**Early epithelial invasion by *Salmonella enterica* serovar
Typhimurium in the swine ileum**

by

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A thesis submitted to the graduate faculty in partial fulfillment of the
requirements for the degree of
MASTER OF SCIENCE

Major: Veterinary Microbiology

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2001

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ABSTRACT

The purpose of the experiment was to define the early cellular invasion of *Salmonella enterica* serovar Typhimurium in a swine gut-loop model. Ileal gut-loops were surgically prepared in 4-5 week old pigs and inoculated with sterile media or *Salmonella* serovar Typhimurium for 0 to 60 minutes. Loops were harvested and prepared for both scanning and transmission electron microscopy. Preferential bacterial adherence to M cells was observed by 5 minutes postinoculation and apical invasion was seen in M cells, goblet cells and enterocytes by 10 minutes postinoculation. This nonspecific invasion was observed throughout the course of infection. The results suggest early invasion by *Salmonella* serovar Typhimurium is not cell-specific and calls into question the concept of cell-specific invasion sites. In addition, scanning electron micrographs showed a specific affinity by *Salmonella* serovar Typhimurium to sites of cell extrusion. Transmission electron micrographs depicted bacteria around and in the crevices formed by extruded cells and the adjacent cells, and in the cytoplasm immediately beneath extruded cells. The combination of scanning and transmission electron micrographs suggests *Salmonella* serovar Typhimurium may utilize sites of cell extrusion for early invasion in swine.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Salmonellosis is an important disease in pigs and a production concern for pig producers (80). It can cause increased production costs and direct animal losses (89). Additionally, *Salmonella enterica* serovar Typhimurium is a zoonotic pathogen that can enter the food chain through pork products (2). Emerging antibiotic resistance creates an increased urgency about finding novel means of controlling this disease (19). A necessary component of that process is a critical understanding of the pathogenesis of salmonellosis.

Studies of *Salmonella* epithelial invasion have often been carried out in mice (17, 52). In mice, early cellular invasion is seen primarily in the M cell of the ileum. The results from murine studies have been inferred to other species often without sufficient examination (9). Previous studies that examined *Salmonella* invasion in pigs have yielded mixed and conflicting results (9, 77, 80). Additionally, the studies utilized time points that ranged in hours while recent work suggests invasion may occur within minutes (26). *Salmonella* also have the capacity to invade epithelial cells from the basolateral membrane (52). Lack of early time points makes definitive statements

concerning apical cell invasion impossible. To define the cells involved in *Salmonella* invasion, early time points are critical.

The experimental design was to evaluate time points from zero to sixty minutes postinoculation utilizing a porcine gut-loop model. Tissues were examined by scanning electron microscopy and transmission electron microscopy.

Thesis Organization

Chapter one is a general introduction that contains an introduction to and a literature review of *Salmonella* invasion. Chapter two of the thesis is a manuscript submitted to *Infection and Immunity* that details the work of assessing early sites of cell invasion by *Salmonella* serovar Typhimurium. In chapter three is a discussion of the results and proposals for future research. The appendix contains additional electron micrographs that were not included in the original manuscript. This is followed by a general list of references for the thesis.

Literature Review

Salmonella enterica is a gram-negative, motile bacillus of the Enterobacteriaceae family (13). *Salmonella enterica* is a facultative anaerobe and a gastrointestinal pathogen. A wide range of species can

be infected by or act as a reservoir for *Salmonella enterica*. This includes most domestic animals, wildlife and humans (10, 11, 89).

Salmonella enterica is ubiquitous in the world ecosystem because of many potential hosts (11). In addition, factors such as persistent host infection, vectors and resilience to environmental degradation play a role in its ubiquity.

Following the resolution of clinical signs, some infected hosts may persistently shed *Salmonella* into the environment for an extended period of time. Bacteriological evaluation of pigs following oral inoculation with *Salmonella enterica* serovar Typhimurium indicated that 50% of the pigs remained culture positive twenty weeks after challenge (108). Fecal pellets from mice have been shown to harbor up to 2×10^5 colony-forming units of *Salmonella enterica* serovar Enteritidis. The same study suggested that *Salmonella* could persist in the mouse population for up to ten months (43).

Vectors may harbor and transmit *Salmonella* in persistently contaminated environments. In a bacteriological survey of cockroaches, 4.1% were positive for *Salmonella*. The authors observed an association between the serovars isolated in the cockroaches and in isolates from humans and animals at the same location. The authors concluded that cockroaches act as a significant

environmental reservoir for *Salmonella* (18). Veterinarians have also been reported to act as vectors for *Salmonella* (36).

Salmonella enterica is a hardy organism and can reside in the environment for extended periods of time. *Salmonella enterica* serovar Choleraesuis has been shown to survive in wet pig feces for three months and dry pig feces for thirteen months (38). Persistent *Salmonella* have been implicated in nosocomial equine infections acquired at veterinary hospitals in spite of ample cleaning and disinfection (88). *Salmonella*'s ability to persist in the environment by various means can play an important role in the clinical setting.

In order to discuss *Salmonella*, it is important to understand the terminology. The current nomenclature for *Salmonella* is complex and controversial (46). It has evolved from the one serovar-one species format. In that system, *Salmonella enterica* was further defined according to serovar based on the identification of the O and H antigens. What once was simply referred to as "*Salmonella typhimurium*" is now formally called "*Salmonella enterica* subspecies *enterica* serovar Typhimurium". The complete name may be abbreviated "*Salmonella enterica* serovar Typhimurium" or "*Salmonella* serovar Typhimurium". This nomenclature system has been accepted for use by the World Health Organization, Centers for Disease Control and Prevention, and by the American Society for Microbiology (10, 46).

Salmonellosis can be described clinically in two general forms: a localized gastrointestinal disease or a systemic typhoid-like disease (3). In the localized disease, the infection from the gut is relatively contained by the immune system at the regional lymph nodes. In the systemic form, the infection overwhelms the immune system resulting in generalized, multiorgan disease.

Two major factors affecting the clinical disease presentation are the immune status of the host and the serovar-host adaptation (3). The host's immune status plays an important role as stressed animals experience more frequent and severe clinical disease (89). In swine, infection with *Salmonella enterica* serovars Typhi, Typhimurium or Choleraesuis leads to different clinical disease based on host-adaptation (67, 80). Serovar-host adaptation also plays role in clinical disease manifestation.

In swine, most clinical cases of salmonellosis are caused by *Salmonella enterica* serovars Choleraesuis and Typhimurium (80, 89). *Salmonella* serovar Choleraesuis is host-adapted to swine and causes a systemic, typhoid-like disease. Clinical signs include anorexia, lethargy, pyrexia and some pigs additionally exhibit cough, dyspnea, diarrhea or cyanosis of the extremities. Rarely, meningitis, encephalitis and abortion have been reported. Gross lesions can often include: diffuse lung congestion and pneumonia, fibrinous

enterocolitis, swollen and edematous mesenteric lymph nodes, splenomegaly, hepatomegaly containing miliary foci of necrosis, and occasionally, petechial hemorrhages on the renal cortex.

Microscopically, foci of coagulative hepatocellular necrosis associated with clusters of macrophages and neutrophils are called paratyphoid nodules. Fibrinous thrombi can be seen in the gastroenteric mucosa, skin, glomerular capillaries and lung. Hyperplasia of reticular cells of the spleen and lymph nodes is common. The lungs often show a diffuse histiocytic interstitial pneumonia. A multifocal necrotizing enterocolitis can also be seen in many pigs.

Salmonella serovar Typhimurium is not host-adapted to swine and infection can cause a localized enterocolitis. Clinically, pigs may exhibit diarrhea, pyrexia, inappetence, and dehydration. Gross lesions include focal to diffuse necrotizing enteritis, colitis or typhlitis. The focal necrosis may be present in the form of button ulcers. A small number of animals can develop rectal strictures as a result of the mucosal necrosis. Microscopically, there is a focal to diffuse enterocyte necrosis. Neutrophil infiltration predominates in very early infection. This is followed by infiltration of numerous macrophages and lymphocytes. Paratyphoid nodules can be present in the liver, but not with the consistency or severity of necrosis as seen with *Salmonella* serovar Choleraesuis infection.

Salmonellosis is an economically important disease of pigs (80). It increases expenses by decreased production efficiency, increased veterinary medical costs and individual pig losses. *Salmonella* infection results in the loss of millions of dollars to pig producers annually (82, 89).

Salmonella serovar Typhimurium is a concern to the swine industry not only by direct economic losses but also by acting as a food borne pathogen for humans. In the United States, an estimated 1.4 million cases of human salmonellosis occurs annually (66). Recent work estimates that up to 25% of the food-borne cases of human salmonellosis are directly associated with pork products (5, 6). Even more critical to human infection is the emergence of multi-antibiotic resistant strains such as *Salmonella* serovar Typhimurium phagetype DT104 (2, 37). The DT104 phagetype is associated with penta-antibiotic resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline. By 1996 greater than 30% of *Salmonella* serovar Typhimurium human isolates were thought to be phagetype DT104 (37). Some *Salmonella* serovar Typhimurium DT104 clinical isolates are now resistant to fluoroquinolones and third generation cephalosporins, a first line of defense for systemic salmonellosis in adults and children (46, 69). Ceftriaxone (a third-generation cephalosporin) resistance has risen from 0.1% of human

Salmonella isolates in 1996 to 0.5% of isolates in 1998 (19). Emerging antibiotic resistance creates sense of urgency about finding novel methods to control salmonellosis. A necessary component of that process is a critical understanding of the pathogenesis of salmonellosis.

Salmonella transmission has traditionally been considered to be by the fecal-oral route, although aerosol transmission has been shown in the calf and mouse (102). The mouse model has frequently been used to define *Salmonella* infection. The site of infection in the mouse was described as the distal ileum and possibly the cecum using intragastric inoculation of *Salmonella enterica* serovar Enteritidis. The infection was almost exclusively associated with the Peyer's patch epithelium of the ileum (14). Oral inoculation in mice with *Salmonella* serovar Typhimurium produced similar foci of infection on the epithelium of the Peyer's patch of the ileum (45). *Salmonella* serovar Typhi inoculated murine gut-loops also showed foci of infection on the ileal Peyer's patch (57). Dogma that the Peyer's patch of the distal ileum is the primary site of early entry is still prevalent, although recent work in esophagotomized pigs suggests that *Salmonella* serovar Typhimurium invasion does not require access to the ileum (22).

The epithelium of the ileum can be morphologically classified into absorptive epithelium (AE) and follicle-associated epithelium

(FAE). The AE is composed primarily of enterocytes and goblet cells. The FAE is composed of enterocytes and M cells with an absence of goblet cells (31). The continuous ileal Peyer's patch is a long segment of lymphoid tissue in the pig ileum (83). Overlying the continuous ileal Peyer's patch are villi with either AE or FAE. The FAE villi are often shorter than the adjacent AE villi and appear more pyramidal than cylindrical in histological sections (4, 15).

M cells are found overlying gut-associated lymphoid tissue such as the Peyer's patch region and are thought to be differentiated epithelial cells originating in the crypts (42, 55, 85). The M cell terminology comes from its microfolds or membranous apical surface first identified by electron microscopic studies of human Peyer's patches. (73). The M cell cannot be differentiated from enterocytes by light microscopy. However, by electron microscopy, M cells are easily discerned by fewer, thicker and more irregular microvilli compared to adjacent enterocytes (31). Additionally, the M cell has a less electron-dense cytoplasm than enterocytes (15).

The M cell is thought to function as an antigen-sampling site for the mucosal immune system. The sampled antigen is transferred to the underlying immune cells and may induce immunologic tolerance or stimulate an immune response (56). M cells are often associated with intraepithelial lymphocytes (IEL's) and macrophages (30). The

appearance of M cells in severe combined immunodeficient mice following syngeneic bone marrow transplantation suggests that lymphoid cells may participate in the development of M cells in mice (90). Intestinal microflora and pathogens may also contribute to M cell development (42, 86).

In addition to antigen-sampling, M cells can take up pathogenic bacteria such as *Shigella flexneri*, *Vibrio cholerae*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Campylobacter jejuni*, *Listeria monocytogenes* and *Salmonella* serovar Typhimurium (35, 39, 49, 52, 74, 101). This uptake pathway by the M cell facilitates the pathogen's translocation across the epithelial barrier. Recently, in *Saccharomyces cerevisiae* inoculated pig gut-loops, the tracer yeast was translocated to the basolateral space and subsequently the lamina propria within one hour of M cell uptake (4). A similar uptake pathway was also seen in another pig study using ferritin as the tracer molecule (60). The rapid and accessible M cell uptake pathway can be used by pathogens (52).

The murine M cell plays an important role in early *Salmonella* infection. An early study of *Salmonella* serovar Typhi inoculation in murine gut-loops showed the foci of infection was located at M cells of the FAE (57). The M cell as a primary portal of entry in early *Salmonella* serovar Typhimurium invasion was demonstrated when

two different groups observed M cells as a preferential site of adherence and early invasion murine gut loops by electron microscopy (17, 52). The M cell invasion was described as a remodeling of the apical cell membrane into lamellipodia, also called membrane ruffles, leading to engulfment of the bacteria. The bacteria caused death and extrusion of the M cell. The rapid loss of M cells led to bacterial exposure of the basement membrane. Bacteria could then invade underlying lymphoid tissue or the basolateral membrane of adjacent cells (52, 95).

The concept that M cells are the primary cellular site of entry during early infection of mice is often applied to other species without sufficient evaluation (8, 9). Characterization of invasion by *Salmonella enterica* serovars Typhimurium and Dublin in bovine gut-loops showed no difference in recovery of bacteria from ileum with or without Peyer's patches. This suggested the Peyer's patch FAE and M cells may not act as an exclusive invasion site in the bovine model (104). The same laboratory group later showed preferential interaction between *Salmonella* serovar Typhimurium and M cells during early invasion. However, both enterocytes and M cells contained internalized bacteria within minutes of inoculation (26). Previous work with *Salmonella* serovar Typhimurium in inoculated rabbit ileal-loops has showed bacterial invasion of enterocytes by two hours

postinoculation (99). Similar findings were seen *in vitro* using rabbit ileal-explants at three hours postinoculation. The authors concluded that the invasion of enterocytes did not depend on the invasion of M cells (8).

After translocating the epithelial barrier, *Salmonella* organisms can spread to the regional lymph nodes and other organs (14). Knockout mice (CD18⁻) have been used to show that CD18⁺ phagocytes are important in the dissemination of the organism to the spleen and liver (97). Two leading cell candidates for this systemic transport are the macrophages and dendritic cells (98). Recent *in vitro* work has suggested that dendritic cells are capable of producing tight junction proteins to penetrate the epithelium and sample luminal bacteria including *Salmonella* (81).

A critical component in *Salmonella* invasion of intestinal epithelial cells is injection of proteins by a type-III secretion system (47, 52). This type-III secretion system and the *Salmonella* invasion protein's (Sip's) are located at a specific locus called Salmonella Pathogenicity Island (SPI)-1 (100). SipA, a protein product of the SPI-1, is important for early invasion (50). SipA binds actin to reduce the critical concentration for actin polymerization and stabilizes the actin to prevent depolymerization (109). This facilitates apical membrane rearrangements by *Salmonella* outer membrane protein (Sop)E. SopE

and SopE2 are two proteins that are involved with cellular invasion (27, 93). These proteins are injected into the cell and act on the Rho family of G-proteins. This induces a rearrangement of the actin cytoskeleton resulting in apical lamellipodia and filopodia formation (41, 59). SptP, another injected protein of the type-III secretion system, participates in the resolution of the induced filopodia and lamellipodia from the cell membrane by inactivating the Rho GTPases Cdc42 and Rac (29).

Inactivation of another SPI-1 invasion gene (*inv*) in *Salmonella* serovar Typhimurium increases the 50% lethal dose (LD₅₀) in BALB/c mice, but does not completely eliminate invasion (28). The reduction of invasion was also seen for invasion mutants placed in bovine gut-loops (104). In contrast, intraperitoneal administration of wild type or invasion mutant strains in BALB/c mice resulted in a similar LD₅₀. This suggests that invasion genes play a less significant role once the bacteria are internalized across the epithelial barrier.

Additional *Salmonella* proteins are important for invasion. After *Salmonella* enter an M cell, the mouse model suggests that it induces M cell death and subsequent extrusion thereby creating a gap in the epithelium for translocation to the basement membrane (49, 52). Experiments using knockout mice suggested that the caspase-1 pathway is important for the induction of M cell death (70). Caspase-1

is a cysteine protease that is activated by SipB to induce apoptosis in macrophages (44). Recently in a bovine gut-loop model, a *Salmonella* serovar Typhimurium *sopB* mutant was used to assess the significance of cell death to induce enteric inflammation and disease. The study showed SopB induction of cell death was not required to activate intestinal inflammation and neutrophil migration (84).

Early intestinal inflammation is considered a key feature of pathogenesis for enteric forms of salmonellosis. Neutrophil recruitment and transmigration across the epithelium is considered the most significant component (65). Caspase-1 can act as a proinflammatory agent by cleaving interleukin-1 beta and interleukin-18 into active molecules (21). SipA has also been shown to contribute to the inflammatory response by activation of phosphokinase C (58). *Salmonella*-induced activation of inflammatory mediators such as nuclear factor-kappa beta and phosphokinase C results in basolateral secretion of interleukin-8 and apical secretion of pathogen-elicited epithelial chemoattractant (20, 58). These molecules act as chemokines promoting the transepithelial migration of neutrophils into the intestinal lumen (33).

The invasion of apical versus basolateral membranes may play a role in the inflammatory response. *In vitro* study of flagellin from *Salmonella* serovar Typhimurium showed it to be a proinflammatory

molecule by inducing interleukin-8 secretion (34). Induction of inflammation was effective only with basolateral presentation of the flagellin. The flagellin was shown to bind to Toll-like receptor 5 that is exclusively expressed on the basolateral surface (32). In recent work, the presence of flagella on *Salmonella* serovar Typhimurium was shown to be required for the full induction of neutrophil influx into the intestines (87).

One difficulty in defining tropism of cell invasion by *Salmonella* serovar Typhimurium is that rapid apical invasion may be followed by lateral invasion of adjacent cells (26, 52, 95).

Interaction between *Salmonella* and host cells can occur rapidly. Recent work in a bovine gut-loop model suggests *Salmonella* serovar Typhimurium may interact with M cells by five minutes postinoculation. Furthermore, bacteria adhered to enterocytes by fifteen minutes postinoculation. By thirty minutes enterocytes with internalized bacteria had exfoliated into the lumen (26). Previous studies on mouse gut-loop models have demonstrated invasion can occur in less than thirty minutes postinoculation (17, 49, 52). Early time points are critical to assess early invasion.

Early work in guinea pigs suggested *Salmonella* serovar Typhimurium has the capacity to directly invade the region of the cellular tight junctions. The bacteria were enclosed by the adjacent

cell membranes and could then invade those cells. This resulted in a bacterium-containing vacuole that was identical in morphology and subsequent cellular migration as bacteria that invaded apically (95). Similar results were seen in mouse gut-loops. Following M cell invasion bacteria were translocated to the basolateral space. Furthermore, if the M cell had exfoliated leaving a gap in the epithelium then the bacteria could directly reach the basolateral space. From the basolateral space bacteria could invade adjacent cells at the basolateral membrane (52). The capability of *Salmonella* serovar Typhimurium to invade within minutes of inoculation compounded by its ability to invade adjacent cells through the basolateral membrane makes early time points critical to definitively assess sites of cellular invasion.

The pig is a good model to study the pathogenesis of enteric and systemic salmonellosis. The pig is susceptible to both host-adapted and non host-adapted serovars that clinically manifest as distinctly different diseases (80, 105). Few studies have examined *Salmonella* cellular invasion in the pig ileum. Recently, intranasal inoculation of four-week old pigs with the human-adapted *Salmonella* serovar Typhi yielded no clinical signs or lesions. Recovery of *Salmonella* serovar Typhi from tonsils three weeks post infection did suggest carriage of the organism (67). In another study, six-week old pigs at twenty-four

hours after oral inoculation, *Salmonella* serovar Typhimurium was observed in phagocytic vacuoles of ileal enterocytes and in the interepithelial space between enterocytes. No evidence of labeled *Salmonella* serovar Typhimurium was found within or adhered to M cells. However, *Salmonella* serovar Choleraesuis was seen in vacuoles of enterocytes and M cells (77). Using inoculated ileal gut-loops, *Salmonella enterica* serovars Heidelberg and Typhimurium organisms were seen intracellularly at two hours postinoculation. The intracellular organisms were located in the apical cytoplasm of enterocytes, but apical penetration by the organisms was not demonstrated. Furthermore, examination of *Salmonella* serovar Choleraesuis inoculated loops revealed no intracellular bacteria in ileal epithelium or phagocytes until eight-hours post inoculation (79, 80). Recently, investigators have shown that *Salmonella* serovar Choleraesuis was internalized in M cells, absorptive enterocytes and goblet cells of pigs within ninety minutes postinoculation. The authors concluded that *Salmonella* serovar Choleraesuis could be internalized by enterocytes removed from active M cell foci; however, definitive statements concerning apical invasion or cellular kinetics of infection could not be made (9).

The absence of early time points make it difficult to definitively conclude apical invasion occurred by the mere presence of

intracellular *Salmonella*. In order to accurately assess cellular invasion portals, early and frequent time points are critical. The purpose of the following study was to document early sites of cellular invasion by *Salmonella* serovar Typhimurium in ileal epithelium using the swine gut-loop model.

CHAPTER 2. Early epithelial invasion by *Salmonella enterica* serovar Typhimurium DT104 at sites of cell extrusion in the porcine ileum

A paper submitted to the journal *Infection and Immunity*

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Abstract

Salmonella enterica serovar Typhimurium is an important intestinal pathogen in swine. The study was performed to document the early cellular invasion of *Salmonella* serovar Typhimurium in the swine ileum. Ileal gut-loops were surgically prepared in 4-5 week old pigs and inoculated for 0 to 60 minutes. Loops were harvested and prepared for both scanning and transmission electron microscopy (SEM and TEM, respectively). Preferential bacterial adherence to M cells was seen at 5 minutes and by 10 minutes bacterial invasion of the apical membrane was seen in M cells, goblet cells and enterocytes. This multicellular invasion was observed throughout the course of infection. Our results suggest that early cellular invasion by *Salmonella* serovar Typhimurium is non specific in swine and reduces

support for the concept of specific cell-invasion portals. In addition, SEM revealed a specific affinity of *Salmonella* serovar Typhimurium to sites of cell extrusion. Using TEM, bacteria in these areas were around and in the crevices formed by the extruding cell and the adjacent cells, and in the cytoplasm immediately beneath an extruding cell. The combination of SEM and TEM data suggests *Salmonella* serovar Typhimurium may utilize sites of cell extrusion for early invasion.

Introduction

The pig is a good model for studying the pathogenesis of systemic and enteric human salmonellosis since it is susceptible to both host-adapted and non host-adapted serovars that clinically manifest as distinctly different diseases (80, 105). In swine, most clinical cases of salmonellosis are caused by *Salmonella enterica* serovars Choleraesuis or Typhimurium (80, 89). *Salmonella* serovar Choleraesuis is host-adapted to swine causing a systemic, typhoid-like disease. *Salmonella* serovar Typhimurium is not host-adapted to swine and infection leads to a localized enterocolitis.

Sites of early *Salmonella* cellular invasion in the pig ileum are not well defined. Previous work has shown that *Salmonella* serovar Typhimurium can be seen in phagocytic vacuoles of ileal enterocytes

and in the interepithelial space 24 hours following oral inoculation. No bacteria were seen within or adherent to M cells (77). In inoculated swine ileal gut-loops, *Salmonella enterica* serovars Heidelberg and Typhimurium organisms were found intracellularly by two hours postinoculation. The intracellular organisms were in the apical cytoplasm of enterocytes, but apical entry by the organisms was not demonstrated. Examination of the 2, 4, and 6-hour *Salmonella* serovar Choleraesuis-inoculated loops revealed no intracellular bacteria in ileal epithelium or phagocytes (79, 80). In another study at 90 minutes postinoculation, *Salmonella* serovar Choleraesuis organisms were documented in M-cells, absorptive enterocytes and goblet cells. The authors concluded that *Salmonella* serovar Choleraesuis could be internalized by enterocytes removed from active M cell foci; however, definitive statements concerning apical invasion or cellular kinetics of infection could not be made (9).

The previous studies assessing invasion in swine have used time points of 90 minutes or more. However, a bovine gut-loop model suggests invasion can occur within minutes (26). In addition, it has been suggested that following apical invasion *Salmonella* spp. have the capacity for basolateral invasion of adjacent cells (52, 95). In the pig, lack of early time points has prevented definitive statements from being made concerning *Salmonella* cellular invasion. The purpose of

this study was to identify sites of early cellular invasion by *Salmonella* serovar Typhimurium in ileal epithelium of the swine gut-loop model.

Materials and methods

Bacteria preparation

Swine isolates included *Salmonella* serovar Typhimurium phagetype DT104 (NVSL 98-745) which is a clinical swine isolate obtained from the National Veterinary Services Laboratories (Ames, Iowa) and *Salmonella* serovar Choleraesuis var. kunzendorf strain χ 3246 (courtesy of Dr. Roy Curtiss III, Washington University, St. Louis, Missouri). Luria-Bertani (LB) cultures (5 ml) were inoculated from 20% glycerol stocks stored at -70° C. Cultures were grown at 37° C with shaking at 220 rpm for 7 hours. Next, ten microliters of the 7-hour growth was inoculated into 5 ml LB broth and grown statically for 16 hours at 37° C. At this time, the cultures were placed on ice and a sample was taken for serial dilutions and subsequent plating on tryptose agar. The final inoculum concentration for *Salmonella* serovar Typhimurium was 8×10^8 CFU/ml and for *Salmonella* serovar Choleraesuis it was 6×10^8 CFU/ml.

Animals

Ten mixed breed pigs were obtained at ~10 days of age and housed in isolation facilities at the National Animal Disease Center

(NADC, Ames Iowa). The pigs were rectal-culture negative for *Salmonella* in the weeks prior to the experiment. For the experiment, 4-5 week old pigs were fasted 18 hours prior to the surgery. The animal protocol was approved by the Animal Care and Use Committee at NADC.

Surgical procedures

Anesthesia for the terminal surgery was induced by intramuscular injection of Telazol™ (tiletamine/zolazepam, 18 mg/kg, Fort Dodge Labs, Ft. Dodge, Iowa), Ketaset™ (ketamine, 24 mg/kg, Fort Dodge Labs, Ft. Dodge, Iowa) and Rompun™ (xylazine, 12 mg/kg, Bayer Animal Health, Shawnee Mission, Kansas). After the surgical plane of anesthesia was reached and the abdomen prepared, a midline abdominal incision was made and the ileocecal junction (ICJ) exteriorized. Surgical loop procedures were adapted from previous descriptions (79, 104). Briefly, within 10-15 cm of the ICJ and working cranially, the ileum was ligated (#2 silk) to form 6 cm test loops each separated by 2 cm spacer loops. Care was taken to not compromise the blood supply and to ensure presence of the Peyer's patch. The test loops were injected with 6 ml of sterile LB or bacterial inoculum. The ileum was then replaced and the abdomen closed. At the appropriate time point the abdomen was reopened and the loops

exteriorized. Each loop was injected to moderate distension with fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer [pH 7.2]). The loop was harvested and placed into a fixative bath. The pig was then euthanized with intravenous sodium pentobarbital.

For the modified loop procedure the ileum was exteriorized and the ileum gently occluded by digital pressure near the ICJ and ~ 60 cm cranially. To facilitate adequate cellular exposure, the *Salmonella* serovar Typhimurium DT104 inoculum was increased to 20 ml, injected into the lumen and the ileum gently rocked. The functional occlusions were removed and the ileum replaced into the abdomen. After 30 minutes the ileum was exteriorized and at 30 cm cranial to the ICJ a 6 cm portion of the ileum was formed into a loop and immediately injected with fixative. The loop was harvested and placed into fixative.

TEM procedures

After 1-2 hours in fixative, the loops were opened and a 3-4 mm wide cross-section of the loop removed and placed into fresh fixative. The samples were refrigerated (4° C) until further processing, usually within 48 hours. Sample preparation was performed as previously described (1). Samples were viewed on a Philips 410 transmission electron microscope.

SEM procedures

After 1-2 hours in the fixative the tissues were removed and opened. A 1x1 cm section of both Peyer's patch and non-Peyer's patch ileum was removed and lightly agitated in fixative to wash off debris. This was kept cool (4° C) until further processing, usually within 48 hours.

SEM sample preparation was modified from previous descriptions (54, 63). Briefly, the tissues were postfixated with 1% osmium tetroxide and further treated with 1% thiocarbohydrazide. These steps were repeated twice. The samples were dehydrated through series of graded ethanol baths and then critical point dried. Tissues were mounted on aluminum stubs with colloidal silver and sputter coated with gold-palladium for a final coating of approximately 300 angstroms. Samples were viewed at the Bessey Microscopy Facility (Iowa State University, Ames, Iowa) on a JSM-5800LV (Japanese Electronic Optical Laboratories) scanning electron microscope at 10 kilovolts.

Experimental design

Ten pigs were randomly assigned to a time point (0, 5, 10, 15, 20, 25, 30 [n=2], 45 & 60 minutes postinoculation). Four, 6-cm loops were prepared in each pig (except 0 and 30 minute modified gut loop)

to accommodate both SEM and TEM processing. Two of the loops received sterile LB and the other two received *Salmonella* serovar Typhimurium DT104. A time "0-minute" point was defined as a loop being injected with fixative immediately after formation. An additional 30-minute time point was allotted for the modified gut-loop procedure described above. For the 60-minute time point, two additional loops were made for *Salmonella* serovar Choleraesuis SEM and TEM processing.

Results

SEM examination

In the pig, the follicle-associated epithelium (FAE) villi were shorter than adjacent absorptive epithelium (AE) villi. FAE villi were found by looking in gaps between AE villi and also on the cut edge of the sample. On the FAE, M cells and enterocytes were the predominant cell types with a noted absence of goblet cells. The M cells were distinguished by the microvilli, which often appear thicker and more irregular compared to adjacent enterocyte microvilli (Fig. 1A).

Bacteria were seen in the intervilli space at all time points (5-60 minutes) of the infected loops and rarely in the controls. Cellular extrusion was seen at the luminal tip of some villi. Additionally,

scattered individual cellular extrusion sites marked by severe cell swelling were noted at various heights of the villi and could be seen in both the FAE and AE. This type of cellular extrusion was found with similar frequency in the both sterile LB and *Salmonella* serovar Typhimurium loops. For the 0-minute time point, a special emphasis was placed on evaluation for sites of cellular extrusion. Sites were seen similar in frequency and character to the 5-minute time point (Table 1). The extrusion rate over 0-60 minutes increased from 0.35 to 1.45 extrusions per villus for the LB loops while the *Salmonella enterica* serovar Typhimurium loops similarly increased from 0.35 to 1.7 extrusions per villus.

At 5 minutes, bacteria were preferentially adherent to a small number of M cells (Fig. 1B). Bacteria were seen on a few FAE enterocytes adjacent to M cells with adherent bacteria, but not on AE enterocytes. At sites of cell extrusion bacteria were preferentially located at the base of the extruding cell (Fig. 1C).

At 10 minutes, M cells with adherent bacteria had filopodia and early bleb formation. Rarely, an isolated patch of AE enterocytes with adherent bacteria was seen. Each patch contained 5-10 bacteria with some filopodia manifested only at the site of attachment.

At 15-30 minutes, M cells with attached bacteria had surface rearrangements including filopodia, lamellipodia and surface

blebbing; however most M cells had no adherent bacteria. Enterocytes with attached bacteria exhibited filopodia and occasional lamellipodia formation at the point of contact with the bacterium (Fig. 1D). Apical membrane reaction sites, with no adherent bacteria, exhibited elongated and degenerated microvilli. Bacteria were present in extrusion zones near the AE villi tips (Fig. 1E). The bacteria were adherent at the edges of cells beginning to degenerate and slough. At sites of individual cellular extrusion the bacteria were most evident near the base of the extruding cell and appeared to be migrating through the crevices (Fig. 1F).

At 45 and 60 minutes, the reacting FAE villi exhibited severe cellular swelling and blebbing near the tip, while lower on the villi normal M cells could be seen with no visible reaction or bacterial adherence. The frequency of extrusion sites with adherent bacteria increased to 56% and the bacterial count averaged 2.74 bacteria per site (Table 1). For comparison, a *Salmonella* serovar Choleraesuis inoculated loop was examined in detail and only two extrusion sites could be found with adherent bacteria.

In the 30-minute modified gut-loop experiment, emphasis was placed on searching for sites of cellular extrusion. Sites found were similar in character to standard loops and bacteria were preferentially adhered to many of these extrusion sites.

TEM examination

In the FAE, scattered among enterocytes were M cells associated with intraepithelial lymphocytes. M cells were distinguished from enterocytes by thicker, more irregular microvilli and a less electron dense cytoplasm (Fig. 2A). The AE contained enterocytes with intermittent goblets cells.

At 5 minutes, bacteria could rarely be seen in the lumen. A few M cells were seen initiating filopodia development, however no bacteria were associated in close proximity to those reacting sites.

At 10 minutes, internalized bacteria were observed apically in the M cells, enterocytes and goblet cells (Fig. 2B). M cells expressed filopodia and luminal bacteria were often associated with these sites. Affected enterocytes and goblet cells had focal apical disruptions unlike the M cells in which the entire apical membrane was rearranged.

At 15-30 minutes, multiple bacteria were seen in individual M cells. The internalized bacteria were within vacuoles of 1-2 bacteria per vacuole. The bacteria were located predominantly in the apical cytoplasm with a few that had progressed to the basolateral space (Fig. 2C). Granulocytes could be seen near the basement membrane. On AE, goblet cells and enterocytes containing bacteria occurred in patches on the villi. Bacteria could be seen at individual extrusion

sites especially along the base of the exiting cell and in the crevices formed by the extruding cell and adjacent enterocytes (Fig. 3A). Some bacteria were located in cytoplasm beneath the exiting cells (Fig. 3B). The swollen cells extended into the lumen and contained large vacuoles with degenerate mitochondria.

At 45-60 minutes, bacterial invasion continued via apical membranes of M cells, enterocytes and goblet cells. A few bacteria were present within apical membrane invaginations of enterocytes that exhibited little microvilli disruption (Fig. 3C). Enterocytes with internalized bacteria exhibited complete resolution of the apical membrane rearrangements and expressed normal microvilli morphology. Bacteria were also observed in the paracellular space after invasion near the tight junction (Fig. 3D). Bacteria were rarely evident in the lamina propria immediately below the basement membrane.

Discussion

Identification of the definitive sites of early cellular invasion by *Salmonella* serovar Typhimurium is the major goal of this experiment. In prior swine studies, definitive statements concerning cellular kinetics of invasion could not be made due to lack of short time points (9, 77, 79, 80). This work demonstrates early preferential adherence

to M cells at 5 minutes and by 10 minutes apical invasion occurs in M cells, enterocytes and goblet cells. These observations are similar to observations in a bovine model that showed *Salmonella* invasion occurs within minutes of inoculation (26). However, the bovine study suggested a halt of bacterial interaction with the FAE by 60 minutes. We saw continued invasion at 60 minutes of FAE and AE cells. The difference between the bovine and swine models might represent differences in protocols between the two experiments or a difference in the kinetics of infection between the species. This study suggests *Salmonella* serovar Typhimurium invasion is not limited by cell type in the swine ileum and may diminish the concept of specific cell sites for invasion.

Early preferential adherence to the M cell may be due to lack of a physical barrier compared to other cells (72). The filamentous brush border glycocalyx of rabbit enterocytes can be a barrier up to 500 nm thick while the variable M cell glycocalyx barrier may be as small as 20 nm (25). Another consideration is the mucus layer secreted by goblet cells. Goblet cells are abundant on the AE and absent on FAE potentially reducing the mucus covering on the FAE. (7, 31). The short time difference between invasion of M cells and other cell types may be a reflection of the absence of these barriers on M cell apical membranes thereby increasing the efficiency of invasion.

A critical component in *Salmonella* cellular invasion of the intestine is injection of proteins by a type-III secretion system (47, 52). These injected proteins act on the Rho family of G-proteins causing a rearrangement of the actin cytoskeleton resulting in apical lamellipodia and filopodia formation (41, 59). *Salmonella* bind to the disrupted apical surface and are engulfed by the cell (17, 24, 52). SipA is a secreted protein critical to early cellular invasion *in vitro* (50). In this study, the *Salmonella* induced rearrangements in the entire apical membrane of M cells while in enterocytes and goblet cells the rearrangement was focal in nature with much of the apical membrane retaining its normal morphology. Previous work has demonstrated that enterocytes can exhibit focal membrane disruptions with eventual resolution (95). Recent work with a *Salmonella* serovar Typhimurium *sptP* mutant suggests that SptP participates in the cell membrane resolution (27). In the murine model, ruffle formation and invasion by *Salmonella* serovar Typhimurium leads to M cell death (17, 52). In our study, evidence of M cell cytotoxicity associated with bacterial invasion is not as profound as the mouse model. This may be due to different M cell kinetics or invasion mechanisms in the swine ileum.

In addition to finding a lack of cell specificity for invasion, our observations also suggest another potential mechanism for entry.

Scanning electron micrographs show *Salmonella* serovar Typhimurium preferentially adheres at sites of cell extrusion. Bacteria appear to migrate into the crevices formed by the extruding cell and the adjacent enterocytes. Transmission electron micrographs confirm bacterial migration through the crevices and bacteria are located in the cytoplasm beneath of the extruding cells. To evaluate the specificity of this preferential adherence, SEM examination of *Salmonella* serovar Choleraesuis inoculated loops show minimal bacterial affinity to these sites. The culmination of these observations suggests *Salmonella* serovar Typhimurium may utilize these sites of cell extrusion as an additional mechanism for host invasion.

Bacteria associated with cell extrusion sites have been previously observed by SEM in *Salmonella* serovar Typhimurium infected bovine gut-loops, but the significance of this affinity was not known (26, 104). Similar effects are observed *in vitro*. *Clostridium difficile* toxins A and B applied to HT-29 cells results in apical cell rounding and increased permeability of tight junctions. This is associated with preferential adherence by *Salmonella* serovar Typhimurium to these sites and an increase in paracellular invasion (23). In comparing *Salmonella enterica* serovars Typhimurium and Typhi invasion through HeLa cells, *Salmonella* serovar Typhimurium is believed to be more efficient at invasion because of increased

adherence to cells with apical membrane rearrangements (68). Other *in vitro* studies show that *Salmonella* serovar Typhimurium preferentially adheres to and invades epithelium when exposed to the lateral surface of enterocytes (106, 107). The exposed lateral membrane may contain surface markers that can facilitate invasion. In polarized MDCK-1 cells, apical infection with *Yersinia pseudotuberculosis* leads to tight junction permeability and attachment to basolateral beta-1 integrins for subsequent paracellular translocation of the bacteria (94). The increased permeability of the tight junctions and subsequent exposure of basolateral markers may play a role in this specific affinity.

The increased affinity to these sites may also be, in part, a result of chemotaxis. Increased *Salmonella* serovar Typhimurium attachment to acid damaged HeLa cells was proposed to be via chemotaxis by components from the damaged cell. The authors suggested that factors increasing cell permeability could act as an attractant to *Salmonella* serovar Typhimurium *in vivo* (96). Additionally, the anaerobic environment in the gut may facilitate tight junction affinity by aerotaxis. *Salmonella* serovar Typhimurium grown in low oxygen conditions has a greater ability of *in vitro* mucus adhesion and invasion of immobilized murine enterocytes (92). Furthermore, the intestinal lumen is considered a low oxygen

environment and increased permeability of tight junctions may allow for an oxygen tension gradient (40). *In vitro* studies of aerotaxis during low oxygen tension suggested oxygen was an attractant for *Salmonella* serovar Typhimurium (91). The environment of the gut promotes expression of virulence traits and may encourage migration to sites of permeability by chemotaxis.

Salmonella serovar Typhimurium invasion occurring at sites of enterocyte loss may be especially relevant as swine and other species have been suggested to have a type-3 cell loss mechanism (64, 71). This type of cell loss tends to occur in isolated individual enterocytes allowing for breeches of integrity in the tight junction. These cells exhibit morphological changes similar to oncosis and are often associated with intraepithelial lymphocytes and possibly neutrophils (62, 71, 76, 103). In the mouse, individual cell loss can occur over the entire villus and may exhibit a circadian variation (78). In this experiment, the extrusion frequency increases over time similarly in both treatment and control loops. Concern that the extrusions are induced by the loop procedure was addressed by a time zero loop. Scanning electron micrographs demonstrate extrusion sites similar in frequency and character to the 5-minute level. This suggests the extrusion sites are not solely surgery induced and may play a normal role in the physiology of the swine ileum. In addition, SEM

examination of the 30-minute modified loop procedure shows bacteria preferentially accumulate at sites of extrusion. This procedure minimizes potential issues such as inadequate blood flow, increased lymphatic hydrostatic pressure or tissue damage. These results suggest *Salmonella* serovar Typhimurium adherence to the sites of extrusion is an event independent of the gut-loop procedure. While anesthesia and the associated drugs could potentially play a role in the extrusions, eliminating their effects in a humane manner is almost impossible.

SEM highlights the FAE as an active area of infection by the obvious lesions of cell swelling and blebbing compared to the less noticeable AE lesions of minor filopodia and lamellipodia formation. The SEM is useful to assess a wide area of tissue and discern the differences in adherence to the sites of extrusion. With TEM, these sites of invasion could be interpreted as bacterial-induced lesions rather than a pre-existent entity that preferentially adheres bacteria. SEM and TEM are complementary in assessing the intestinal epithelium invasion by the *Salmonella*.

The swine gut-loop model demonstrates *Salmonella* serovar Typhimurium preferential adherence to M cells after 5 minutes and apical invasion of M cells, enterocytes and goblets cells after 10 minutes. Our work suggests *Salmonella* serovar Typhimurium

invasion to be non cell-specific in the pig ileum and minimizes the concept of specific cellular invasion portals. This work also provides the first *in vivo* evidence of invasion by *Salmonella* serovar Typhimurium at sites of cell extrusion. The significance of this mechanism in natural infection is unknown.

Acknowledgments

We thank Judy Stasko, John Mattila and Tracy Pepper for electron microscopy sample preparation and examination. Thanks also to Missy Yungclas, Ruth Willson and Chuck Greiner for technical assistance.

Table

Table 1. Observed number of cell extrusions and associated bacteria over time (minutes).

	Time (inoculum)					
	0 (none)	5 (LB)^a	15 (ST)^b	30 (ST)	45 (ST)	60 (ST)
Average # extrusions per villus^c	0.35	0.35	0.65	1.05	1.45	1.70
Extrusions associated with bacteria (%)^d	–	–	23%	38%	41%	56%
Average # bacteria per random extrusion^e	–	–	0.38	0.57	0.72	2.74

^a LB = Luria-Bertani broth

^b ST = *Salmonella* serovar Typhimurium

^c Twenty random villi were examined and cellular extrusions counted.

Total extrusions were divided by 20.

^d Cellular extrusions associated with bacteria were divided by the total number of cell extrusions.

^e Total number of bacteria associated with extrusions was divided by the total number of extrusions.

Figures

Figure 1. Scanning electron micrographs of LB (A) and *Salmonella* serovar Typhimurium (B-F) inoculated loops, bar = 5 microns. (A) Apical surface of M cells (m) and enterocytes (e) in 5-minute loop. (B) M cell (m) with adherent bacteria (arrows), 5-minute loop. (C) Cell extrusion with bacteria (arrows) near its base, 5-minute loop. (D) Enterocytes associated with bacteria exhibit filopodia (arrows) formation, 25-minute loop. (E) Villus tip, adherent bacteria (arrows) preferentially located at the lateral border of the sloughing cells, 20-minute loop. (F) Bacteria at the crevices formed by the extruded cells, 15-minute loop.

Figure 2. Transmission electron micrographs of LB (A) and *Salmonella* serovar Typhimurium (B-C) inoculated loops. (A) M cell (m) of FAE, 20-minute loop, 3,600x. (B) A bacterium engulfed by an enterocyte, 10-minute loop, 14,500x. (C) Bacteria (arrows) traffic via an M cell to cross the basolateral membrane and enter the paracellular space, 25-minute loop, 3,600x.

A

B

C

Figure 3. Transmission electron micrographs of *Salmonella* serovar Typhimurium (A-D) inoculated loops. (A) Bacteria (arrows) in the lumen and in the crevices formed by extruded cells, 20-minute loop, 2,800x. (B) An unvacuolated bacterium (arrow) in the cytoplasm beneath a site of extrusion, 25-minute loop, 4,300x. (C) Bacterium invading an enterocyte with minimal apical membrane reaction, 60-minute loop, 11,500x. (D) Bacterium in paracellular space between enterocytes, 45-minute loop, 17,000x.

CHAPTER 3. GENERAL CONCLUSIONS

General Discussion

Identification of the sites of early cellular invasion by *Salmonella* serovar Typhimurium was the major goal of this experiment. In prior swine studies, definitive statements concerning cellular kinetics of invasion could not be made due to lack of short time points (9, 77, 79, 80). This work demonstrates early preferential adherence to M cells by 5 minutes, and by 10 minutes apical invasion occurs in M cells, enterocytes and goblet cells. These observations are similar to observations in a bovine model that showed *Salmonella* invasion occurs within minutes of inoculation (26). However, the bovine study suggested a halt of bacterial interaction with the FAE by 60 minutes. We saw continued invasion at 60 minutes of FAE and AE cells. The difference between the bovine and swine models might represent differences in protocols between the two experiments or a difference in the kinetics of infection between the species. The early epithelial invasion seen is consistent with recent work showing natural *Salmonella* infection can occur rapidly in pigs. In that study, pigs exposed to a *Salmonella* serovar Typhimurium contaminated environment became infected within two hours (48). Our study suggests *Salmonella* serovar Typhimurium invasion occurs within ten

minutes postinoculation and is not limited by cell type in swine ileal loops. This may diminish the concept of specific sites for cell invasion by *Salmonella* in the pig.

Early preferential adherence to the M cell may be due to lack of a physical barrier compared to other cells (72). The filamentous brush border glycocalyx of rabbit enterocytes can be a barrier up to 500 nm thick while the variable M cell glycocalyx barrier may be as thin as 20 nm (25). Another consideration is the mucus layer secreted by goblet cells. Goblet cells are abundant along the AE and absent on FAE potentially reducing the mucus covering on the FAE. (7, 31). The short time difference between invasion of M cells and other cell types may be a reflection of the absence of these barriers on M cell apical membranes thereby increasing the efficiency of invasion.

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Recent studies have documented *Salmonella* serovar Typhimurium strains that can express proteases to cleave the tight junctions thereby causing cell exfoliation and decreased integrity of

the epithelial layer (12, 61). Additionally, *Salmonella* serovar Typhimurium is associated with localized tight junction dysfunction and decreased epithelial integrity in polarized MDCK cells (51). Since *Salmonella* serovar Typhimurium strains have mechanisms to disrupt the tight junction and epithelial permeability, it would be reasonable to assume that they also have a mechanism to preferentially locate to and take advantage of the affected locations.

Salmonella serovar Typhimurium invasion occurring at sites of enterocyte loss may be especially relevant as swine and other species have been suggested to have a type-3 cell loss mechanism (64, 71). This type of cell loss tends to occur in isolated individual enterocytes allowing for breeches of integrity in the tight junction. These cells exhibit morphological changes similar to oncosis and are often associated with intraepithelial lymphocytes and possibly neutrophils (62, 71, 76, 103). The affected enterocytes could be seen at all locations of the villus. In the mouse, individual cell loss can occur over the entire villus and may exhibit a circadian variation (78).

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minute time point. This suggests the extrusion sites are not solely surgery induced and do play a normal role in the physiology of the swine ileum. In addition, SEM examination of the 30-minute modified loop procedure shows bacteria preferentially accumulate at sites of extrusion. This procedure minimizes potential issues such as inadequate blood flow, increased lymphatic hydrostatic pressure or tissue damage. These results suggest *Salmonella* serovar Typhimurium adherence to the sites of extrusion is an event independent of the gut-loop procedure. While anesthesia and the associated drugs could potentially play a role in the extrusions, eliminating their effects in a humane manner is almost impossible.

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Conclusion

The swine gut-loop model demonstrates *Salmonella* serovar Typhimurium preferential adherence to M cells after 5 minutes and apical invasion of M cells, enterocytes and goblets cells after 10 minutes. Our work suggests *Salmonella* serovar Typhimurium invasion to be non cell-specific in the pig ileum and minimizes the concept of specific cellular invasion portals. This work also provides the first *in vivo* evidence of invasion by *Salmonella* serovar Typhimurium at sites of cell extrusion. The significance of this mechanism in natural infection is unknown.

Recommendations for Future Research

The results of this research highlight numerous interesting areas for future examination. One direction is to further define organs of *Salmonella* cell-invasion outside the ileum. Previous work has shown the pig ileum is not essential to infection (22). The non cell-specific invasion seen in the ileum may be observed at other locations. To examine site-specific invasion, isolation of the selected tissue would be necessary. Some potential tissues to examine include tonsils, nasal-associated lymphoid tissue, trachea/bronchi, colon and other regions of small intestine.

Our experiments were done with surgically prepared gut-loops. It would be appropriate to try to establish an oral inoculation procedure that could consistently predict the arrival of bacteria to the ileum. This would more closely mimic natural infection and avoid gut-loop procedures with anesthetic and surgical manipulation.

A significant finding that deserves attention is the invasion at sites of cell extrusion. The *Salmonella* showed an affinity to these locations. Two possible explanations for this were chemotaxis and exposure of lateral surface receptors.

To address the role of chemotaxis *Salmonella* mutants could be used to identify specific pathways of attraction. Use of established *in vitro* chemotactic mutants would be a place to start. A key feature for the *Salmonella* mutant would be the development of one that is only sensory defective. This is important because chemotactic mutants can be defective in motility (53). Another method of assessing the chemotaxis is to eliminate the gradient. For example, if glycine was hypothesized to be the chemotactic molecule, it could be added to the inoculum to eliminate any potential concentration gradients. Lack of affinity would suggest glycine participated in the preferential interaction.

To address the role of surface markers, *Salmonella* mutants defective in expression of specific surface antigens could be examined

in the gut-loop model for adherence. Additionally, *in vitro* invasion assays in cell lines with varying expression and polarity of surface molecules could help address the issue.

In vitro bacterial affinity for sites of cell extrusion has been attributed to increased tight junction permeability. To examine the role this may play in natural infection, additives that inhibit tight junction permeability could be supplemented in the diet. Two such examples include genistein, a soybean isoflavone, and the amino acid glutamine (75, 107). Oral *Salmonella* challenge to this group and controls might provide insights to frequency, severity or distribution of infection. Furthermore, collection and testing of the extracellular fluid (ECF) for chemotactic molecules would be appropriate. This could help discern if the putative chemotactic molecule is an innate ECF component or a byproduct of the affected epithelial cells.

APPENDIX. ELECTRON MICROGRAPHS

Figure I. Scanning electron micrograph of LB inoculated loop,
bar = 100 microns. Non-infected villi of ileum, 45-minute
loop.

Figure II. Scanning electron micrograph of *Salmonella* serovar Typhimurium DT104 inoculated loop, bar = 1 micron. Bacteria congregate near base of cell extrusion, 25-minute loop.

Figure III. Scanning electron micrograph of *Salmonella* serovar Typhimurium DT104 inoculated loop, bar = 1 micron. Multifocal microvilli degeneration (arrows) with associated bacteria on absorptive epithelium, 60-minute loop.

Figure IV. Transmission electron micrographs of *Salmonella* serovar Typhimurium DT104 inoculated loop. (A) M cell with apical membrane lamellipodia and internalized bacterium, 15-minute loop, 9,100x. (B) Goblet cell with internalized bacterium at the apical surface, 60-minute loop, 11,500x.

A **B**

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ACKNOWLEDGMENTS

First and foremost, I would like to thank my LORD and Savior Jesus Christ, "in whom are hidden all the treasures of wisdom and knowledge" (Colossians 2:3). He repeatedly reminds me of what is truly important. As Jeremiah 9:23-24 states, "Let not the wise man boast of his wisdom or the strong man boast of his strength or the rich man boast of his riches, but let him who boasts boast about this: that he understands and knows me, that I am the LORD, who exercises kindness, justice and righteousness on earth, for in these I delight, declares the LORD."

Next, I want to express my love and appreciation for my wife Karen who has been a patient helpmate and encourager. She is a great friend. And I thank Elizabeth, Abbey, and Isaac for their patience in sharing my free time. We will try to wrestle more this year.

Special thanks to Dr.'s Ronald Griffith and Thomas Stabel for patiently and persistently participating in the roles of my Co-Major Professors. Also thanks to Dr.'s Mark Ackermann and Michael Wannemuehler for participating as committee members. In addition, I would like to thank: Ruth Willson, Missy Yungclas, Judy Stasko, John Mattila, Tracy Pepper, Dr. Steve Carlson, Dr. Tim Smith and Dr. Melvin Swenson.