maintenance, transmission, and dissemination of

Salmonella choleraesuis in the environment. Although the concept of an *S. choleraesuis* carrier state is not new, unlike *S. typhimurium*[7,8], specific details remain unknown.

The minimum dose of *S. choleraesuis* required to establish a persistent infection in swine has not been studied. There is also a lack of information regarding the minimum dose required to produce clinical disease. Only one report of moderate disease following a 10^6 inoculation of *S. choleraesuis* is available[9].

It has been shown that intranasal inoculation of *S. choleraesuis* in swine results in more severe clinical disease and dissemination of the organism to more tissues, as compared to a gastric challenge[10,11]. Pneumonia caused by *S. choleraesuis* has been induced by intranasal inoculation[12]. The occurrence of pneumonia associated with *S. choleraesuis* infection in swine has increased in recent years[13], implicating the respiratory tract as an important route of inoculation.

We intranasally inoculated pigs with 3 different doses of *S. choleraesuis* and then monitored the magnitude and duration of shedding, distribution of organisms in the tissue and the immune response of swine over a 15 week period.

Materials and Methods

Bacterial strains and challenge cultures. Wild type *S. choleraesuis* var. Kunzendorf ,3246[14] (3246), kindly provided by the laboratory of Roy Curtiss III, Washington University, St Louis, MO, was used as the challenge strain.

Challenge cultures were prepared by inoculating 10 ml of LB broth with 100 µL of a -70°C frozen stock culture of 3246. The culture was grown overnight at 37°C on an orbital shaker at 150 rpm. A 1% inoculum was transferred into fresh LB broth and grown for 3.5 hours at 37°C and 220 rpm. The culture was centrifuged, the pellet was resuspended in 1/2 volume of phosphate buffered saline (PBS), and adjusted to an final concentration of $1x10^9$ (OD_{600} 0.832), $1x10^6$ (1/1000 dilution of $1x10^9$) or $1x10^3$ (1/1000 dilution of $1x10^6$) CFU/ml in PBS. Final concentrations were confirmed by plate counts.

Swine. Source sows were monitored prior to weaning for presence of *Salmonella* by bacteriology as described below. Nineteen 10 to 14 day old pigs were weaned and transported to isolation facilities at the National Animal Disease Center. Pigs were raised in isolation as previously described[15]. Fecal pools and swabs were obtained from pigs prior to challenge at 2, 4, 6 and 8 weeks of age and cultured to determine if *Salmonella spp.* were present.

Experimental design. At 7 weeks of age, 19 *Salmonella* culture negative pigs were randomly divided into four groups. Each group was housed in separate isolation facilities and allowed to acclimate to the new environment for 1 week. Pigs were challenged at 8 weeks of age (day 0). Group 1 (n=5) was challenged intranasally (INT) with 1 ml (0.5 ml in each nostril dropwise alternating nostrils) of 3246 at $1x10^9$ CFU/ml. Groups 2 (n=5) and 3 (n=5) were challenged using the same procedures with 1 ml at $1x10^6$ CFU/ml and $1x10^3$ CFU/ml, respectively. Group 4 (n=4) served as controls and were sham inoculated with sterile PBS using the same procedures described above.

After inoculation, pigs were observed for clinical signs and rectal temperatures were taken daily for 14 days then weekly for 15 weeks. Antemortem bacteriologic examinations, which included separate tonsil (T), nasal (N), rectal (R) swabs and fecal pools, were performed on days 0,1,2,3,5,8 and weekly thereafter throughout the 15 week period. Fecal pools were obtained from the pens of each group by collecting fresh 1-2 g samples of feces randomly from 10 different areas. Blood samples for serum collection were obtained on day 0, then weekly through 12 weeks and at week 15.

Three pigs from groups 1, 2 and 3 and one control pig from group 4 were euthanized and necropsied at 6 weeks PI. Two pigs from group 3 and 1 control pig were euthanized and

necropsied at 10 weeks PI. At 15 weeks PI two pigs from groups 1 and 2 and 2 control pigs were euthanized and necropsied. Tissues were aseptically collected for bacteriologic examination. Tissues collected included the tonsil, lung, spleen, liver, ileal-colic junction (ICJ), ileal-colic lymph node (ICLN), cecum, cecal contents (Cec Cont), and colon.

Bacteriologic examinations. All T,N,R swabs, fecal pools and tissues (collected at necropsy) were incubated at 37°C in GN-Hajna[a] (GN) broth for 18 to 24 hours then streaked on brilliant green agar with sulfadiazine[a] and 200 μg/ml streptomycin (BGSS). Additionally, at 18 to 24 hours 100 μL was transferred to Rappaport-Vassiliadis (RV) medium[16], incubated at 37°C for 18 hours, then streaked to BGSS. All BGSS plates were incubated 24 hours at 37°C. Colonies having the appearance typical of *Salmonella* were picked and inoculated into triple sugar iron and lysine iron agar slants. Positive isolates were confirmed as group C by agglutination with *Salmonella* antiserum group C_1O[a]. Representative isolates were serotyped at the National Veterinary Services Laboratory, Ames, IA. Quantitative bacteriology was conducted using the 5 tube most probable number (MPN) method[17] with GN, BGSS and RV mediums as described above. Results are reported as the mean of the respective group for each necropsy day.

Erythrocyte sedimentation rate. Erythrocyte sedimentation rates (ESR) were determined as previously described[18]. Blood samples for ESR's were collected in EDTA tubes on days 0, 2, 3, and 8 PI.

Antigen preparation. Salmonella choleraesuis 3246 LPS antigen was prepared by the method of Westphal[19] and lyophilized. Soluble antigen from *S. choleraesuis* 3246 was prepared by growing 1 liter of culture in super broth (20 g tryptone, 10 g yeast extract, 20 g NaCl, pH 7.0) for 5 hours at 37°C and 220 rpm. The culture was centrifuged (12000 rpm for 10 min) and resuspended in 20 ml sterile distilled H_2O. The resuspended pellet was placed in an Omni Mixer[R;b] and homogenized on setting 6 for 20 minutes. The homogenate was removed, placed in an Erlenmeyer flask and heated in a 65°C water bath for one hour. The cells were removed by centrifugation at 19000 rpm for 6 minutes and the supernatant was removed and filtered through a Millex-GV[c] 0.22 μm filter and stored at -20°C until use.

Endotoxin from *S. choleraesuis* 3246 was prepared by the method of Morrison[20] and lyophilized for use in the lymphocyte blastogenesis assays.

Protein determination. Total protein was determined by the method of Bradford[21] using bovine serum albumin as the standard.

ELISA. Serum titers were determined by ELISA in 96-well Immulon[d] microtiter plates. Immulon 1 plates were coated with 3 μg per well of 3246 LPS antigen diluted in 0.01 M PBS (pH 7.2) and Immulon 2 plates were coated with 3 μg/well of 3246 soluble antigen diluted in 0.01M PBS (pH 7.2). Following addition of antigen, plates were incubated overnight at 4°C then washed 3 times with PBS. Wells were blocked with 3% bovine serum albumin[e] (BSA) for thirty minutes at room temperature then washed 3 times with PBS. Plates were allowed to dry at room temperature and stored at 4°C for up to 48 hours.

Serum samples were diluted two-fold in diluent buffer (PBS, 0.04% Tween 20[f], 0.5% BSA) in 96-well microtiter dilution plates. Immunoglobulin titers for IgM and IgG in sera were determined by incubating diluted sera on the antigen coated plates for 1 hour at 37°C. Following incubation the plates were washed 6 times with wash buffer (WB; PBS, pH 7.2, Tween-20 0.04%). The washes were followed by the addition of 50 μL/well goat anti-swine IgM (mu specific) peroxidase conjugated antibody[g] diluted 1/1000 or goat anti-swine IgG (gamma specific) peroxidase conjugated antibody[g] diluted

1/1500. All plates were incubated 1 hour 37°C. Plates were washed 6 times with WB before adding 25μL/well of TMB microwell substrate[g] (1 component) per well. The reaction was stopped by the addition of 50 μL/well of 1% sulfuric acid.

All plates were read at 410nm on a 96 well microplate reader[d]. Titers were determined by taking the reciprocal of the highest dilution having an optical density greater than the highest optical density of the day 0 serum sample. Titers are reported as the mean of the experimental value minus the mean of the respective group 4 control value.

Lymphocyte Blastogenesis Assay. Blastogenesis assays were performed on day 0 and at weeks 2, 4 and 6 PI on all pigs. Thirty ml of heparinized peripheral blood was collected from each pig and diluted with 30 ml of Hank's balanced salt solution without calcium or magnesium (HBSS). Thirty ml of diluted blood was then layered over 9 ml Ficoll-Paque[h] and centrifuged at 400 x g for 30 min. The mononuclear cell layer was removed and washed once with HBSS. Cells were resuspended in 10 ml of HBSS and 10 ml of lysing solution (0.013 M Na_2HPO_4, pH 7.2), incubated for 40 seconds at room temperature and returned to isotonicity with 20 ml of restoring solution (0.1 M PBS, pH 7.4). Cells were centrifuged (450 x g), washed twice in HBSS and resuspended to 2.5 x 10^6 cells/ml in RPMI 1640 (containing 2 mM L-Glutamine)[i], 10% fetal bovine serum,

25 mM HEPES° and 50 μg/ml gentamicin°. Cells were dispensed into 96-well microtiter plates (100 μL/well in triplicate) containing 100 μl of 3246 endotoxin diluted to 50 μg/ml in RPMI media.

The plates were incubated at 37°C with 5% CO_2 for 48 hours. At the end of the incubation period, cells were pulsed with 0.5 μCi of ^{3}H-thymidine per well, incubated for 18 hours and harvested. Values are reported as the mean stimulation index (SI; defined as experimental values divided by negative control values) plus or minus the standard error of the mean for the necropsied pigs in each group. Negative control wells contained media and cells only. Positive control wells contained concanavalin A°(ConA) at 10.0 μg/ml.

Results

Clinical response to exposure. Pigs in group 1 elicited a marked febrile response beginning 48 hours PI which lasted approximately 2 weeks. The response peaked on day 3 at 41.2°C. Group 2 also elicited a mild febrile response beginning on day 3 PI which also lasted approximately 2 weeks (peak day 3; 40.8°C). Pigs in group 1 were slightly lethargic on day 2 PI. Lethargy and depression progressed from mild to severe on day 3 persisting until day 10 PI. Mild to severe diarrhea was observed in group 1 which lasted until

2 weeks PI. Mild depression and lethargy, but no diarrhea, was noted in pigs in group 2 between days 5 and 8. No febrile response or clinical signs were detected for groups 3 or 4.

Erythrocyte sedimentation rates. Increased ESR values were observed for group 1 on day 2 (12.6 mm) and 3 (13.8 mm) PI. Group 2 had an increased ESR on day 3 (4.9 mm) PI (data not shown). The increased ESR values observed in groups 1 and 2 returned to normal by 1 week PI. No increase in ESR values were observed for groups 3 and 4.

Antemortem bacteriologic examination. Sows from which experimental pigs were obtained were culture negative for *Salmonella spp.* Prior to challenge all pigs were also culture negative for *Salmonella spp.* Group 4 control pigs remained free of *Salmonella spp.* throughout the experiment. Following challenge, *S. choleraesuis* was not recovered at any time on T, N, or R swabs or from fecal cultures of pigs in group 3. The frequency of recovery of *S. choleraesuis* on swabs and fecal cultures from groups 1 and 2 is presented in Table 1. *Salmonella choleraesuis* was recovered from both groups 1 and 2 beginning on day 1 PI. At least 1 positive swab was obtained from group 1 throughout the 15 week period with the exception of week 13. Positive swabs were obtained from nearly all of the pigs in group 1 at each time point through 10 weeks PI.

Fecal pools of group 1 were positive through 8 weeks PI and at weeks 10, 11 and 15.

Nearly all of the pigs in group 2 had positive swabs in the first week PI (4/5). The frequency of recovery of *S. choleraesuis* from swabs declined rapidly after 1 week PI. One positive rectal swab was obtained at 9 weeks PI from group 2 (this was the last positive swab from the group). Sporadic recovery of *S. choleraesuis* from fecal pools of group 2 was observed until 9 weeks PI.

The magnitude of fecal shedding for groups 1 and 2 is presented in figures 1a and 1b, respectively. The magnitude of fecal shedding peaked for group 1 at $4.9x10^3$ CFU/g on day 5 PI. A decline to $9.7x10^1$ CFU/g was observed at 3 weeks PI, followed by an increase to $7.9x10^2$ CFU/g at 5 weeks PI. After dropping to very low numbers (10^{-1}CFU/g), another smaller increase ($4x10^1$ MPN/g) was observed at 10 weeks PI. The peak magnitude of shedding in group 2 was $4.2x10^1$ CFU/g on day 5 PI, which decreased rapidly at 1 week PI to 10^{-1}/g. Further decline to undetectable levels at 5 weeks PI was noted. A slight increase in fecal shedding was observed on weeks 7 and 8 for group 2.

Postmortem bacteriologic examination. The frequency of recovery of *S. choleraesuis* is presented in Table 2. The recovery from group 2 tissues was infrequent with only 1

positive ICLN found at 6 weeks PI. In contrast, group 1 only had 1 negative tissue (spleen) at 6 weeks PI. Two of the 3 pigs had a positive tonsil, ICJ, ICLN, cecum, cec cont, and colon at 6 weeks PI. At 15 weeks PI, *S. choleraesuis* was found in the tonsil, cecum, cec cont, and colon of both pigs necropsied. One ICJ and ICLN were also positive. *Salmonella choleraesuis* was not recovered from any tissues from group 3 or group 4 pigs.

Quantitative bacteriology was performed on the tonsil, ICJ, ICLN, and Cec Cont and is presented in Table 3. The level of infection in group 2 was not quantifiable at 6 weeks PI. At 6 weeks PI, group 1 tissues had a minimum of 2 logs of *S. choleraesuis/g* of tissue, the tonsil had the highest concentration of infection (4.78 $\log_{10}$CFU/g). At 15 weeks PI the tonsil was the only tissue from group 1 that had quantifiable numbers of *S. choleraesuis* (average 3.16 $\log_{10}$CFU/g).

Serum antibody responses. The IgM response of group 1 to LPS antigen peaked 3 weeks PI at a (titer of 19) then decreased to undetectable numbers at 7 weeks PI (Fig 2). Groups 2 and 3 had low IgM titers to 3246 LPS (Fig 2).

The IgM titer of group 1 to soluble antigen rose rapidly after inoculation to a peak of 563 at 3 weeks PI, then decreased to 115 at 5 weeks PI. A more gradual decrease in

titer was observed after 5 weeks PI (Fig 3a). The IgM titer of group 2 to soluble antigen was low throughout the 15 weeks (peak titer 20, 15 weeks PI; Fig. 3b). Group 3 IgM titers to soluble antigen increased to 19 at 1 week PI. The titers remained low ($\leq$ 10) until 6 weeks PI when a marked rise in IgM response was observed for group 3 (peak 48). The titer then decreased until week 10 when the 2 remaining group 2 pigs were euthanized.

Group 1 IgG titers to LPS antigen did not increase until weeks 6 and 7 PI. After week 7, the titer rose steadily to a peak titer of 1536 at week 12, which persisted until 15 weeks PI. The IgG titer of group 1 to soluble antigen increased steadily to 2112 (excluding week 6) until week 7 PI, then leveled off and remained at an approximate titer of 2100 until 15 weeks PI (Fig 4a).

Group 2 IgG titers to LPS were low ($\leq$ 10) until 6 weeks PI when a peak titer of 116 was observed (Fig 4b). The IgG titer decreased to 10 at week 11 but then increased at weeks 12 and 15 (peak titer 132 at 15 weeks). Group 2 IgG titers to soluble antigen were consistently low (less than 20) throughout the experiment (Fig 4b). The group 3 titers to LPS antigen also remained low until 6 weeks PI when a peak of 58 was observed. Group 3 titers to soluble antigen were also low throughout the experiment (peak titer 20 7 weeks PI;Fig. 4b). Group 4 control pig titers, which were subtracted out of the

reported values, deviated from the day 0 bleed by a maximum titer of 8 (data not shown).

Lymphocyte blastogenic responses. Groups 1 and 2 showed increased lymphocyte proliferation in response to 3246 endotoxin as compared to the control group. Group 3 showed no increased sensitization of lymphocytes to 3246 endotoxin. Interestingly, the SI's of group 1 pigs remained below group 2 SI's indicating some form of suppression of the proliferative response to 3246 endotoxin (Fig 5).

The response of ConA stimulated peripheral blood lymphocytes was normal and not affected by INT exposure to 10^6 or 10^3 CFU of (groups 2 and 3) *S. choleraesuis* (data not shown). However, data indicate a suppression of the ConA stimulated lymphocyte mitogenic response following a 10^9 (group 1) INT dose of *S. choleraesuis* (data not shown).

Discussion

Although there is a considerable amount of information regarding acute *S. choleraesuis* infection in swine[1,22], there is little information regarding the carrier state and immune status of persistently infected swine. Reports suggest the main source of new *S. choleraesuis* infections is asymptomatic carrier swine[1]. Our study demonstrates an apparent dose

dependent correlation with the carrier state.

Acute paratyphoid can be a difficult disease to reproduce in swine, requiring doses of 10^8 to 10^{11} CFU of *S. choleraesuis* for production of acute disease[1]. Strain differences and differences in challenge protocols are likely responsible for some interstudy differences. One study reports moderate disease following a 10^6 CFU oral drench[9]. Gray et al.[10,11] showed that intranasal inoculation of 10^8 CFU *S. choleraesuis* results in more severe clinical disease and greater magnitude of fecal shedding than gastric inoculation at the same dose. Therefore intranasal inoculation was chosen in this study in order to maximize response to the pathogen.

It is not surprising that group 1 pigs exhibited marked clinical signs indicative of acute *S. choleraesuis* infection. In comparison, group 2 pigs exhibited only mild clinical disease which, in a large production setting, would likely not have been noticed. Interestingly, group 3 pigs exhibited no clinical signs of disease. Gray et al.[10,11] also demonstrated that a 10^8 intranasal challenge using strain 3246 results in severe clinical disease. Together these results demonstrate that experimental infection of swine with a dose of 10^8 CFU or higher will result in severe clinical disease and a long term carrier state. At moderate doses, less than 10^7 but above 10^3, inoculation will likely result in mild clinical disease and a short term carrier state of at least 9 weeks. A dose of less

than 10^3 will be cleared with no apparent shedding of the organism.

The magnitude of fecal shedding for groups 1 and 2 indicate a direct relationship between dose and shedding magnitude. Interestingly, regardless of dose, the pattern of shedding was similar between groups. Peak shedding was observed on day 5 for both groups and declined by 1 week PI. Groups 1 and 2 demonstrated an increase in shedding at 5 weeks and 7 weeks, respectively. *S. choleraesuis* was only sporadically recovered from fecal pools after 7 weeks for group 1 and 5 weeks for group 2. These data indicate that regardless of dose, an intermittent paradigm of shedding occurs in carrier animals. These data also indicate that carrier swine may shed *S. choleraesuis* for at least 15 weeks PI as observed in group 1. Furthermore, the results also show that negative fecal cultures are not a reliable measure of herd or individual animal status. This underscores the need for a reliable test or multiple testing under current methodologies to identify infected animals. Similar results have been shown with naturally occurring *S. typhimurium* infections, where shedding was observed after prolonged periods of negative fecal cultures[4,23,24].

The acute phase response induced by exposure to *S. choleraesuis* has been described by Kramer et al[25]. The ESR assay was used in this study as a non-specific measurement of

acute phase response. We observed that increased sedimentation rates of groups 1 and 2 correlated with the febrile response and clinical signs caused by acute *S. choleraesuis* infection. The ESR values returned to normal as pigs recovered from acute infection. However, no correlation with clearance of the bacteria could be made.

The post mortem bacteriologic data did not indicate that a persistent infection occurred after challenge with a 10^3 dose of *S. choleraesuis*. However, IgM and IgG antibody titers observed for group 3 indicate that bacteria were present for a sufficient period of time to induce at least a weak immune response. Interestingly, a blastogenic response to *S. choleraesuis* antigens was not observed for group 3. This may be attributed to assay sensitivity and thus mild responses may have been missed. In addition, a blastogenic response may have been observed with more frequent sampling and/or continued sampling beyond 6 weeks PI.

A 10^6 dose resulted in infection for at least 6 weeks PI and fecal shedding was detected until 9 weeks PI. However, *S. choleraesuis* could not be recovered from tissue or feces from group 2 pigs after 9 weeks PI. A measurable antibody response was also observed in this group, as well as an antigen specific blastogenic response. These results indicate that experimental infection with 10^6 CFU of *S. choleraesuis* results in a carrier state with detectable shedding for at least 9

weeks PI. The cellular and humoral immune response observed appear to correlate with clearance of the organism.

A dose of 10^9 resulted in a long term carrier state. At 15 weeks PI the pigs in group 1 continued to shed *S. choleraesuis* and had multiple tissues positive for *S. choleraesuis*. In addition, we observed an average of 3 logs of *S. choleraesuis*/g of tonsil at 15 weeks PI. Gray et al.[11] has shown that high tonsil populations of *S. choleraesuis* is indicative of the carrier state. The carrier state observed in this group of pigs was present despite antibody titers and a measurable cellular response to *S. choleraesuis* antigens. This suggests that the immune system may be overwhelmed by such a large dose.

At low doses (10^3), *S. choleraesuis* is unable to stimulate a measurable antigen-specific lymphocyte response; however, normal mitogenic responses were observed. At moderate doses, between 10^3 and 10^7 optimal antigen stimulation of lymphocytes occurs as well as normal mitogenic responses. At these moderate doses the immune response observed here and in other studies[11] is apparently successful at greatly reducing the pathogen load in the host.

In contrast, at higher doses (10^9), *S. choleraesuis* causes some degree of antigen-specific and non-specific suppression of lymphocyte activation. One reason for the increased shedding and level of infection observed in group 1

may be the observed lymphocyte suppression. A long term carrier state occurred in group 1 despite a measurable humoral response and a suppressed cellular response. The observed immunosuppression is likely an important factor in the pathogenesis of *S. choleraesuis* in swine and our observations suggest that it may play an important role in development of a carrier state. Immune suppression by *S. typhimurium* infection in chickens and its effect on the carrier state has been described by Hassan and Curtiss[26].

In conclusion, these data indicate that the magnitude of shedding and persistence of infection after *S. choleraesuis* infection is dose dependent. Low doses of *S. choleraesuis* can be easily cleared, moderate doses can persist for at least 2 months, and high doses result in a long term carrier state. Regardless of dose, cellular and humoral immune activation occur after inoculation. However, at high doses, *S. choleraesuis* causes signs of lymphocyte suppression, which may affect the ability of the immune system to eliminate the bacteria. These data provide an important model for future investigation of *S. choleraesuis* infection in swine.

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Acknowledgements

The authors thank Dr. Greg Mahairas, Dr. Roy Curtiss III and Sandra Kelly of Washington University, St. Louis, MO. for providing the original bacterial culture. The authors also

thank Nels Nord, Ruth Willson, Kathy Foss, Brad Chriswell, Jeannie Frey, Nathan Adair and Jenny Emerson for excellent technical assistance.

The authors also thank Dr. Ted Kramer for sharing his expertise regarding *S. choleraesuis* in swine.

This research was completed as partial fulfillment of requirements for the degree Doctor of Philosophy at Iowa State University, Department of Microbiology, Immunology and Preventative Medicine.

Figure Legends

Fig 1. Quantitative recovery of *S. choleraesuis* from fecal pools (a) group 1 (10^9 CFU) or (b) group 2 (10^6 CFU). Reported values are the MPN/g of feces for each group.

Fig 2. Serum IgM response of swine to *S. choleraesuis* LPS antigen following challenge with *S. choleraesuis* at a 10^9 (group 1), 10^6 (group 2) or 10^3 (group 3) CFU dose. Reported values are the mean ELISA titer minus the mean uninoculated control value. Vertical bars represent the standard error of the mean.

Fig 3. Serum IgM response of swine to *S. choleraesuis* soluble antigen following challenge with *S. choleraesuis* (a) 10^9

(group 1), (b) 10^6 (group 2) or 10^3 (group 3) CFU dose. Reported values are the mean ELISA titer minus the mean uninoculated control value. Vertical bars represent the standard error of the mean.

Fig 4. Serum IgG response of swine to *S. choleraesuis* LPS or soluble antigen following challenge with *S. choleraesuis* (a) 10^9 (group 1), (b) 10^6 (group 2) or 10^3 (group 3) CFU dose. Reported values are the mean ELISA titer minus the mean uninoculated control value. Vertical bars represent the standard error of the mean.

Fig 5. Lymphocyte blastogenic response of swine to *S. choleraesuis* endotoxin following challenge with 10^9 (group 1), 10^6 (group 2)or 10^3 (group 3) CFU dose or uninoculated control (group 4). Assays were performed at days 7,14,28 and 42 post inoculation. Error bars represent the standard error of the mean.

Table 1 - Number of samples positive for *S. choleraesuis* from tonsil, nasal, rectal swabs and fecal pools

		Day						Week													
GROUP	Sample	D0	D1	D2	D3	D5	D8	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Tonsil	0[a]	5	3	4	4	4	3	2	3	4	4	2	2	0	2	1	1	0	1	1
	Nasal	0	4	2	4	5	4	1	0	0	2	0	1	0	0	0	0	0	0	0	0
	Rectal	0	4	2	0	4	4	4	1	2	2	1	0	0	0	0	1	0	0	1	0
	n=	5[b]	5	5	5	5	5	5	5	5	5	5	2	2	2	2	2	2	2	2	2
	Fecal[c]	-[d]	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+
2	Tonsil	0	2	4	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Nasal	0	1	1	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Rectal	0	0	1	2	3	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	n=	5	5	5	5	5	5	5	5	5	5	5	2	2	2	2	2	2	2	2	2
	Fecal	-	-	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-

a- Number of swabs positive for *S. choleraesuis*
b- Number of pigs sampled
c- Ten sample fecal pool; qualitative results only
d- Results reported as *S. choleraesuis* present(+) or absent(-)

Table 2 - Qualitative recovery of *S. choleraesuis* from the tissue of experimentally infected swine.

Number of positive samples/Number of pigs necropsied

Group	1		2		3	
Tissue	6[a]	15[b]	6[a]	15[b]	6[a]	10[c]
Tonsil	2[c]/3	2/2	0/3	0/2	0/3	0/2
Lung	1/3	0/2	0/3	0/2	0/3	0/2
Spleen	0/3	0/2	0/3	0/2	0/3	0/2
Liver	1/3	0/2	0/3	0/2	0/3	0/2
ICJ	2/3	1/2	0/3	0/2	0/3	0/2
ICLN	2/3	1/2	1/3	0/2	0/3	0/2
Cecum	2/3	2/2	0/3	0/2	0/3	0/2
Cec Cont	2/3	2/2	0/3	0/2	0/3	0/2
Colon	2/3	2/2	0/3	0/2	0/3	0/2

a - 6 week necropsy
b - 15 week necropsy
c - 10 week necropsy

Table 3 - Mean populations of *S. choleraesuis* recovered from tissues of pigs challenged with 10^9 CFU (group 1).

S. choleraesuis $\log_{10}$g/tissue

Week	Tonsil	ICJ	ICLN	Cec Cont
6[a]	4.77	2.24	2.10	2.51
15[b]	3.16	NQ-P[c]	NQ-P	NQ-P

a. n=3
b. n=2
c. NQ-P - not quantifiable but positive by qualitative bacteriology

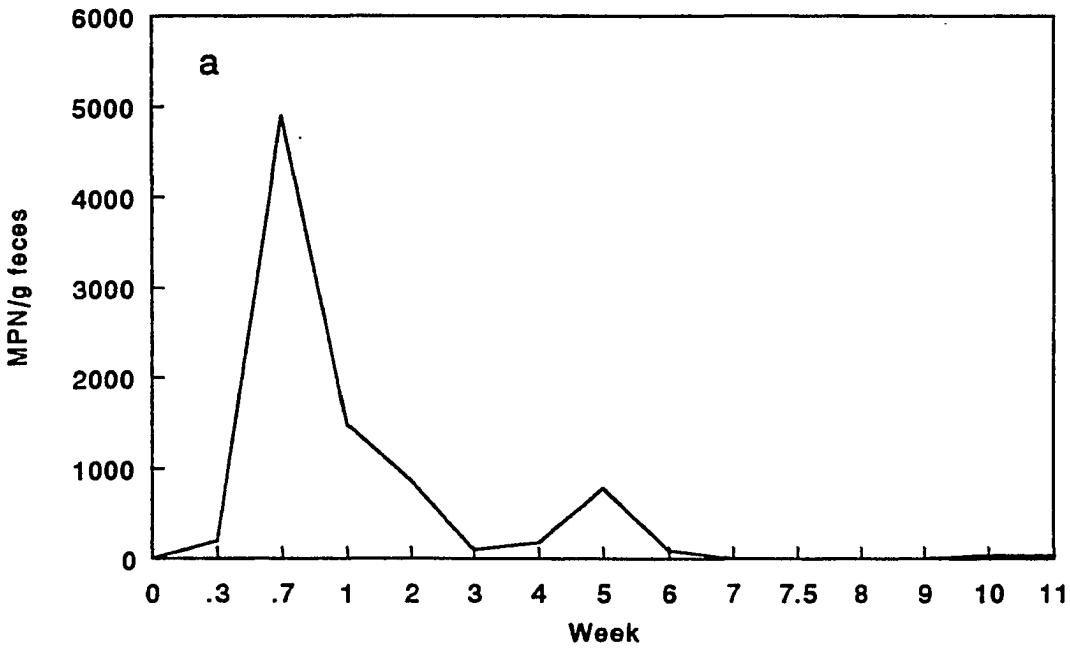
a
6000
5000
4000
3000
2000
1000
0
MPN/g feces
0
.3
.7
1
2
3
4
5
6
7
7.5
8
9
10
11
Week

Figure 1a.

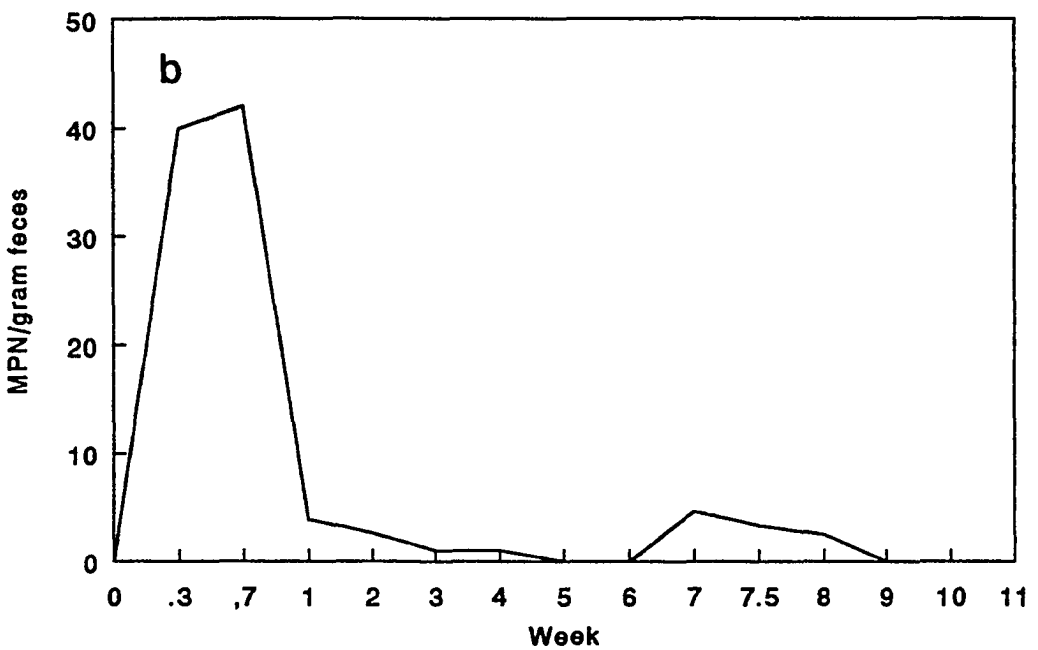

Figure 1b.

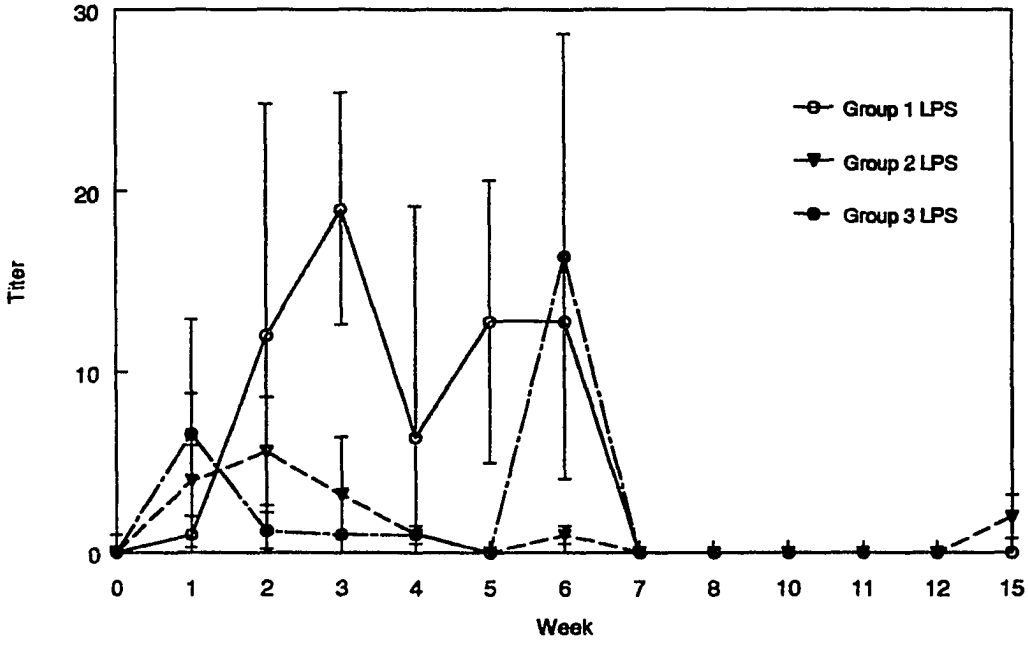
30
20
10
0
Titer
0
1
2
3
4
5
6
7
8
10
11
12
15
Week
Group 1 LPS
Group 2 LPS
Group 3 LPS

Figure 2.

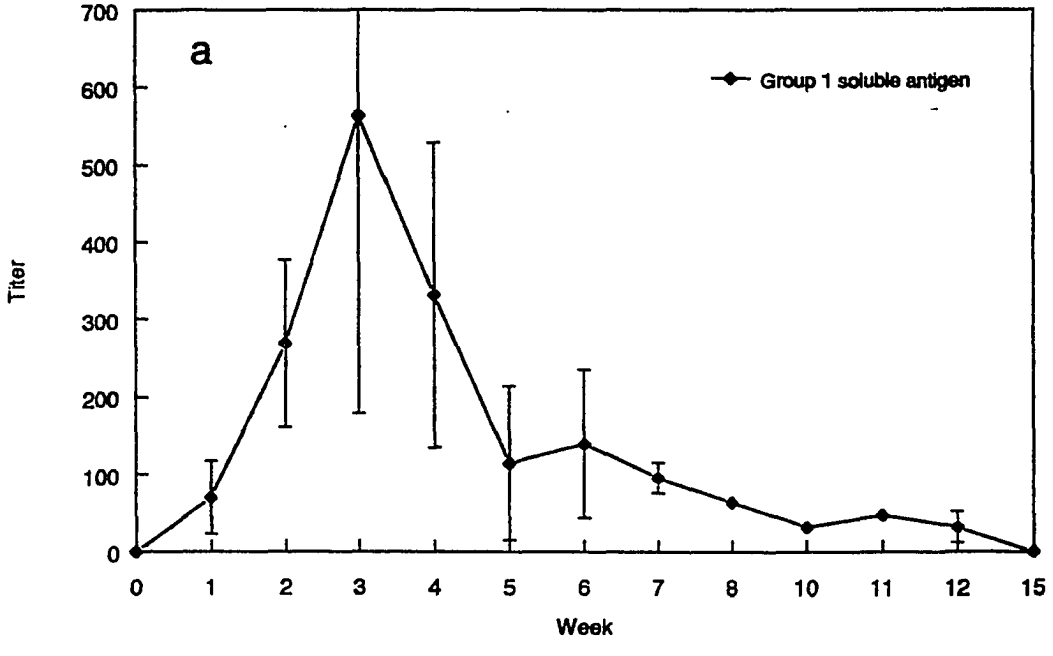
a
Group 1 soluble antigen
Titer
700
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
10
11
12
15
Week

Figure 3a.

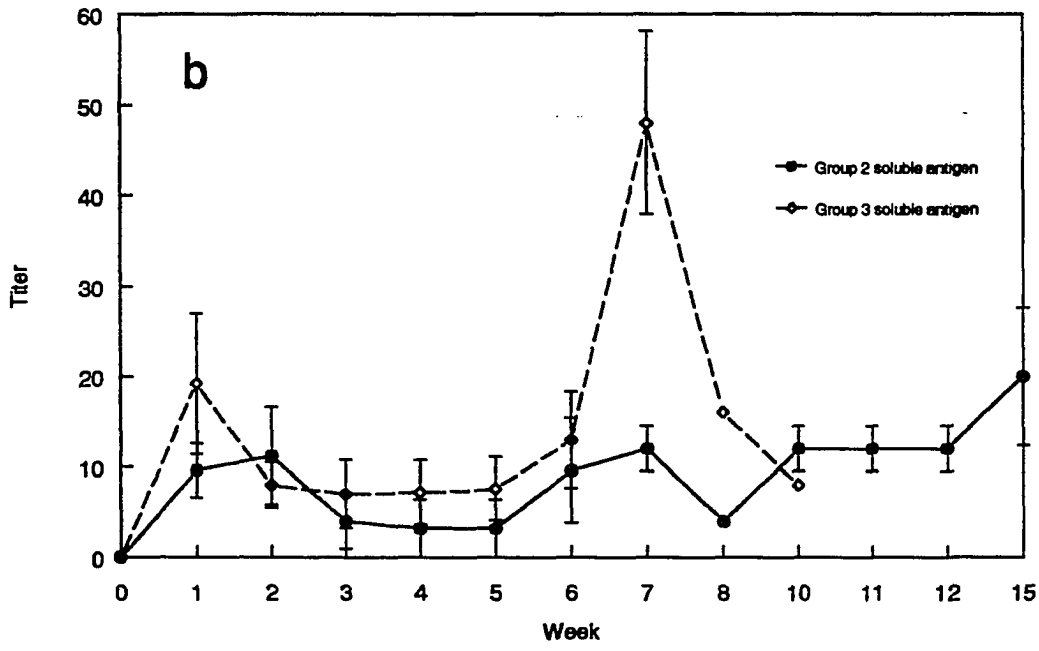
b
Titer
Week
Group 2 soluble antigen
Group 3 soluble antigen
0
10
20
30
40
50
60
0
1
2
3
4
5
6
7
8
10
11
12
15

Figure 3b.

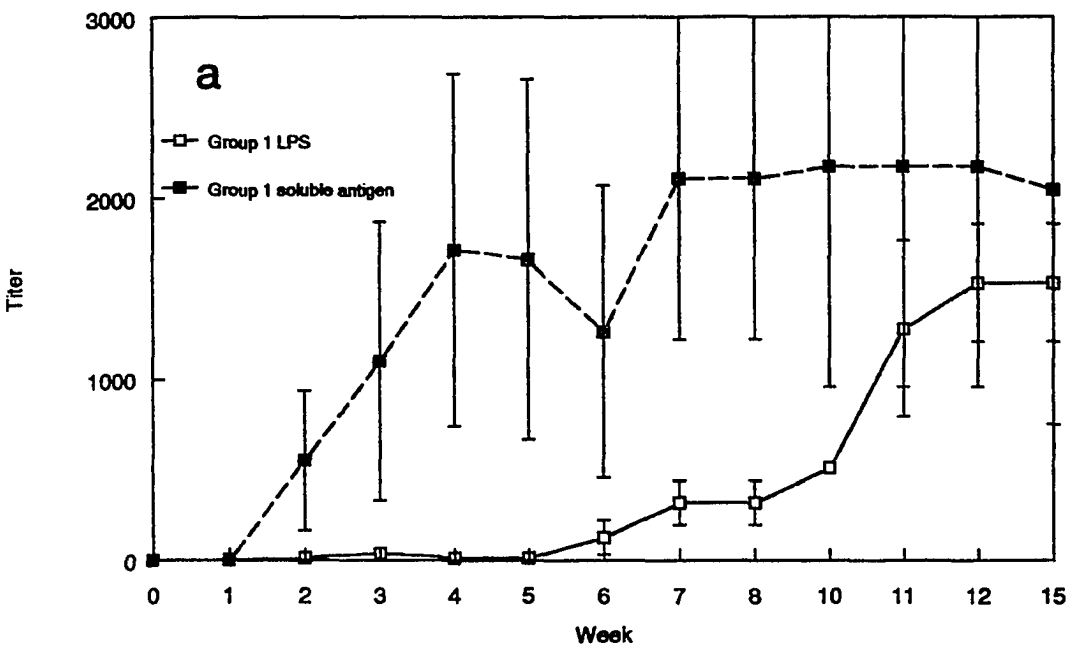
a
Group 1 LPS
Group 1 soluble antigen
Titer
3000
2000
1000
0
0
1
2
3
4
5
6
7
8
10
11
12
15
Week

Figure 4a.

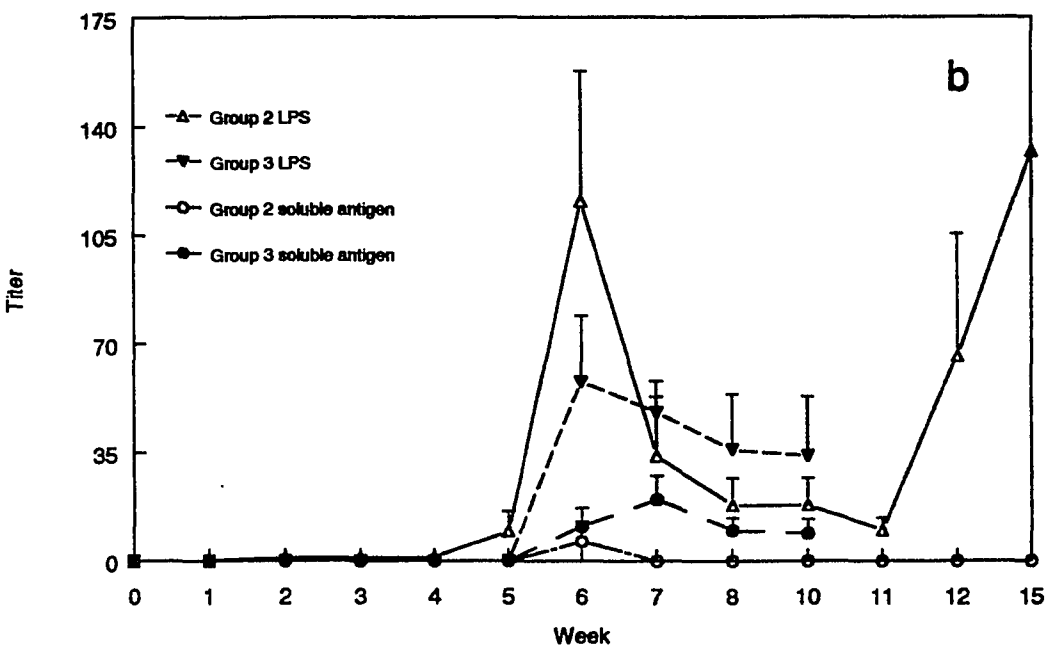
b
Group 2 LPS
Group 3 LPS
Group 2 soluble antigen
Group 3 soluble antigen
Titer
175
140
105
70
35
0
0
1
2
3
4
5
6
7
8
10
11
12
15
Week

Figure 4b.

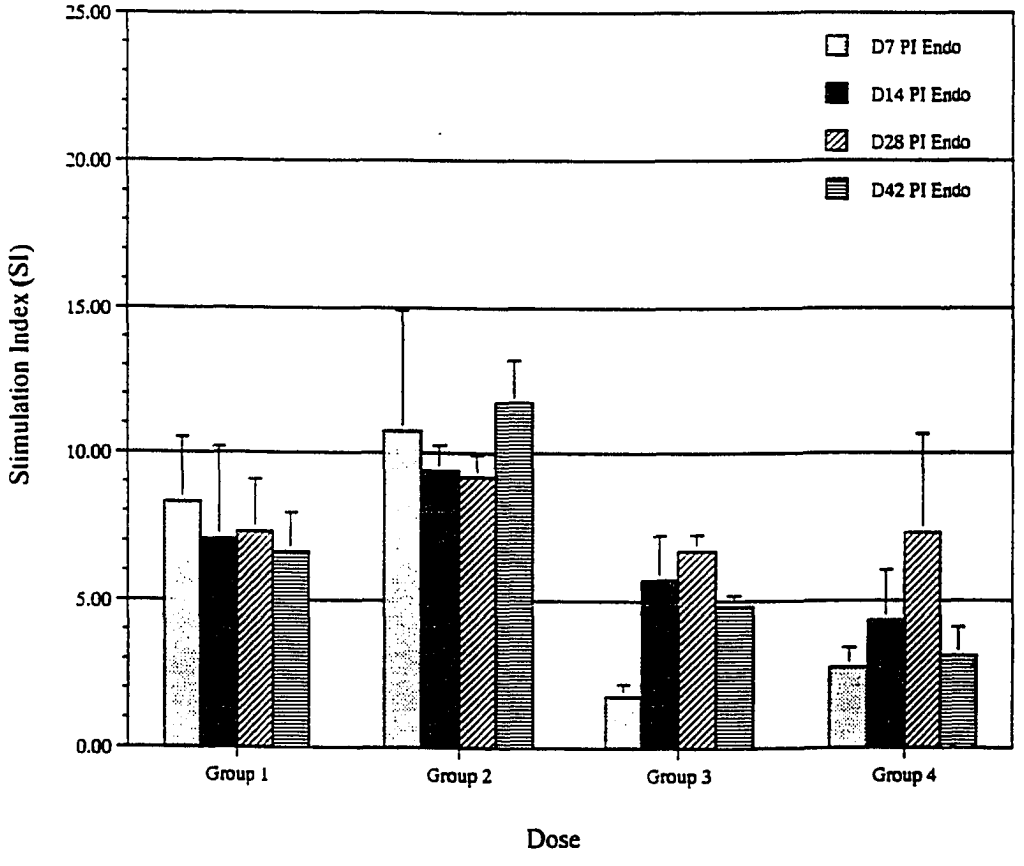
25.00
20.00
15.00
10.00
5.00
0.00
Stimulation Index (SI)
D7 PI Endo
D14 PI Endo
D28 PI Endo
D42 PI Endo
Group 1
Group 2
Group 3
Group 4
Dose

Figure 5.

CHAPTER 4: THE NATURAL TRANSMISSION OF *SALMONELLA CHOLERAESUIS* IN SWINE

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Submitted to

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Running title: Transmission of *Salmonella choleraesuis*

Summary

The purpose of this experiment was to study the natural transmission of *S. choleraesuis* in swine. Group 1 (n=12) was challenged with 10^8 *S. choleraesuis* by intranasal inoculation. One day post inoculation (PI) group 2 (n=24) was commingled with group 1. Pigs were necropsied at 1,2,4,6,9 and 12 weeks PI. Pigs in group 1 were shedding 2.61 $\log_{10}$ CFU/g feces prior to commingling. Swabs and group fecal pools indicated that group 2 pigs were shedding *S. choleraesuis* 1 day after commingling. At 1 week PI 32/32 group 1 tissues were positive for *S. choleraesuis* while 39/62 tissues were positive for group 2. At 12 weeks PI only group 2 had positive tissues. These data indicate that naive swine exposed to swine shedding a relatively low amount of *S. choleraesuis* can be infected and shed *S. choleraesuis* within 24 hours of exposure. However, under well managed conditions, the level of *S. choleraesuis* can be significantly reduced in naturally exposed swine.

Introduction

Salmonella choleraesuis is a host adapted, facultative intracellular pathogen that causes swine paratyphoid (26). It is the most frequent *Salmonella* serotype recovered from swine (10) and was isolated from >95% of swine salmonellosis outbreaks in Iowa in 1989 (20). The National Animal Health Monitoring Survey estimated that swine salmonellosis is responsible for 28 million dollars in annual production losses in Iowa and 100 million in losses nationwide (20).

Although *S. choleraesuis* is the most frequent porcine isolate, it is rarely isolated from swine feed or non-porcine *Salmonella* reservoirs. The source of *S. choleraesuis* seems to be limited to carrier pigs and facilities previously contaminated with this serotype (26). The carrier state of *S. typhimurium* (27, 31), as well as *S. choleraesuis* (12), in swine have been described after experimental infection. Epidemiological studies have indicated crowding and contact with infected feces increased infection rates (14, 17). However, these studies did not provide specific data involving infection levels of individual animals. Fedorka-Cray et al. (7) studied the natural transmission of *S. typhimurium* to swine and observed a short term carrier state after exposure to low concentrations of *S. typhimurium* in the environment. Similar information involving *S. choleraesuis* infection is not

available. While there are no data available describing the carrier state after natural transmission of *S. choleraesuis* in swine, Hinton (15) studied naturally occurring *Salmonella* infections in cattle and chickens. He concluded that naturally occurring infection patterns were different from those observed for experimentally infected animals and suggested that natural infection models are important for understanding salmonellosis.

The purpose of this experiment was to study shedding patterns, tissue colonization and the immune response after natural transmission of *S. choleraesuis* in swine and determine if a carrier state can develop following contact with experimentally infected pigs.

Materials and Methods

Bacterial strains and challenge cultures. Wild type *S. choleraesuis* var. *kunzendorf* χ3246 (16), kindly provided by the laboratory of Roy Curtiss III, Washington University, St Louis, MO, was used to challenge a 12 week old pig by intranasal inoculation and redesignated 3246pp as previously described (12). The strain has a naturally acquired resistance to streptomycin. Challenge cultures were prepared by inoculating 10 ml of LB broth with 100 μL of a -70 frozen stock culture of 3246pp. The culture was grown overnight at

37°C on an orbital shaker at 150 rpm. A 1% inoculum was transferred into fresh LB broth and grown for 3.5 hours at 37°C and 220 rpm. The culture was centrifuged, the pellet was resuspended in 1/2 volume of phosphate buffered saline (PBS) and adjusted to a final concentration of $1x10^8$ CFU/ml (OD_{600}= 0.123) in PBS.

Experimental design. After farrowing, source sows were tested and confirmed to be negative for *Salmonella spp.* by bacteriologic culture as described below. At two weeks of age, 40 pigs were weaned and transported to isolation facilities as previously described (8). Tonsil (T), nasal (N) and rectal (R) swabs were taken from pigs at 2, 3, 4 and 6 weeks and cultured for *Salmonella spp.* At 6 weeks of age the pigs were randomly divided into three groups. Pigs were housed in a climate controlled, fully enclosed environment on concrete floors. Each group was housed in separate isolation facilities and allowed to acclimate to the new environment for 1 week. Feces was physically removed and the pen was washed with water once daily throughout the experiment. At 7 weeks of age (day 0) the group 1 pigs (n=12) were inoculated intranasally (INT) with 1 ml (0.5 ml in each nostril dropwise on inspiration, alternating nostrils) of 3246pp at $1x10^8$ CFU/ml. One day post inoculation (PI) group 2 naive pigs (n=24) were commingled with the group 1 pigs. Group 3 (n=4)

served as uninoculated controls.

Feces was collected (10-20 g samples) from individual pigs (≥60% of the pigs per group) in group 1 and 2 and swabs (T, N, R) on all pigs were obtained on days 1 through 6, 8, 9, 11, 13, 16, 19, 23, 26, 30 then weekly. Fecal pools for each group were obtained by combining approximately 2 grams of feces from each pig per group. The *S. choleraesuis* environmental load was measured by scraping all available feces into a pile, mixing well and taking a 20-30 g sample.

Two pigs from group 1 and 4 pigs from group 2 were euthanized and necropsied at 1, 2, 4, 6, 9 and 12 weeks PI. One pig from group 3 was necropsied at 1, 4, 9 and 12 weeks PI. Tissues were collected aseptically for bacteriologic examination (sterile gloves and instruments were used for each tissue). Tissues included the turbinate (1 g; approximate weight), tonsils (4 g), thymus (3 g), mandibular lymph nodes (mandib-LN; 3 g), trachea (1 g), esophagus (1 g), lungs (8 g), bronchial lymph nodes (bronch-LN; 2 g), stomach wall (stom wall; 2 g), spleen (5 g), liver (5 g), middle ileum (ileum-mid; 4 g), ileocolic junction (ICJ; 6 g), ICLN (3 g), cecum (4 g), and colon (4 g); stomach contents (stom cont; 20 g) and cecal contents (cec cont 25 g).

Clinical signs. Rectal temperatures and clinical signs were monitored 2 times daily until day 3 PI for pigs in all

groups. Between days 4 and 14 PI, pigs were monitored once daily then once weekly after day 14 PI.

Bacteriologic examinations. Tissue samples and fecal pools were processed as previously described (12). All T, N, R swabs, fecal pools and tissues (collected at necropsy) were incubated at 37°C in GN-Hajna (GN; Difco, Detroit, MI) broth for 18 to 24 hours then streaked on brilliant green agar with sulfadiazine (BGS; Microdiagnostics, Lombard, IL). Additionally, at 18 to 24 hours 100 µl of the GN broth was transferred to Rappaport-Vassiliadis (RV) medium (24), incubated at 37°C for 18 hours, then streaked to BGS. All BGS plates were incubated 24 hours at 37°C. In order to facilitate recovery and identification of the challenge strain, streptomycin sulfate (200 µg/ml; Sigma, St. Louis, Mo) was added to media used for group 1 and 2 pigs.

Colonies having the typical appearance of *Salmonella* were picked and inoculated into triple sugar iron and lysine iron agar slants. Positive isolates were confirmed as serogroup C by agglutination with *Salmonella* antiserum group C_1O (Difco, Detroit, MI). Representative isolates were serotyped at the National Veterinary Services Laboratory. Quantitative bacteriology was conducted using the 5 tube most probable number (MPN) method (30) with GN, BGS-S and RV media as described above and are reported as the mean of the respective

group for each necropsy day. Differences between groups were evaluated by the χ^2 test.

Antigen preparation. *Salmonella choleraesuis* 3246 LPS antigen was prepared by the method of Westphal(25) and lyophilized for use in ELISA assays.

Endotoxin from *S. choleraesuis* 3246pp was prepared by the method of Morrison (18) and lyophilized for use in the lymphocyte blastogenesis assays. Heat extract antigen (HE) from *S. choleraesuis* 3246pp was prepared as previously described (12).

Protein determination. Total protein was determined by the method of Bradford (2) using bovine serum albumin as the standard.

Intestinal antibody. Local intestinal antibody secretion was measured in intestinal wash collected as previously described (6).

Antibody responses. Antibody responses in serum and intestinal washes were determined by ELISA assay as previously described (12) except that serum samples were diluted 1/10 in diluent buffer. Values are reported as optical densities plus or minus the standard error of the mean for the pigs in each

group and not as titers.

Lymphocyte Blastogenesis Assays. Blastogenesis assays were performed at weeks 1, 2, 4, 6, 9 and 12 weeks PI on 4 pigs from group 1 (2 pigs selected for necropsy plus 2 others; except at 12 weeks when only 2 pigs were available), 4 pigs from group 2 (pigs selected for necropsy) and all available group 3 pigs. Assays were performed as described previously (12) except that lymphocytes were incubated with the respective antigen for 48 hours and 5 day incubations were not performed. Lymphocytes were incubated with either *S. choleraesuis* endotoxin (18) at 50.0 μg/ml or HE antigen (12) at 10.0 μg/ml. Positive control wells contained concanavalin A (ConA; Sigma) at 5.0 μg/ml. Negative control wells contained cells and media only. Values are reported as the mean stimulation index (SI; defined as experimental values divided by negative control values) plus or minus the standard error of the mean for the pigs in each group.

Results

Clinical signs. No clinical signs were observed on day 1 PI (day of commingling). Pigs in group 1 first elicited a febrile response on day 2 which peaked at 41.2°C 4 days PI and persisted until day 11 PI. Group 2 pigs also developed a

febrile response on day 3 PI (day 2 post-commingling, PC) which peaked at 41.4°C on day 5 PI and persisted until day 8 PI. Severe clinical signs including diarrhea, chills, respiratory distress and severe depression were observed in both groups between days 2 and 10 PI. Clinical signs in both groups were resolved by day 14 PI.

Antemortem bacteriologic results. All source sows were culture negative for *Salmonella spp.* All pigs were also culture negative for *Salmonella spp.* prior to challenge. Following challenge, T, N, R swabs indicated that all of the group 1 pigs were shedding *S. choleraesuis* on day 1 PI. A combination of R swabs and fecal cultures indicated at least 16% (4/24) of the pigs in group 2 were shedding *S. choleraesuis* by day 2 PI or 24 hours PC. By day 4 PI at least 50% (12/24) of group 2 pigs were shedding *S. choleraesuis* and 88% (21/24) of the group 2 pigs were confirmed to be shedding *S. choleraesuis* (antemortem culture) by day 11 PI.

The magnitude of fecal shedding for groups 1 and 2 and the environmental load of *S. choleraesuis* is presented in Table 1. At the time of commingling the environmental load was 2.61 $\log_{10}$ CFU *S. choleraesuis*/g feces. Fecal shedding in group 1 peaked on day 8 PI (3.65 $\log_{10}$ CFU/g), while group 2 peaked on day 9 PI (1.51 $\log_{10}$ CFU/g; day 8 PC). With the exception of days 6, 8, and 9 PI, the environmental fecal

levels followed the group 1 fecal levels closely. The last positive fecal culture was observed at 8 weeks PI for group 2.

Postmortem bacteriologic examination. The frequency of recovery of *S. choleraesuis* from individual tissues is presented in Tables 2 and 3 for groups 1 and 2, respectively. *Salmonella choleraesuis* was recovered from nearly all of the tissues cultured from group 1 at the 1 (100%) and 2 (81%) week PI necropsies. At the 4, 6 and 9 week necropsies the numbers of tissues found to be infected declined to numbers observed previously (31%,47%,19%, respectively)(12). Similarly, at weeks 1 and 2 (when clinical disease was observed) *S. choleraesuis* could be found at high frequency in many tissues for group 2 pigs and 4/4 pigs were positive for both the 1 and 2 week necropsy . The tissues in which *S. choleraesuis* was most frequently recovered in group 2 were the ICJ (50%), ICLN (42%), cec cont (42%) and cecum (33%). During the subclinical carrier state (4-12 weeks) the tissues most frequently positive for *S. choleraesuis* in group 2 were the ICJ (25%), ICLN (19%), cec cont, colon and CLN (13%).

Nearly all of the group 1 pigs were tissue positive for *S. choleraesuis* (10/12). However, the 2 pigs necropsied at 12 weeks were negative. All of the group 2 pigs (8/8) were tissue positive at the 1 and 2 week necropsies and 3/4 were positive at the 4 week necropsy. One group 2 pig was tissue

positive at each of the 6, 9 and 12 week necropsies.

Quantitative bacteriology for groups 1 and 2 is presented in Table 4. Relatively high populations of *S. choleraesuis* were found in the tonsils, lung, ICLN, ICJ and cec cont at 1 week for both group 1 and 2. The lung of group 2 was the lowest (1.49 $\log_{10}$ CFU/g) and, except for the lung, the populations were similar between groups. High populations ($\geq$ 3.96 $\log_{10}$ CFU/g) were observed for group 1 in the tonsils through 9 weeks PI (excluding week 2). The ICLN, ICJ and cec cont were also highly populated through 6 weeks PI. For group 2 populations $\geq$1.47 $\log_{10}$ CFU/g were observed in the lung through 2 weeks PI. Quantifiable amounts of *S. choleraesuis* were observed in group 2 in the cec cont through 4 weeks PI, the ICJ through 9 weeks PI (excluding 6 weeks PI) and the ICLN through 6 weeks PI.

Serum and intestinal antibody responses. Serum antibody responses to *S. choleraesuis* LPS for groups 1 and 2 are presented in figure 1a and 1b for IgG and IgM, respectively. High serum IgG responses to *S. choleraesuis* LPS were observed beginning 2 weeks PI for both groups which continued to rise until 5 and 6 weeks for groups 2 and 1, respectively. The group 2 response, although slightly lower, paralleled the group 1 response closely. The serum IgM response for group 1 was observed within 1 week PI while the group 2 IgM response

was observed within 2 weeks PI. The IgM response peaked for both groups at 2 weeks PI. As observed for the IgG response, the group 2 response paralleled the group 1 response closely. Serum IgA responses were not observed for either group.

Intestinal IgG, IgM, and IgA responses to 3246pp LPS are presented in figures 2a, 2b and 2c, respectively. A low mucosal IgG response was observed for group 1, 1 week PI which increased markedly, peaked at 6 weeks PI, and declined steadily until 12 weeks PI. The intestinal IgG response for group 2 was not observed until 2 weeks PI and then remained constant from 4 to 12 weeks PI. An IgM response was observed for both groups 1 and 2 beginning at 1 week PI and continuing throughout the experiment. The highest response was observed 6 weeks PI for group 1 and at 12 weeks PI for group 2.

Figure 2c indicates a low intestinal IgA response to *S. choleraesuis* LPS antigen for both groups beginning at 1 week PI which declined and continued until 12 weeks PI. Although higher responses may have occurred at weeks 1 and 12 PI the large error bars make this result unclear.

Blastogenic responses. Lymphocytes from group 1 and group 2 pigs showed similar increased proliferation to *S. choleraesuis* HE antigen beginning at 4 weeks PI as compared to controls. Increased sensitivity to HE antigen persisted through 9 weeks PI before returning to normal levels at 12

weeks PI (figure 3). Similar trends were observed with *S. choleraesuis* endotoxin.

Discussion

This experiment was conducted to study the natural transmission of *S. choleraesuis* within swine herds, determine the effect of natural transmission on the carrier state and compare the natural disease syndrome to experimentally infected pigs.

Pigs infected with *S. choleraesuis* usually begin to show clinical signs between 36 and 48 hours after infection (12,19). We designed this study so that pigs were commingled before clinical disease was evident. This represented a real world scenario mimicking field conditions such as when new stock is purchased or when swine are moved and regrouped into new pens and exposed to infected swine which are not manifesting clinical signs.

It has been shown that experimentally infected swine shed between 10^3 and 10^6 CFU/g of feces during peak clinical disease (12,21). It is unclear however, how this magnitude of shedding relates to that of natural infections and subsequently to the transmission of *S. choleraesuis* to naive swine. We have demonstrated that naive swine exposed to a population shedding as little as 2.61 $\log_{10}$ CFU/g of feces will

begin shedding *S. choleraesuis* within 24 hours of exposure. In addition, severe clinical signs were observed in group 2 within 3 days of exposure to the experimentally infected population. This indicates that exposure to relatively low concentrations of *S. choleraesuis* may result in high morbidity and initiate a severe outbreak of swine paratyphoid within a relatively short period of time. This not only indicates the rapidity of *S. choleraesuis* transmission on the farm but may also translate to transmission and spread of *Salmonella spp.* during transport and time in lairage before slaughter.

Nearly all (88%) of the naive pigs were confirmed by swabs or fecal cultures to be positive for *S. choleraesuis* by 11 days PI indicating that the majority of swine exposed to a population shedding between 2 and 3 logs of *S. choleraesuis* per g of feces will become short term *S. choleraesuis* shedders.

Interestingly, although the clinical signs of group 2 were similar to group 1 in severity and duration, peak shedding was at least 2 logs lower (1.5 $\log_{10}$ CFU/g of feces for group 2 vs. 3.6 $\log_{10}$ CFU/g for group 1). This indicates that the high levels of shedding observed after experimental infection may not be indicative of shedding magnitude after natural infection.

The tissue distribution of *S. choleraesuis* in swine after experimental infection has been described for clinical

infection (4, 19, 21, 28) and the carrier state (12). The tissue distribution observed for group 1 was similar to other studies for both clinical and subclinical infection periods. However, in comparison to experimental infection some differences were observed for the naturally infected group 2 pigs. High concentrations of *S. choleraesuis* were observed in the tonsils for both groups at 1 week PI. This suggests that either the tonsils became colonized due to a septicemia or that it is a primary site of invasion for *S. choleraesuis*. Gray et al. demonstrated that tonsil populations declined to zero by 2 weeks PI then increased during the later time points of the experiments following experimental *S. choleraesuis* challenge (12). This result was also observed at weeks 2, 4, 6 and 9 PI for group 1 in this experiment (Table 4). The tonsils have been described as an important subclinical carrier state tissue for *S. choleraesuis* (12,13) and *S. typhimurium* (31) after experimental infection. Interestingly, in this study after the 1 week necropsy, tonsil populations were not observed in the group 2 pigs. It is unclear whether tonsil populations during subclinical disease (>2 weeks PI) is reserved as an artifact of high dose experimental infection or is triggered by an unknown factor not present in the group 2 model. The results presented here suggest that positive tonsil cultures may also indicate a very recent exposure to *Salmonella spp.*

Pneumonia associated with *S. choleraesuis* infection has been previously described (1) and a recent increase in *S. choleraesuis* associated pneumonia has been reported (23). It is unclear whether this predilection for the lung is due solely to the pathogen, poor ventilation in large confinement buildings or some combination of these and other factors. Experimental infection models have not provided good answers because positive lung samples have been regarded as an artifact of intranasal or per os inoculation. The data presented here indicates that the lung is a site of initial infection of *S. choleraesuis* in naturally exposed pigs. We recovered *S. choleraesuis* from 6/8 lungs of the naturally infected, group 2 pigs during the first two weeks of infection. The concentrations of *S. choleraesuis* in the lung remained at 1.5 $\log_{10}$ CFU/g through 2 weeks PI in the naturally exposed group.

Swine have a large number of alveolar macrophages in the lung (29). Fedorka-Cray et al. (9) hypothesized that swine alveolar macrophages may have an impaired ability to contain *Salmonella* within the early hours after infection. In addition, the alveolar macrophages may act as a vehicle for dissemination of *Salmonella*. Our data demonstrates that after natural infection there is a population of *Salmonella* which resides in the lung for at least 2 weeks after exposure possibly residing in alveolar macrophages and acting as a

continual source of infection.

Overall the tissues in which *S. choleraesuis* can most frequently be found in naturally infected pigs are the ICJ, ICLN, cec cont and cecum. During clinical disease ($\leq$ 2 weeks PI) *S. choleraesuis* was isolated from many of the same tissues in group 2 as is observed with field outbreaks and experimental infections (26). During the subclinical carrier state the tissues of predilection are the ICJ, ICLN, cec cont, colon, CLN and cecum in the naturally infected, group 2 swine. This is similar to the tissue distribution which has been described after experimental infection of swine with either *S. typhimurium* (27, 31) or *S. choleraesuis* (12). The sole exception is that the tonsils were not positive in the subclinical group 2 pigs.

The data presented here indicates that only 1 of 4 group 2 pigs was positive at each of the 6, 9, and 12 week necropsies suggesting two things. First, even after a severe clinical outbreak of swine paratyphoid the numbers of *S. choleraesuis* carried in deep tissue and present in the environment was markedly reduced in the group 2 pigs. A significant ($P < 0.01$) number of naive pigs were able to clear *S. choleraesuis* between 9 and 12 weeks PI, indicating that not all pigs will become long term carrier animals. Although no management protocols were tested here, this data suggests that good management practices can control and significantly reduce

S. choleraesuis in swine herds.

Secondly, these data suggests that a small fraction of carrier pigs is responsible for maintenance of this pathogen in the swine population. It has been shown that *S. choleraesuis* is a serotype that is rarely isolated in feeds or the environment (26) implicating subclinical carrier swine in new outbreaks. It remains a mystery, however, as to the factors which induce carrier swine to shed levels necessary to initiate a new outbreak.

Experimental infection models have used doses ranging from 10^6 to 10^{11} CFU of *S. choleraesuis* (26). Gray et al. (13) and others (5) have shown that experimental infection with a dose of 10^6 CFU causes moderate disease. In contrast, a dose of 10^3 CFU of *S. choleraesuis* is cleared with no clinical signs and no apparent shedding (13). In this study, group 2 swine were exposed to a population shedding a maximum of 3.6 $\log_{10}$ CFU/g of feces with a maximum environmental load equal to 3.0 $\log_{10}$ CFU/g of feces. In comparison to experimental models, the group 2 swine would have needed to ingest between 250 g (10^6 CFU dose) and 25000 g (10^8 CFU dose) of feces each to manifest the severe clinical signs observed in group 2. This amount of coprophagia is unlikely.

Previous studies have shown swine have a dose dependant immune response to *S. choleraesuis* (13). In the present study the humoral and cellular immune responses were similar between

the group 1 and group 2 pigs suggesting they received nearly the same dose or the organism was able to replicate to nearly the same concentrations in the host. Taken as a whole, these data indicate that during natural transmission the infectious dose of *S. choleraesuis* is much lower than experimental models have previously described. It may also be that pigs are exposed to a large dose of *S. choleraesuis* by a mechanism other than fecal oral transmission. The latter possibility would necessitate large populations of *S. choleraesuis* are excreted by infected swine by a route other than feces such as mucous or urine. However these high concentrations cannot be confirmed in the environmental samples. Fedorka-Cray et al. (9) has hypothesized that routes other than fecal/oral are important in the pathogenesis of swine salmonellosis.

A more likely hypothesis is that there are factors involved in the natural transmission of *S. choleraesuis* which are not reproduced in experimental infection. Some of these factors may include the virulence of the *S. choleraesuis* following passage through the host which may allow lower infectious doses and may allow the organism to replicate to high concentrations in the host. *In vitro* studies have shown the importance of the expression of induced antigens for intracellular survival (3,11,22). Other factors involved in the transmission of *S. choleraesuis* may include the unknown affect of multiple inoculations with smaller doses and

inoculation mechanisms not reproduced during experimental infections. Some combination of these and other factors is likely.

As discussed previously the humoral, mucosal and cellular immune response observed for the group 2 swine closely paralleled that of group 1. This indicates that in terms of immune parameters, the experimental infection may be an appropriate model for study of this response. In addition, this indicates that serum collected from experimentally infected swine provide a useful tool for the development of serologically based Salmonella detection tests.

In conclusion, this model for *S. choleraesuis* transmission provides a dramatic example of the difference between infection with *Salmonella* grown the laboratory versus the natural host and should be seen as a basis for design of future animal studies.

Acknowledgements

The authors thank Dr. Greg Mahairas, Dr. Roy Curtiss III and Sandra Kelly of Washington University, St. Louis, MO. for providing the original bacterial culture. The authors also thank Nels Nord, Ruth Willson, Kathy Foss, Brad Chriswell and Nathan Adair for excellent technical assistance.

The authors also thank Dr. Evelyn Nystrom for her

consultation on mucosal antibody response and Dr. Mark Ackermann for consultation on histopathology. In addition, Dr. LeeAnn Thomas and Kathy Ferris are to be extended sincere gratitude and appreciation for serotyping the isolates.

This research was completed as partial fulfillment of requirements for the degree Doctor of Philosophy at Iowa State University, Department of Microbiology, Immunology and Preventative Medicine.

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Table 1 - Magnitude of fecal shedding for Group 1 (intranasally challenged), Group 2 (naturally exposed) and environmental (envir) load of *S. choleraesuis*.

	Feces (log_{10} CFU/g)		
Day	Group 1	Group 2	Envir
D1	2.61	NA[a]	2.61
D2	1.55	+[b]	2.55
D3	2.55	+	1.86
D4	ND[c]	0.64	2.29
D5	3.26	+	2.98
D6	3.35	0.46	1.6
D8	3.65	0.83	2.03
D9	2.97	1.51	2.25
D11	2.7	0.31	1.62
D13	1.73	0.43	1.0
D16	2.08	1.11	1.81
D19	1.72	0.49	1.35
D23	2.45	-[d]	1.08
D26	1.11	0.59	1.11
D30	2.25	-	2.1
D34	1.77	-	0.55
6 week	-	-	-
7 week	0.75	-	1.11
8 week	-	0.63	-
9 week	-	-	-

a- not applicable
b- positive by enrichment only
c- not done
d- negative

Table 2 - Number of *S. choleraesuis* positive tissues from 12 Group 1 intranasally challenged pigs.

	Necropsy week (n=2)						
Tissue	1	2	4	6	9	12	% Pos.
Turbinate	2	2	0	0	0	0	33%
Tonsils	2	0	1	1	1	0	42%
Thymus	2	0	0	0	0	0	17%
Mandib-LN	2	2	1	1	0	0	50%
Lung	2	2	0	0	0	0	33%
Bronch-LN	2	2	0	0	0	0	33%
Stom wall	2	0	0	0	0	0	17%
Spleen	2	2	0	0	0	0	33%
Liver	2	2	1	1	0	0	50%
Ileum-mid	2	2	0	1	0	0	42%
ICJ	2	2	2	2	1	0	75%
ICLN	2	2	2	2	0	0	67%
Cecum	2	2	0	1	1	0	50%
Cec Cont	2	2	2	2	1	0	75%
Colon	2	2	1	2	1	0	67%
CLN	2	2	0	2	1	0	58%
% Pos.	100%	81%	31%	47%	19%	0	
Pigs Pos.	2	2	2	2	2	0	

Table 3 - Number of *S. choleraesuis* positive tissues from 24 Group 2 naturally exposed pigs.

	Necropsy week (n=4)						
Tissue	1	2	4	6	9	12	% Pos
Turbinate	0	0	0	0	0	0	0
Tonsils	3	1	0	0	0	0	17%
Thymus	0	0	0	0	0	0	0
Mandib-LN	0	0	0	0	0	0	0
Lung	4	2	0	0	0	0	25%
Bronch-LN	3	3	0	0	0	0	25%
Stom wall	0	1	0	0	0	0	4%
Spleen	4	1	0	0	0	0	21%
Liver	4	3	0	0	0	0	29%
Ileum-mid	0	2	0	0	0	0	8%
ICJ	4	4	3	0	1	0	50%
ICLN	3	4	2	1	0	0	42%
Cecum	4	3	1	0	0	0	33%
Cec Cont	4	4	2	0	0	0	42%
Colon	3	0	0	1	0	1	21%
CLN	3	1	0	1	0	1	25%
Percent Pos	61%	44%	13%	5%	2%	3%	
Pigs Pos	4	4	3	1	1	1	

Table 4 - Mean populations of *S. choleraesuis* recovered from tissues of pigs challenged by intranasal (group 1) or by natural exposure (group 2).

Log_{10} CFU/g tissue *S. choleraesuis*

Week	Tonsils	Lung	ICLN	ICJ	Cec Cont
Group 1; n=2					
1	4.05	4.69	6.73	5.56	4.35
2	-[a]	1.20	4.70	5.43	3.67
4	4.47	-	1.32	3.79	2.17
6	3.96	-	2.00	4.98	1.56
9	5.35	-	-	0.62	0.06
12	-	-	-	-	-
Group 2; n=4					
1	4.68	1.49	4.33	5.13	2.98
2	+[b]	1.47	3.99	4.26	1.50
4	-	-	1.83	1.36	-0.28
6	-	-	1.08	-	-
9	-	-	-	0.50	-
12	-	-	-	-	-

a- negative
b- positive by qualitative bacteriology only

Figure Legends

Figure 1. Serum antibody response of swine to *S. choleraesuis* LPS following challenge with *S. choleraesuis* (group 1), after natural exposure to a challenged group (group 2) or uninoculated controls (group 3). Reported values are the mean ELISA $O.D._{410}$ of either IgG (panel a) or IgM (panel b) isotype specific assays.

Figure 2. Intestinal antibody response of swine to *S. choleraesuis* LPS following challenge with *S. choleraesuis* (group 1), after natural exposure to a challenged group (group 2) or uninoculated controls (group 3). Reported values are the mean ELISA $O.D._{410}$ of either IgG (panel a), IgM (panel b) or IgA (panel c) isotype specific assays.

Figure 3. Two-day *in vitro* blastogenic response to *S. choleraesuis* HE antigen of peripheral blood lymphocytes from swine following challenge with *S. choleraesuis* (group 1), after natural exposure to a challenged group (group 2) or uninoculated controls (group 3).

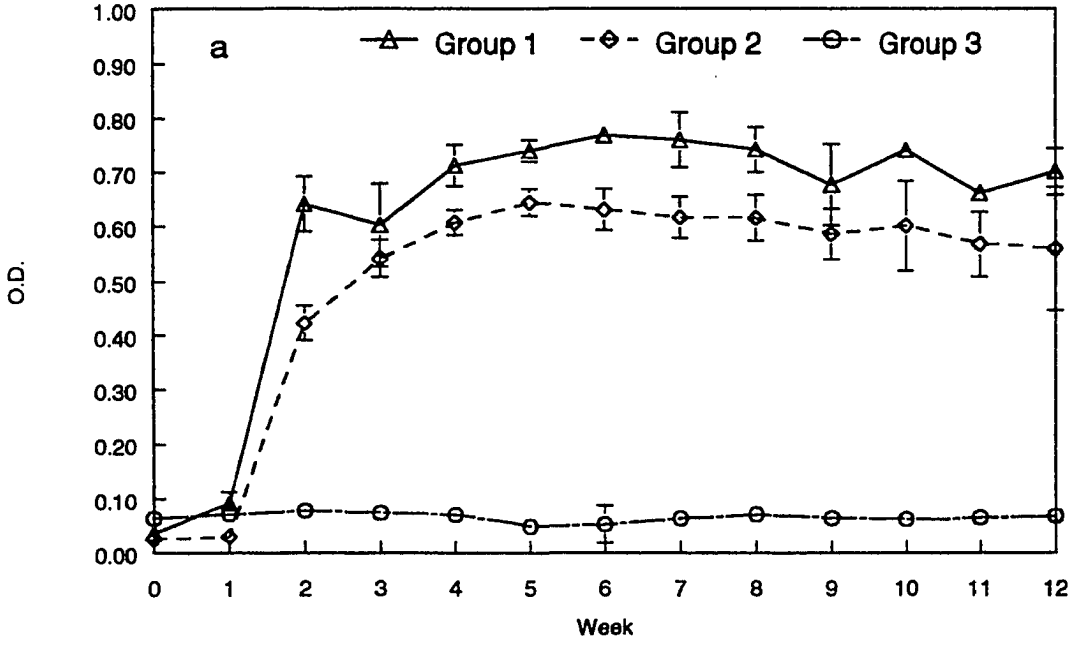
a
Group 1
Group 2
Group 3
O.D.
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
1
2
3
4
5
6
7
8
9
10
11
12
Week

Figure 1a.

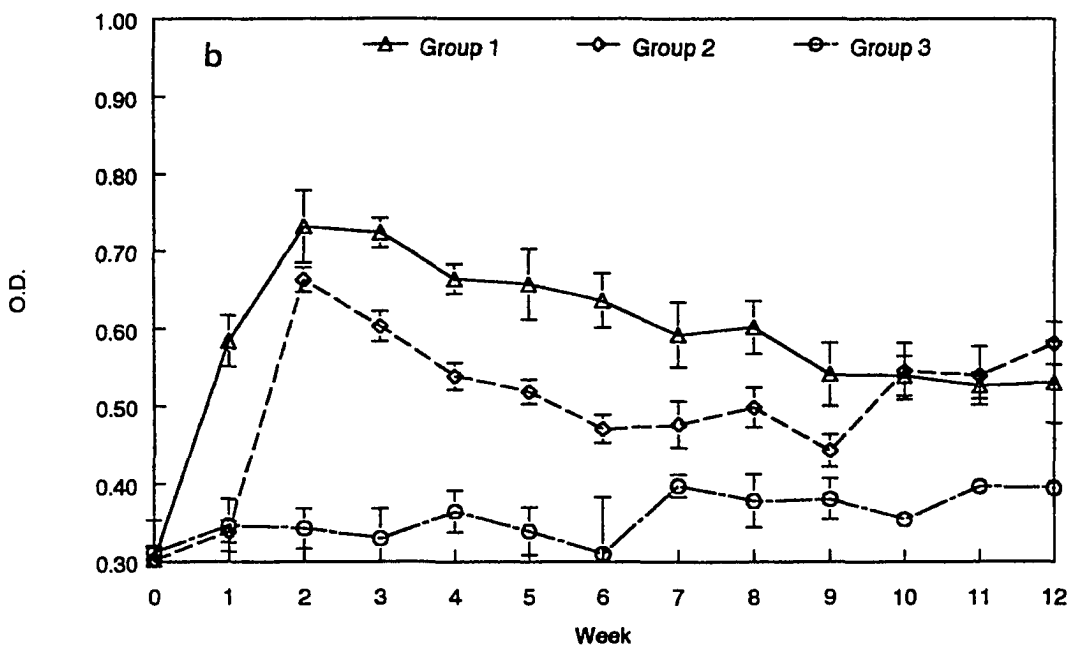
b
Group 1
Group 2
Group 3
O.D.
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0
1
2
3
4
5
6
7
8
9
10
11
12
Week

Figure 1b.

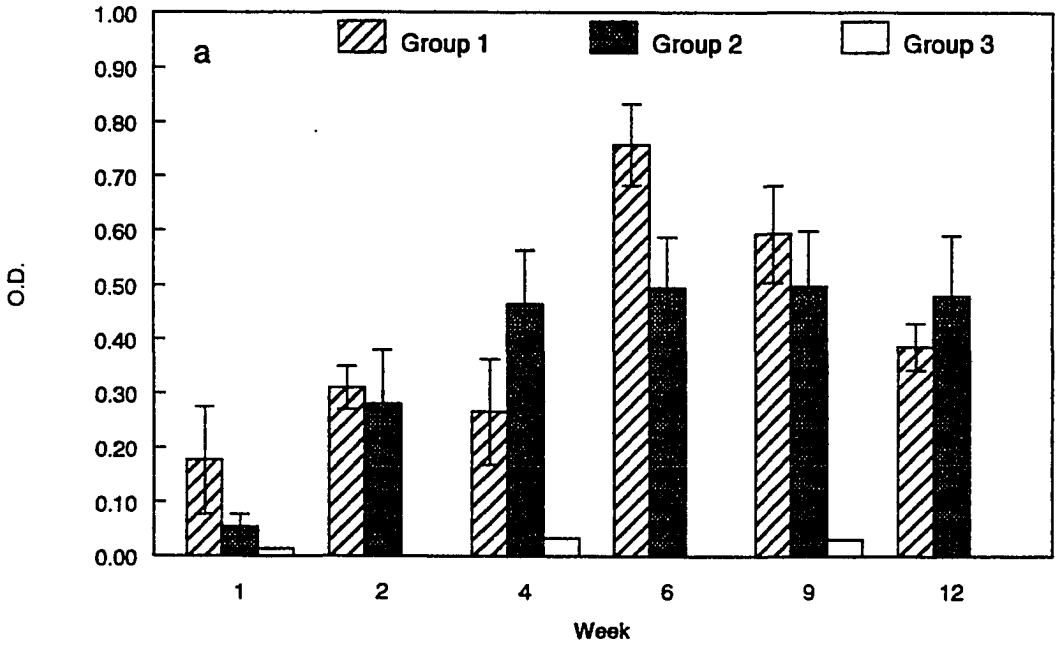
a
Group 1
Group 2
Group 3
O.D.
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
1
2
4
6
9
12
Week

Figure 2a.

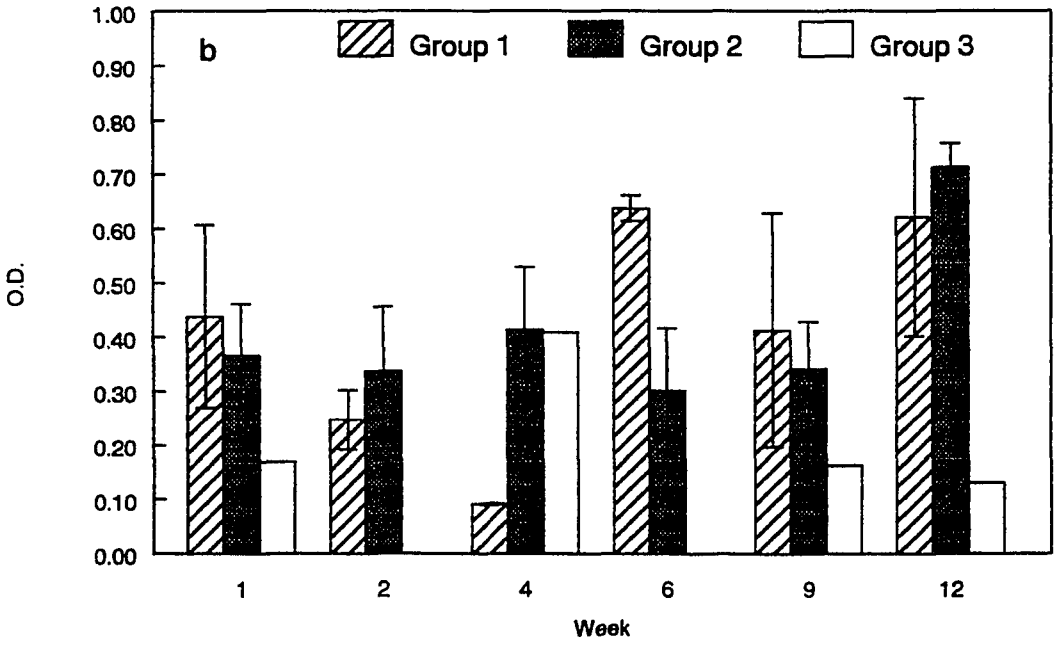
b
Group 1
Group 2
Group 3
O.D.
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
1
2
4
6
9
12
Week

Figure 2b.

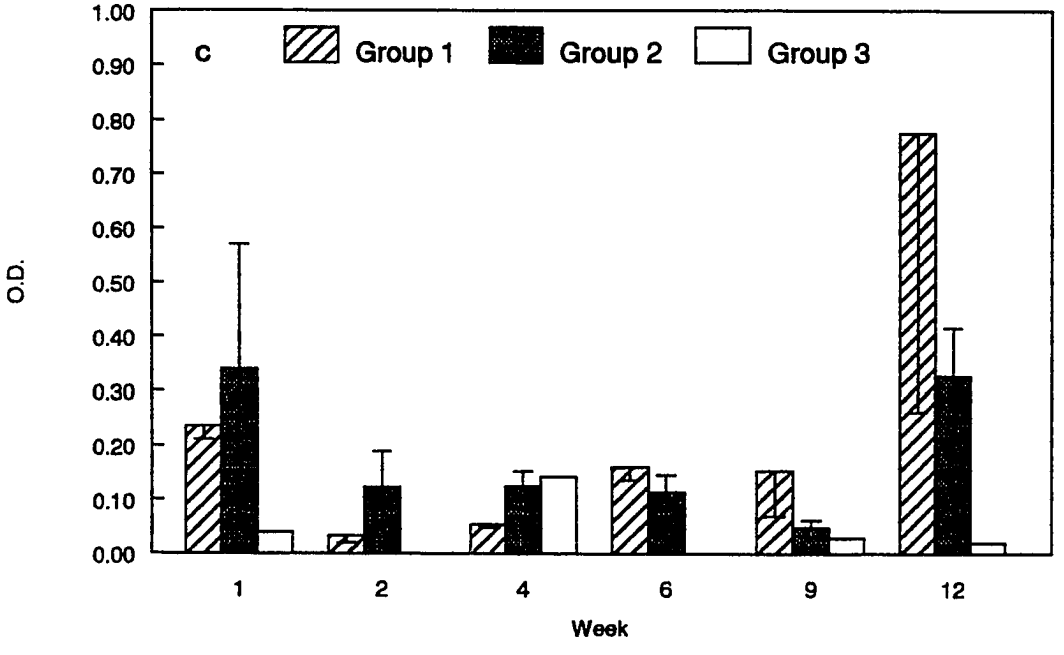
c
Group 1
Group 2
Group 3
O.D.
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
1
2
4
6
9
12
Week

Figure 2c.

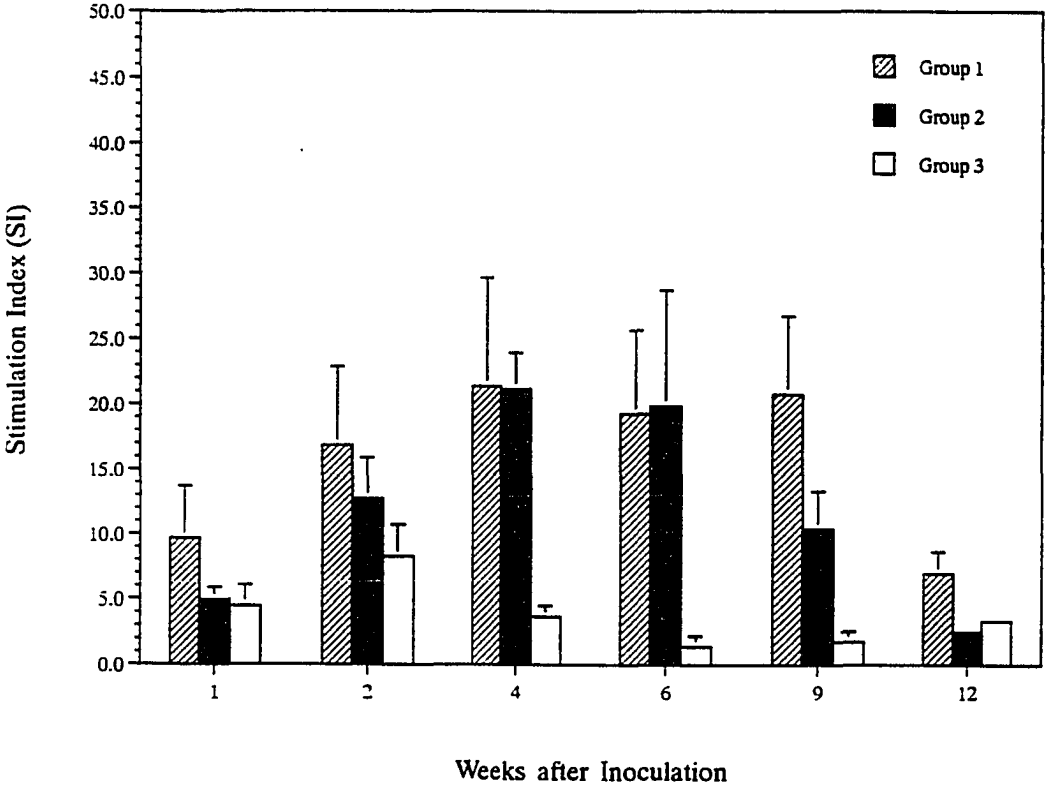
Stimulation Index (SI)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Group 1
Group 2
Group 3
1
2
4
6
9
12
Weeks after Inoculation

Figure 3.

SUMMARY AND CONCLUSIONS

These experiments demonstrate that a carrier state exists in swine infected with *Salmonella choleraesuis*. Results of these studies also indicate that regardless of route of infection, *S. choleraesuis* can persist in the tonsil, ICLN, ICJ and colon and can be excreted in feces of experimentally infected pigs for at least 12 weeks PI. Therefore, upon infection with 10^8 organisms and after resolution of initial clinical signs, the bacteria persist at relatively low numbers in clinically normal pigs establishing a carrier state.

Infection of swine with *S. choleraesuis* in the present studies indicates that the tissue distribution was similar to that reported for *S. typhimurium* except that there was no predilection for *S. choleraesuis* to colonize the tonsil during acute disease. As the subclinical infection progresses the numbers of *S. choleraesuis* in the tonsil increase markedly.

The organism may persist in the tonsil of intranasally infected pigs until 19 weeks post challenge. This suggests that the tonsils are a critical site for maintenance of *S. choleraesuis* in a herd. Although *S. choleraesuis* is often associated with pneumonia in swine, the pathogen was only recovered from the lung up to 6 weeks PI. This suggests that recovery from the lung, but not the tonsil, is indicative of acute disease, while recovery from the tonsil is more likely

to be indicative of a carrier animal. Additionally these data indicate that the tissues in which *S. choleraesuis* is most likely to be found at necropsy are the ICJ (68% recovery; groups combined) and ICLN (65% recovery; groups combined) suggesting that they should be included in slaughterhouse surveys and necropsies where *S. choleraesuis* is a potential isolate.

However, these experiments also indicate that in comparison to experimental infection some differences were observed in naturally infected pigs. High concentrations of *S. choleraesuis* are observed in the tonsils for both naturally and experimentally infected pigs at 1 week PI. This suggests that either the tonsils became colonized due to a septicemia or that it is a primary site of invasion for *S. choleraesuis*. Interestingly, tonsil populations are not observed in naturally infected pigs after 1 week PI. It is unclear whether tonsil populations during subclinical disease (>2 weeks PI) is reserved as an artifact of high dose experimental infection or is triggered by an unknown factor not present in the group 2 pigs. The results presented here suggest that positive tonsil cultures may also indicate a very recent exposure to *Salmonella spp.*

Pneumonia associated with *S. choleraesuis* infection is common. It is unclear whether this predilection for the lung is due solely to the pathogen, poor ventilation in large

confinement buildings or some combination of these and other factors. Experimental infection models have not provided good answers because positive lung samples have been regarded as an artifact of intranasal or per os inoculation. The data presented here indicates that the lung is a site of initial infection of *S. choleraesuis* in naturally exposed pigs. *Salmonella choleraesuis* was recovered from 6/8 lungs of the naturally infected pigs during the first two weeks of infection. The concentrations of *S. choleraesuis* in the lung remained at 1.5 $\log_{10}$ CFU/g through 2 weeks PI in the naturally exposed group.

The effect of route of inoculation was also studied. The intranasal route of inoculation results in more lesions and more severe clinical disease suggesting that *S. choleraesuis* is more virulent in swine following exposure by this route. In these studies the group infected intranasally have a greater magnitude and duration of fecal shedding when compared to the group challenged by gastric route resulting in more environmental contamination. In addition, a larger number of *S. choleraesuis* positive tissues are observed after intranasal inoculation for the first 6 weeks PI. These results suggest that respiratory exposure affects the pathogenesis and maintenance of *S. choleraesuis* in swine.

There is a paucity of information regarding the magnitude and duration of fecal shedding of *S. choleraesuis*. These

studies have shown after a challenge of 10^8 CFU in 1 ml PBS marked clinical illness will occur. The magnitude of shedding after this dose was 10^3 CFU/g which peaked between 72 hours and 3 weeks PI. Beyond 3 weeks PI *S. choleraesuis* was only detected in the feces in low numbers. However, shedding was detected regardless of route of inoculation throughout the 12 week period. The T, N and R swab data and fecal cultures indicate that although these samples may be sufficient to detect *S. choleraesuis* within a herd, they are not effective in detection of individual carrier animals. However, since *S. choleraesuis* is mainly found in swine and swine operations, isolation from any sample suggests that an asymptomatic carrier is present, thereby increasing the likelihood for an outbreak within the herd. These data indicate that carrier animals shed low numbers of *S. choleraesuis* in feces and suggest that suspected shedders should be isolated from naive animals to control outbreaks involving *S. choleraesuis* in confinement units.

There is a direct relationship between dose and shedding magnitude after a 10^9 or 10^6 dose of *S. choleraesuis* is given to pigs. Interestingly, regardless of dose, the pattern of shedding was similar between groups. Peak shedding was observed on day 5 for both groups and declined by 1 week PI. Regardless of dose, pigs demonstrated an increase in shedding at 5 weeks and 7 weeks, respectively. These data indicate

that regardless of dose, an intermittent paradigm of shedding occurs in carrier animals. These data also indicate that carrier swine may shed *S. choleraesuis* for at least 15 weeks PI as observed after administration of a 10^9 dose.

Studies on the humoral immune response of *S. choleraesuis* carrier pigs have shown the development of a sequence of antibody isotype titers indicative of a classic humoral response to bacterial pathogens. The trends in isotype responses observed in the serum were also evident in intestinal immunoglobulin responses, regardless of route of inoculation.

Both routes of inoculation are equally effective at stimulating peripheral blood B-cells. However the intranasal route is more effective, although slower, at activating peripheral blood T-cells.

It has been proposed that adequate immunity to *Salmonella spp.* requires both humoral and cellular responses in the mouse model. This study indicates that swine can harbor *S. choleraesuis* for at least 12 weeks in the presence of a relatively high IgG to *S. choleraesuis* antigens. In general, a combined humoral, mucosal and cellular immune response to *S. choleraesuis* antigens was observed. The response coincided with the reduction in overall *S. choleraesuis* infection. This suggests that adequate immunity to *S. choleraesuis* in swine requires both humoral and cellular immunity.

The minimal dose required to produce a persistent infection of *S. choleraesuis* was also explored. Together these results demonstrate that experimental infection of swine with a dose of 10^8 CFU or higher will result in severe clinical disease and a long term carrier state. At moderate experimental doses, less than 10^7 but above 10^3, inoculation will likely result in mild clinical disease and a short term carrier state of at least 9 weeks. A dose of less than 10^3 will be cleared with no apparent shedding of the organism.

These experiments also demonstrate a dose dependent response after experimental infection with regards to establishment of a carrier state, shedding and the immune response. The post mortem bacteriologic data did not indicate that a persistent infection occurred after challenge with a 10^3 dose of *S. choleraesuis*.

A 10^6 dose results in infection for at least 6 weeks PI and fecal shedding is detected until 9 weeks PI. However, *S. choleraesuis* could not be recovered from tissue or feces from these pigs after 9 weeks PI. A measurable antibody response is also observed in this group, as well as an antigen specific blastogenic response. These results indicate that experimental infection with 10^6 CFU of *S. choleraesuis* results in a carrier state with detectable shedding for at least 9 weeks PI.

A dose of 10^9 results in a long term carrier state. At

15 weeks PI the pigs in group 1 continued to shed *S. choleraesuis* and had multiple tissues positive for *S. choleraesuis*. In addition, we observed an average of 3 logs of *S. choleraesuis*/g of tonsil at 15 weeks PI. The carrier state observed in this group of pigs was present despite antibody titers and a measurable cellular response to *S. choleraesuis* antigens. This suggests that the immune system may be overwhelmed by such a large dose.

At low doses (10^3), *S. choleraesuis* is unable to stimulate a measurable antigen-specific lymphocyte response; however, normal mitogenic responses were observed. At moderate doses, between 10^3 and 10^7, optimal antigen stimulation of lymphocytes occurs as well as normal mitogenic responses. At these moderate doses the immune response observed, here and in other studies is apparently successful at greatly reducing the pathogen load in the host.

In contrast, at higher doses (10^9), *S. choleraesuis* causes some degree of antigen-specific and non-specific suppression of lymphocyte activation. One reason for the increased shedding and level of infection observed in group 1 may be the observed lymphocyte suppression. A long term carrier state occurs in group 1 despite a measurable humoral response and a suppressed cellular response. The observed immunosuppression is likely an important factor in the pathogenesis of *S. choleraesuis* in swine and our observations

suggest that it may play an important role in development of a carrier state.

In contrast, it was also demonstrated that naive swine exposed to a population shedding as little as 2.61 $\log_{10}$ CFU/g of feces will begin shedding *S. choleraesuis* within 24 hours of exposure. In addition, severe clinical signs are observed in the naturally exposed group within 3 days of exposure to the experimentally infected population. This indicates that exposure to relatively low concentrations of *S. choleraesuis* may result in high morbidity and initiate a severe outbreak of swine paratyphoid within a relatively short period of time. This not only indicates the rapidity of *S. choleraesuis* transmission on the farm, but may also translate to rapid transmission and spread of *Salmonella spp.* during transport and time in lairage before slaughter.

The data presented here indicate that only 1 of 4 naturally infected pigs was positive at each of the 6, 9, and 12 week necropsies suggesting two things. First, even after a severe clinical outbreak of swine paratyphoid, the numbers of *S. choleraesuis* carried in deep tissue and present in the environment is markedly reduced after natural exposure. A significant ($P < 0.01$) number of naive pigs are able to clear *S. choleraesuis* between 9 and 12 weeks PI, indicating that not all pigs will become long term carrier animals. Although no management protocols were tested here, this data suggests that

good management practices can control and significantly reduce *S. choleraesuis* in swine herds.

Secondly, these data suggest that a small fraction of carrier pigs are responsible for maintenance of this pathogen in the swine population. It has been shown that *S. choleraesuis* is a serotype that is rarely isolated in feeds or the environment implicating subclinical carrier swine in new outbreaks. It remains a mystery, however, as to the factors which induce carrier swine to shed levels necessary to initiate a new outbreak.

Overall the tissues in which *S. choleraesuis* can most frequently be found in naturally infected pigs are the ICJ, ICLN, cec cont and cecum. During clinical disease ($\leq$ 2 weeks PI) *S. choleraesuis* is isolated from many of the same tissues in naturally exposed pigs as is observed with field outbreaks and experimental infections. During the subclinical carrier state, the tissues of predilection are the ICJ, ICLN, cec cont, colon, CLN and cecum in the naturally infected swine. This is similar to the tissue distribution which has been described after experimental infection of swine with either *S. typhimurium* or *S. choleraesuis*. The sole exception is that the tonsils were not positive in the subclinical naturally infected pigs.

Interestingly, although the clinical signs of the naturally infected swine were similar to the experimentally

infected swine in severity and duration, peak shedding was at least 2 logs lower (1.5 $\log_{10}$ CFU/g of feces for group 2 vs. 3.6 $\log_{10}$ CFU/g for group 1). This indicates that the high levels of shedding observed after experimental infection may not be indicative of shedding magnitude after natural infection.

In this study, the naturally infected swine were exposed to a population shedding a maximum of 3.6 $\log_{10}$ CFU/g of feces with a maximum environmental load equal to 3.0 $\log_{10}$ CFU/g of feces. In comparison to experimental models, the naturally infected pigs would have needed to ingest between 250 g (10^6 CFU dose) and 25000 g (10^8 CFU dose) of feces each to manifest the severe clinical signs observed in this group. This amount of coprophagia is unlikely.

Experimental infection models have shown that swine have a dose dependant immune response to *S. choleraesuis*. In the natural transmission study the humoral and cellular immune responses were similar between the naturally and experimentally infected pigs suggesting they received nearly the same dose or the organism was able to replicate to nearly the same levels in the host. Taken as a whole, these data indicate that during natural transmission the infectious dose of *S. choleraesuis* is much lower than experimental models have previously described. It may also be that pigs are exposed to a large dose of *S. choleraesuis* by a mechanism other than

fecal oral transmission. The latter possibility would necessitate large populations of *S. choleraesuis* are excreted by infected swine by a route other than feces such as mucous or urine. However these high concentrations cannot be confirmed in the environmental samples.

A more likely hypothesis is that there are factors involved in the natural transmission of *S. choleraesuis* which are not reproduced in experimental infection. Some of these factors may include the virulence of the *S. choleraesuis* following passage through the host which may allow lower infectious doses and may allow the organism to replicate to high levels in the host. Other factors involved in the transmission of *S. choleraesuis* may include the unknown affect of multiple inoculations with smaller doses and inoculation mechanisms not reproduced during experimental infections. Some combination of these and other factors is likely.

As discussed previously the humoral, mucosal and cellular immune response observed for the naturally infected swine closely parallel that of the experimentally infected group. This indicates that in terms of immune parameters, the experimental infection may be an appropriate model for study of this response. In addition, this indicates that serum collected from experimentally infected swine provide a useful tool for the development of serologically based Salmonella detection tests.

In conclusion these studies have demonstrated that route of inoculation impacts on the carrier animal. However, regardless of route of inoculation, a carrier state persists for at least 12 weeks following challenge with 10^8 CFU *S. choleraesuis*. In addition, these data indicate that the magnitude of shedding and persistence of infection after *S. choleraesuis* infection is dose dependent. Low doses of *S. choleraesuis* can be easily cleared, moderate doses persist for at least 2 months, and high doses result in a long term carrier state. Regardless of dose, cellular and humoral immune activation occurs after inoculation. However, at high doses, *S. choleraesuis* causes signs of lymphocyte suppression, which may affect the ability of the immune system to eliminate the bacteria. These data provide an important model for future investigation of *S. choleraesuis* infection in swine. The model for *S. choleraesuis* transmission described in these studies provides a dramatic example of the difference between infection with *Salmonella* grown the laboratory versus the natural host and should be seen as a basis for design of future animal studies.

ACKNOWLEDGEMENTS

I would like to thank all of the individuals in my laboratory including Nels Nord, Ruth Willson, Kathy Foss, Brad Chriswell, Nathan Adair, Jenny Puls and Jenny Emerson. Without this team of individuals none of these studies would have been possible. I would also like to thank Dr. Thomas Stabel his help, expertise and contributions to the immunological aspects of my studies. His efforts strengthened my learning experience.

In addition I would like to thank my Ph.D. POS committee members, Dr. Paula Fedorka-Cray, Dr. Hank Harris, Dr. Ted Kramer, Dr. Eldon Uhlenhopp and Dr. Don Beitz, for their support and guidance during my graduate studies. This diverse and distinguished group of scientists ensured that I was always learning and thinking about my studies in new ways resulting in a very broad based education.

I would like to extend special thanks to Dr. Harris who fully supported my research while allowing me the freedom to explore many avenues within the realm of microbiology and to Dr. Kramer for his collaborations, guidance and friendship as well as always showing a sincere interest in my studies.

I would like to extend my sincere gratitude to my friend and mentor Dr. Fedorka-Cray who made sure I learned what I needed to know and then allowed me the freedom to build on

this knowledge. She has given me the encouragement to develop my own interests and ideas and given me a broad base from which to start my career.

Finally, I would like to thank my family for their support and understanding over the past years and I would especially like to thank my wife Melisse, for her support throughout my graduate studies. She endured my frustrations as well as my successes and always was my strongest supporter.