CHARACTERIZATION OF SUSCEPTIBILITIES AND ASSESSMENT OF RISK TO *LISTERIA MONOCYTOGENES* EXPOSURE IN PREGANT GUINEA PIGS AND PRIMATES

by

DENITA WILLIAMS

(Under the Direction of Mary Alice Smith)

ABSTRACT

Pre-term delivery of a stillborn or neonatal illness can result from exposure of a pregnant woman to *Listeria monocytogenes.* The objectives of this research are to use our previously established guinea pig model to 1) conduct a risk assessment of *L. monocytogenes* using dose-response data from primate and guinea pig models and compare the results to previous assessments 2) investigate invasion of *L. monocytogenes* in tissues at various intervals following maternal ingestion using culturing and microscopy 3) investigate the impact of gestation and dose on tissue invasion with *L. monocytogenes,* fecal shedding and birth outcome and 4) determine whether exposure induces changes in select pro-inflammatory and anti-inflammatory cytokine mRNA expression in fetal liver and brain.

We evaluated risk based on dose-responses from pregnant rhesus monkeys and guinea pigs. The mortality rate was calculated using new dose-response information, and

results show that the mortality rate of $5.9x10^1$ using primates is much different from the predicted rate of $1.3x10^{-7}$ by the FDA/USDA/CDC.

To determine a time course of tissue invasion, guinea pigs were sacrificed on post treatment days (ptd) 2, 6, 9 and 21. Maternal and fetal tissues were invaded at 2 days post treatment. At the highest administered dose of 10^8 cfu the average time to stillbirth was 10 ptd indicating that it takes several days for listeriosis to progress and kill the fetus. Independently, culturing and microscopy may underestimate the number of positive samples but combined can be effective tools for confirmation of *L. monocytogenes*.

When addressing whether gestation and dose affected tissue invasion and fecal shedding following *L. monocytogenes* exposure we found that non-pregnant animals are invaded after *L. monocytogenes* exposure and the likelihood of invasion and shedding is dose dependent. Earlier gestation did not reduce or prevent the risk for tissue invasion or fetal death. At the dose of 10^2 CFU, TNF-α and IL-2 were decreased in fetal brain and liver. At 10^7 CFU, all cytokines were decreased in the fetal brain. However, in the fetal liver, IFN-γ, TNF-α and IL-5 were increased with IL-2 at lower levels compared to controls.

INDEX WORDS: *Listeria monocytogenes*, guinea pigs, nonhuman primates, stillbirths, dose-response, risk assessment, culturing, microscopy, green fluorescent protein (GFP) and cytokine

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DEDICATION

The fulfillment of this degree is dedicated to my mother, Vernessa Davis Williams. You have truly inspired me throughout my entire life. You loved unconditionally, supported without boundaries and listened without judgment. I pray that my life is as fruitful as yours. I hope that through my actions I will be able to touch as many souls as you. I think about you everyday. I love you.

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I would like to thank God for his grace and mercy during my graduate journey. For had it not been for God none of this would even be possible. I give you all of the glory, honor and praise. I request that as I begin this new phase of life, my steps be ordered according to your holy and devine will.

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CHAPTER 1

INTRODUCTION

In the United States, *Listeria monocytogenes* (*L. monocytogenes*) remains a public health concern. Among the foodborne pathogens, *L. monocytogenes* is second only to *Salmonella* in the number of deaths that result annually from exposure. In spite of public health and food industry prevention efforts, the number of cases is not continually declining (CDC, 2008). The primary route of exposure to *L. monocytogenes* is through ingestion of contaminated foods. The ubiquitous nature of *L. monocytogenes* makes it hard to eradicate the pathogen from the food supply; therefore, many humans are likely to be exposed. Yet, certain subpopulations are more susceptible to *L. monocytogenes* infection resulting in adverse health conditions. Fetuses and neonates are among these susceptible subpopulations. Approximately one-third of the cases involving listeriosis are pregnancy-related and carry the risk of feto-placental infection that results in severe health conditions such as septicemia, pneumonia, or meningitis, and in about 25% of cases, the occurrence of pre-term delivery or stillbirth (Mylonakis et al., 2002). Pregnant women are usually asymptomatic or suffer from more subdued symptoms including influenza-like illness with fever, headache, and myalgia (Abram et al., 2002).

Pregnant women experience hormonal and immunological changes that take place during pregnancy to prevent fetal rejection. During the third trimester of pregnancy cell mediated immunity is suppressed. Interestingly, listeriosis most commonly occurs during the third trimester (Mylonakis et al., 2002). It is documented that fetal effects are dependent on the point of gestation of exposure. First trimester exposures can lead to

miscarriages while second- and third-trimester infections can result in premature delivery followed by neonatal illness or pre-term delivery of a stillborn (Farber and Peterkin, 1991; Gellin and Broome, 1989).

In joint efforts to reduce foodborne listeriosis in the United States, in 2003, the Center for Food Safety and Applied Nutrition of the U.S. Food and Drug Administration (FDA), the Food Safety Inspection Service of the U.S. Department of Agriculture (USDA) and the Centers for Disease Control and Prevention (CDC) (FDA/USDA/CDC, 2003) and in 2004, the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) conducted risk assessments for *L. monocytogenes* in selected ready-to-eat foods (FAO/WHO, 2004). The FDA/USDA/CDC relied on dose response data from a mouse study (Golnazarian et al., 1989) for completion of the *Listeria monocytogenes* risk assessment. Adjustment factors were needed to account for the following: 1) the study being conducted in adult, nonpregnant mice as opposed to gestating mice, 2) in the study, mice were exposed intravenously as opposed to ingestion (the primary exposure route in humans) and 3) differences in end points following exposure (death in adult mice as opposed to stillbirths in gestating mice). As a result of these adjustment factors, the LD_{50} calculated was 10^{13} CFU that is high when compared to 10^{6} CFU that was calculated based on an actual human outbreak (FAO/WHO, 2004). Upon completion of these risk assessments, the following were a few of the data gaps identified: 1) a need to characterize susceptible populations and risk factors, 2) lack of dose response information in the low dose region of the dose response curve, 3) lack of dose response information relevant to humans and 4) animal models that assess the effects of *L. monocytogenes* on pregnancy.

In response to these risk assessments, we developed the pregnant guinea pig as an animal model for human listeriosis (Williams et al., 2007). In a previous study using our guinea pig model, we demonstrated that following maternal ingestion, *L. monocytogenes* crosses the intestinal barrier and invades maternal liver and spleen, placenta, fetal liver and brain as well as causes premature delivery of nonviable fetuses (Williams et al., 2007). We have also shown that pregnant guinea pigs and pregnant nonhuman primates (Smith et al., 2003; Smith et al., 2008) have similar LD_{50}s following maternal ingestion (2.0×10^7 and 4.0×10^7 CFU, respectively). Both are comparable to the human LD_{50} of 10^6 CFU (FAO/WHO, 2004) that is based on a previous outbreak among pregnant women. We revised the FDA/USDA/CDC risk assessment using our nonhuman primate and guinea pig data.

Although we have made advances toward elucidating human listeriosis with the development of the guinea pig as an animal model, additional studies are needed to further characterize susceptible populations and investigate the low dose region of the dose response curve. The general population is frequently exposed to low doses of *L. monocytogenes* and is rarely affected. However susceptible subpopulations including fetuses or neonates may suffer adverse effects as a result of a low dose exposure. Also, low dose data is needed to effectively set regulatory guidelines concerning *L. monocytogenes* in foods.

Healthy individuals are usually asymptomatic following exposure to *Listeria* so it is necessary to determine if a systemic infection is occurring or if the pathogen is cleared by fecal shedding. Also, it is important to employ the most sensitive method of detection when confirming the presence of *L. monocytogenes*.

The objectives of the current study are to 1) conduct a risk assessment of *L. monocytogenes* using dose-response data from non-human primate and guinea pig models and compare the results to published *L. monocytogenes* risk assessments 2) investigate the invasion of *L. monocytogenes* in maternal and fetal tissues at various time points following maternal ingestion using two different methods, culturing and microscopic analysis 3) investigate whether gestational age of the fetus impacts the risk of tissue invasion with *L. monocytogenes,* fecal shedding and birth outcome, 4) determine whether exposure to a low dose of 10^2 *L. monocytogenes* CFU can result in tissue invasion and adverse pregnancy outcome and 5) determine whether maternal treatment with *L. monocytogenes* induces changes in select pro-inflammatory and anti-inflammatory cytokine mRNA expression in fetal liver and brain.

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CHAPTER 2

LITERATURE REVIEW

1. Listeriosis and Pregnancy

Almost one-third of the cases involving listeriosis occur in pregnant women and carry the risk of feto-placental infection that could result in severe health effects such as septicemia, pneumonia, or meningitis and in some cases, the occurrence of pre-term delivery or stillbirth (Teberg et al., 1987; Mylonakis et al., 2002). In humans, *L. monocytogenes* has a predilection for the feto-placental unit (Bortolussi et al., 1995 and Buchdahl et al., 1990). Listeriosis most commonly occurs during the third trimester (Buchdahl et al., 1990; Gellin et al., 1989) and is probably related to the major decline in cell-mediated immunity that occurs at 26-30 weeks of gestation (Weinberg, 1984). Infections are rare during the second trimester and even rarer during the first trimester (Buchdahl et al., 1990; Fuchs et al., 1994).

In most listeriosis cases, the pregnant woman is asymptomatic; however, there have been some reports of women experiencing influenza-like illness with fever, headache, and myalgia (Abram, 2002). The mild flu-like symptoms occur in approximately two-thirds of infected pregnant women that are symptomatic and transpire during the period of bacteremia. Severe listeriosis in pregnant women is rare (Sirry et al., 1994), and listeriosis in the pregnant female is usually self-limiting with the delivery of an infected baby. Contrastingly, some animal studies indicate that both the mother and fetus are at risk for serious illness if the mother is infected by *L. monocytogenes* during pregnancy. In a previous study, *L. monocytogenes*-infected pregnant mice did not clear

the pathogen from their spleens and livers as efficiently as infected virgin mice, and pregnant mice had a much higher mortality rate (Schlech et al., 1993; Luft et al., 1982). Also, necropsy of dead pregnant mice revealed that most of the mice had resorbed their fetuses (Decker et al., 1991). Primarily, pregnancy-related listeriosis in humans affects the fetus or neonate. The effect of fetal *Listeria* infection is dependent on the point in gestation time when infection occurs. First-trimester infection leads to spontaneous abortion, whereas second-and third-trimester infections lead to preterm birth followed by neonatal illness or fetal death with preterm delivery of a stillborn (Cruikshank, 1989; Enocksson, 1992; Farber, 1991b). Schlech et al (1993) demonstrated this using pregnant rodents. Rodents that were injected with *L. monocytogenes* during the third trimester had more fetal resorptions, stillbirths, or heavily infected pups than animals treated in early or late gestation. These data suggest that midgestational infection had greater adverse effects on fetal outcome as compared to infection during the earlier or later part of pregnancy.

The changes in hormonal and immunological parameters that take place during pregnancy influence the susceptibility of pregnant women to *L. monocytogenes* infection. There is evidence that pregnancy leads to suppression of cell-mediated immunity and a substantial increase of progesterone production in the mother in order to prevent fetal rejection (Sacks et al., 1999). However cell mediated immunity is necessary for the defense against intracellular pathogens, and therefore suppression of this immune response may predispose the fetus to infection with intracellular pathogens, such as *L. monocytogenes*.

During gestation when cell-mediated immunity is mildly impaired (Weinberg, 1984), pregnant women are prone to develop listerial bacteremia. *Listeria monocytogenes* cells may proliferate in the placenta in areas that appear to be unreachable by usual defense mechanisms. For unknown reasons, maternal CNS infection (the most commonly recognized form of listeriosis in other at-risk groups) is extremely rare during pregnancy in the mother when other risk factors are absent (Gellin et al., 1989; Gellin et al., 1991). Acquired immunity to *L. monocytogenes* is entirely cell mediated and largely dependent on cytotoxic CD8+T cells that recognize and lyse infected cells (Zinkernagel, 1974).

Increases and decreases of cytokine production are necessary for regulating homeostasis in the body during infection. During pregnancy, cytokine production is tightly regulated to avoid deleterious effects to the fetus. T helper 1-type (Th1) cytokines provoke an inflammatory response when activated, and T helper 2-type (Th2) cytokines have an anti-inflammatory response when activated. Th1 cytokines have been shown to have deleterious effects on the placenta and fetus (Raghupathy et al., 2001). As inflammatory mediators, Th1 cytokines also elicit cell-mediated cytotoxic responses, which can have harmful effects on the fetus. Specific Th1 cell products have been recognized; interleukin (IL)-2, interferon (IFN)-λ, tumor necrosis factor (TNF)-β. Th2 cells are elicited for involvement with an anti-inflammatory response via B cell antibody production (Maksheed et al., 2001). IL-4, IL-5, IL-6 and IL-10 are Th2 cytokines that are thought to be up-regulated during pregnancy. Infection from other pathogens during pregnancy show that the normal relative ratio of Th1 and Th2 cytokines might be disrupted leading to deleterious effects to the fetus (Lin et al., 2003).

2. Guinea Pig as Surrogate for Human Listeriosis

A pregnant animal model that expresses similar symptoms as humans is essential to accurately assess the risk of maternal exposure to *L. monocytogenes* on the resulting fetus and/or neonate. In 2003 the FDA conducted a risk assessment for *L. monocytogenes* in ready-to-eat foods relying on a dose response curve obtained from mouse data. Due to the limited quantity and insufficient quality of existing human data, the FDA applied an adjustment factor to mouse data for estimating human risk (FDA/USDA/CDC, 2003). Mice were the only animal data for which there was sufficient information to obtain a dose-response curve as they were the primary animal model for human listeriosis until recently.

In most laboratory studies, oral exposure of mice to *L. monocytogenes* rarely caused severe systemic infection. However, a few published studies showed infection in mice following an oral exposure to *L. monocytogenes*. In 1970, Miller and Burns reported using mice to investigate a 24 hour oral exposure to *L. monocytogenes*-contaminated drinking water (Miller and Burns, 1970). Histopathology showed microgranuloma lesions on the liver of the exposed non-pregnant and pregnant animals sacrificed at post-treatment day seven; however, the lesions were more severe in pregnant mice. In 2003, Czuprynski et al. demonstrated that the use of an anesthetic, sodium pentobarbital, prior to intragastric treatment with *L. monocytogenes* in mice appeared to enhance the ability of the bacteria to invade the liver (Czuprynski et al., 2003). The bacterial counts in the liver and spleen of mice treated with sodium pentobarbital prior to *L. monocytogenes* dosing were significantly higher than non-anesthetized mice receiving the same dose. However, the effects of sodium pentobarbital on susceptibility to *L.*

monocytogenes decreased if anesthetic treatment occurred > 2 hours prior to dosing with *L. monocytogenes* (Czuprynski et al., 2003). Based on further treatments with loperamide (to reduce gastric motility) and sodium bicarbonate (to neutralize gastric acid), it is unlikely that decreased gastric motility or a reduction in gastric acidity resulted in the increased susceptibility to *L. monocytogenes* infection (Czuprynski et al., 2003). In 2004, a more recent study characterized gastritis in mice due to *L. monocytogenes* exposure. Park et al. (2004) reported that there were no bacteria present in the liver or spleen of animals sacrificed at seven days post-treatment (Park et al., 2004).

Based on molecular differences in their receptor protein (E-cadherin) which is essential for transmigration of *L. monocytogenes* across the intestinal barrier and because the adverse health effects are not the same (death in mice opposed to stillbirths/meningitis in humans), mice are not the most appropriate animal model to use in estimating dose response for human listeriosis. A single amino acid shift located at position sixteen in murine E-cadherin (proline to glutamic acid) results in a loss of the ability for *L. monocytogenes* and the receptor to interact (Lecuit et al., 1999). A transgenic mouse that expressed human E-cadherin solely in enterocytes was developed in order to demonstrate the interactions of internalin and E-cadherin *in vivo* (Lecuit et al., 2001); however these receptors are localized within the intestine and may not be associated with other tissues, more specifically, the placenta. Despite this molecular advancement, comparisons between quantitative expression in humans and transgenic mice have not been published.

Previous studies show that pregnant nonhuman primates are susceptible to listeriosis both naturally and experimentally (McClure et al., 1986; Anderson et al., 1993;

Smith et al., 2003; Smith et al., 2008). Furthermore, fetal infections in both humans and nonhuman primates can lead to similar conditions such as abortions, stillbirths, and/or neonatal deaths (Chalifoux et al., 1981; McClure et al., 1986; Smith et al., 2003; Smith et al., 2008). For humans and nonhuman primates, the pathogenesis and morphological findings associated with stillbirths due to *L. monocytogenes* are essentially the same (Anderson et al., 1993; Chalifoux et al., 1981). Nonhuman primates exhibit responses to *L. monocytogenes* that are similar to humans. Yet the number of primates needed to thoroughly examine the low dose region of the dose-response curve and to conduct mechanistic studies is prohibitive, and presently, the sequence of their E-cadherin protein is not published.

Studies conducted in the 1970s used non-pregnant guinea pigs to investigate the interaction between *Listeria* and host cells. Using electron microscopy, the interaction between *L. monocytogenes* and intestinal epithelial cells was investigated after pre-conditioned guinea pigs had been treated with 10^9 CFU through a stomach tube (Racz et al., 1972). These studies showed that *L. monocytogenes* entered the small intestine epithelial cells and multiplied there before being phagocytosed by macrophages (Racz et al., 1972). Pregnant guinea pigs were one of several animals used to characterize *Listeria* isolates (Dutta and Malik, 1981). Following exposure to *L. monocytogenes*, pregnant guinea pigs exhibit similar effects to humans including fetal abortion (Dutta and Malik, 1981) as well as GI tract disturbances (Lecuit and Cossart, 2002). In a previous study, pregnant guinea pigs aborted on the 3rd and 7th day following infection with *L. monocytogenes* by ip injection (Dutta and Malik, 1981), but the experimental design did not include the treatment dose.

Recently, it was discovered that E-cadherin, which mediates transmigration of *L. monocytogenes* into mammalian epithelial cells, has the same sequence in both humans and guinea pigs (Lecuit et al., 1999; Schubert et al., 2002). The sequence of the E-cadherin protein is extremely important in the binding of *L. monocytogenes* to epithelial cells and a single amino acid transition can alter the active site's configuration as previously discussed with the mouse model (Lecuit et al., 1999). Previous studies conducted by our laboratory (Williams et al., 2007; Irving et al., 2008a; Irving et al., 2008b; Jensen et al., 2008; Williams et al., 2009) along with others (Bakardjiev et al., 2004) support using the pregnant guinea pig as a surrogate for human listeriosis. Bakardjiev et al. (2004) used the pregnant guinea pig to study feto-placental transmission of *L. monocytogenes* following invasion of the maternal system through intravenous inoculation. Surprisingly, prior to our study published in 2007, none of the listeriosis studies that used the guinea pig as an animal model used ingestion as the route of exposure. Humans are exposed to *L. monocytogenes* through ingestion, and importantly, experimentally by-passing the gastrointestinal tract probably leads to differences in dissemination and disease symptoms. By using an exposure regimen that is realistic to human exposures, we will be better equipped to estimate maternal and fetal health conditions following exposures to *L. monocytogenes*.

Despite scientific advances made as a result of the establishment of the guinea pig model, recently the guinea pig has been questioned as an animal model of choice for elucidating human listeriosis. The reluctance is based on molecular differences in guinea pig Met receptors compared to human Met receptors (Cabanes et al., 2008). The Met receptor is believed to increase the ability of the pathogen to invade nonphagocytic cells

including epithelial and endothelial cells and hepatocytes. Despite these molecular differences, we have shown that following maternal ingestion of *L. monocytogenes,* guinea pigs exhibit similar effects as human including maternal and fetal tissue invasion as well as delivery of nonviable fetuses (Irvin et al., 2008a; Irvin et al., 2008b; Williams et al., 2009; Williams et al., 2007). The dose-response curves developed using this guinea pig model predict an LD_{50} of approximately 10^7 (Williams et al., 2009) which is comparable to an LD_{50} of 10^6 that was based on a human outbreak involving pregnant women (FAO/WHO, 2004). These data suggest that either the Met receptor is not essential for *L. monocytogenes* invasion, or that *L. monocytogenes* utilizes an alternate pathway for invasion of maternal and fetal tissues in the guinea pig. Some evidence for the latter has recently been published (Mostowy et al., 2009) although how or whether the pathway in guinea pigs is different from humans is not known.

More recently, gerbils have been proposed as an animal model to assess human listeriosis because their E-cadherin active site is the same as humans and their Met receptor contains key amino acids that are required for efficient interactions with the *L. monocytogenes* surface protein, InlB. Disson et al. (2008) demonstrated that gerbils were susceptible to neonatal death following intravenous inoculation. However, there are no dose-response data on the effects of *L. monocytogenes* following oral inoculation in gerbils. Future studies are needed to evaluate whether gerbils are appropriate surrogates for human listeriosis.

3. Pathogenicity of *Listeria monocytogenes*

The genus *Listeria* contains six species: *L. grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeliger*i, and *L. welshimeri*. Of these six, *L. monocytogenes* and *L.*

ivanovii are currently the only species to be classified as pathogenic resulting in an opportunistic infection of humans and animals involving severe clinical manifestations such as meningoencephalitis or septicemia and in pregnancy-related cases abortions, stillbirths, or neonatal deaths. The natural habitat is thought to be the surface layer of soil rich in decaying plant matter. From this habitat they gain access to host via ingestion of contaminated food sources. *L. monocytogenes* can infect a wide range of animal species, including mammals and birds.

L. monocytogenes possesses some unusual characteristics that make it unique from other food borne pathogens and contribute to the organism's ubiquitous nature. *L. monocytogenes* replicates at a broad temperature range (1°C to 45°C). Therefore, *L. monocytogenes* not only survive, but multiply at the normal refrigeration temperature of 4°C. *L. monocytogenes* can also replicate at high salt concentrations (Wing, 2002).

L. monocytogenes is a typical facultative intracellular parasite. It is able to proliferate within macrophages and a variety of normally nonphagocytic cells, such as epithelial and endothelial cells and hepatocytes. Following ingestion, it is advantageous for an intracellular pathogen such as *L. monocytogenes* to invade mammalian cells as efficiently as possible. *L. monocytogenes* crosses the mucosal barrier of the intestine by using its surface protein, internalin, to interact with the host's E-cadherin protein that is located along the intestinal wall. Once in the bloodstream, the organism may disseminate hematogenously to any site; however, *L. monocytogenes* has a particular predilection for the CNS and the placenta (Lorber, 1997).

The most commonly affected human populations include: neonates, elderly, pregnant, and immunocompromised individuals. L. monocytogenes has evolved the

ability to invade and multiply within eukaryotic cells. Except for vertical transmission from mother to fetus and rare instances of cross contamination in the delivery suite or newborn nursery (Farber et al., 1991b), human to human infection has not been documented. Infection most likely begins following an oral exposure to the organism. The oral inoculum required to produce clinical infection is unknown; experiments in healthy non-pregnant mammals including cynomolgus monkeys (Farber et al., 1991a) and goats (Miettinen et al., 1990) indicate that $\geq 10^9$ organisms are required. Data from a previous study conducted through our laboratory indicate that pregnant rhesus monkeys are susceptible to listeriosis and spontaneous abortions occurred following an oral exposure at doses$\geq 10^6$ CFU (Smith et al., 2003). The incubation period for invasive illness is not well established, but evidence from a few cases with exposures by ingestion points to incubation periods ranging from 11 days to 70 days (with mean incubation period, 31 days) (Linnan et al., 1988).

The mechanisms of how *L. monocytogenes* moves from cell to cell avoiding the maternal immune system has been well documented. Internalin, an 80-kD member of the family of leucine-rich repeat proteins, interacts with E-cadherin resulting in induction of phagocytosis (Cossart et al., 1996; Mengaud et al., 1996). Once *Listeria monocytogenes* enters cells, listeriolysin O (LLO) forms pores in the primary vacuole allowing the bacteria to escape. LLO is a toxin and belongs to the class of thiol-activated toxins. They are only active on cholesterol-containing membranes, with cholesterol likely acting as a receptor in the membrane. Colonies of bacteria producing LLO are hemolytic. Nonhemolytic *Listeria monocytogenes* strains are avirulent. The gene that encodes LLO is *hly*. LLO is also a potent inflammatory stimulus, promoting the expression of adhesion

molecules in human umbilical vein endothelial cells and chemokine secretion. The crossing of endothelial cells by *Listeria monocytogenes* may involve the LLO-secreting bacteria and inflammatory cells producing tumor necrosis factor alpha and IL-1 resulting in increased leukocyte-endothelial cell interaction and leading to the influx of leukocytes through the endothelial barrier. This mechanism would be pertinent to *Listeria monocytogenes* invasion of the placenta as well as the blood brain barrier. *plcA* gene is upstream of the *hyl* gene and it encodes the phosphatidylinositol phopholipase C (PI-PLC) which along with LLO is involved in the escape from primary and double membrane vacuoles. These virulence factors enable *Listeria* to avoid intracellular killing (McKay et al., 1991; Portnoy et al., 1992).

Once *Listeria* escapes the primary vacuole and enters the cytoplasm, the cells can divide (doubling time, ~1 hour). Actin polymerization is required to propel *Listeria* to the cellular membrane. Actin polymerization requires expression of the *actA* gene. *actA* encodes the surface protein, ActA which is anchored into the bacterial cytoplasmic membrane. ActA is asymmetrically distributed on the bacterial surface such that it is weakly detectable at one pole, with an increasing concentration toward the other pole, which is the site of comet tail formation. The asymmetrical distribution of ActA appears to be essential for intracellular *Listeria monocytogenes* movement in the direction of the non-ActA-expressing pole. When *L. monocytogenes* reaches the host cell's membrane it pushes against the membrane forming elongated pseudopod-like projections that can be ingested by adjacent cells such as macrophages, enterocytes, and hepatocytes (Tilney et al., 1989; Sanger et al., 1992).

Once *Listeria monocytogenes* is engulfed by a neighboring cell, lysis of the double membrane vacuole must occur. Again, lysis of the double membrane requires LLO and PI-PLC and a broad-spectrum of phospholipases encoded by the plcB gene. plcB is located immediately downstream from actA.

The pathogenic events of *Listeria monocytogenes* described above are controlled by eight genes. Of the eight, six are clustered on the same operon on the chromosome: *prfA, plcA, hly, mpl, actA* and *plB*. *inlA* and *inlB* form another operon. Ironically, all of these genes are maximally expressed at 37°C (normal body temperature) and repressed at low temperatures. They are coordinately regulated by PrfA, the transcriptional activator encoded by *prfA*. *prfA* is downstream from *plcA* and is co-transcribed with it.

Through this life-cycle, *L. monocytogenes* can move from cell to cell without being exposed to antibodies, complement, or neutrophils. The entire cycle is completed in approximately 5 hours.

4. *Listeria monocytogenes* and Food Matrices

Although diseases caused by *L. monocytogenes* occur at a low rate relative to those caused by other food-borne pathogens, the organism is second only to *Salmonella* spp. in the estimated number of food-related deaths in the United States (Mead et al., 1999). Since 1981, epidemiologic investigations have repeatedly indicated that the consumption of contaminated food is the primary vehicle of transmission of listeriosis. Presently, the Food and Drug Administration has classified the following foods as being at high risk for *Listeria* contamination: ready-to-eat and processed foods that support the growth of *L. monocytogenes* such as deli meats, smoked seafood, soft cheeses and dairy products.

The first confirmed food-borne outbreak of listeriosis occurred in 1981 in Canada. A case-control study implicated locally prepared coleslaw as the vehicle, and the epidemic strain was subsequently isolated from an unopened package of this product (Schlech et al., 1983). The next documented outbreak was in Boston, Massachusetts in 1983 and included 49 cases over a two-month period; a case-control study implicated pasteurized milk as the vehicle (Fleming et al., 1985). In 1985 in California, an outbreak of listeriosis with 142 cases was traced to a Mexican-style cheese (Linnan et al., 1988). Ninety-three cases or 65.5% occurred in pregnant women or their offspring, and of the forty-eight total deaths that occurred, twenty were fetuses and ten were neonates. In Illinois in 1994, an outbreak of listeriosis occurred as the result of the ingestion of contaminated chocolate milk. Although no deaths occurred, 45 people were sickened with gastroenteritis (present in 79% of the cases) and fever (present in 72% of the cases). *L. monocytogenes* was isolated from stool specimens as well as unopened cartons of milk. In 1998, hot dogs and deli meats were the implicated vehicles of a listeriosis multistate outbreak that resulted in deaths of six adults and spontaneous abortions in two pregnant women. *L. monocytogenes* was isolated from both open and unopened packages of hotdogs and deli meats from the manufacturing facility. An outbreak occurred in North Carolina in 2000 that ultimately resulted in five stillbirths, three premature deliveries and two infected newborns. Of the twelve patients, eleven were of childbearing age and the other was an immunocompromised male. Ten of the eleven women were pregnant. The results of the investigation implicated noncommercial, homemade, Mexican-style fresh soft cheese produced from contaminated raw milk as the causative agent. In 2002, Pilgrim's Pride issued a recall on 27.4 million pounds of

poultry after illness occurred in 46 individuals and at least 8 deaths. *Listeria* was detected in the floor drains of the manufacturing facility. Another listeriosis outbreak occurred in 2002 of which contaminated ready-to-eat turkey deli meat caused 54 illnesses, 8 deaths and 3 stillbirths in nine states.

Eradicating *L. monocytogenes* from the food supply is also an international concern as can be seen in the numbers of cases resulting from ingestion of the pathogen. *L. monocytogenes*-contaminated soft cheese was responsible for a 4-year (1983-1987) outbreak of 122 cases in Switzerland (Bille et al., 1989) and a contaminated paté caused a 300-case outbreak in the United Kingdom in 1989 to 1990 (Gilbert et al., 1993). In France, contaminated pork tongue in aspic was the principal vehicle of 279 cases of listeriosis in ten months in 1992 (Jacquet et al., 1995), potted pork was associated with 39 cases in 1993 (Goulet, 1995), and soft cheese was the vehicle of 33 cases in 1995 (Loncarevic et al., 1995). The total exposed is not available for any of the above outbreaks. In 2008, a Canadian listeriosis outbreak was linked to Maple Leaf Foods Plant in Toronto, Ontario. Twenty-three people died and there were 57 confirmed cases. The confirmed cases consisted primarily of nursing home residents. *L. monocytogenes* was found in processed meats. The recall cost the company $200 million dollars.

In 2001, FDA acknowledged that the food matrix may influence the pathogenic state of *L. monocytogenes* which could ultimately influence the predicted infectious dose for humans (FDA, 2001). However, there were limited studies available that used animal models to assess the effect of food content on the organism's ability to produce illness. Farber et al (1991) administered sterile whole milk along with Maalox to female cynomolgous monkeys to determine the effect of gastric acidity on infection caused by *L.*

monocytogenes. Observations by Farber et al (1991) were consistent with a study conducted in 1989 by Golnazarian et al. where they administered cimetidine to mice to decrease their gastric acid secretion (Farber et al., 1991; Golnazarian et al., 1989). Golnazarian observed no significant differences between normal and cimetidine-treated mice (Golnazarian et al., 1989). Both studies concluded that acidity did not significantly affect the pathogens ability to produce illness. Several outbreaks of salmonellosis suggest that low infective doses are associated with high fat content foods, such as chocolate (Craven et al., 1975; D'Aoust et al., 1975; Greenwood et al., 1983) cheese (D'Aoust et al., 1985; Hedberg et al., 1994) and paprika-powdered potato chips (Lehmacher et al., 1995). The *Listeria* outbreaks stated above were predominately associated with foods that are high in fat content suggesting that lipid content may be an important factor in the pathogenic state of *L. monocytogenes*.

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CHAPTER 3

RISK OF FETAL MORTALITY AFTER EXPOSURE TO *LISTERIA MONOCYTOGENES* BASED ON DOSE-RESPONSE DATA FROM PREGNANT GUINEA PIGS AND PRIMATES[1]

[1] Williams, D., Castleman, J., Lee, C.C., Mote, B., and Smith, M.A. 2009. *Risk Analysis.* 29:1495-1505. Reprinted here with the permission of publisher.

1. Abstract

One-third of the annual cases of listeriosis in the United States occur during pregnancy and can lead to miscarriage or stillbirth, premature delivery, or infection of the newborn. Previous risk assessments completed by FDA/USDA/CDC *(1)* and FAO/WHO *(2)* were based on dose-response data from mice. Recent animal studies using nonhuman primates *(3, 4)* and guinea pigs *(5)* have both estimated LD_{50}s of approximately 10^7 *L. monocytogenes* colony forming units (CFU). The FAO/WHO *(2)* estimated a human LD_{50} of 1.9×10^6 CFU based on data from a pregnant woman consuming contaminated soft cheese. We re-evaluated risk based on dose-response curves from pregnant rhesus monkeys and guinea pigs. Using standard risk assessment methodology including hazard identification, exposure assessment, hazard characterization and risk characterization, risk was calculated based on the new dose-response information. To compare models, we looked at mortality rate per serving at doses ranging from 10^{-4}-10^{12} *L. monocytogenes* CFU. Based on a serving of 10^6 *L. monocytogenes* CFU, the primate model predicts a death rate of 5.9×10^{-1} compared to the FDA/USDA/CDC (Figure IV-12) *(1)* predicted rate of 1.3×10^{-7}. Based on the guinea pig and primate models, the mortality rate calculated by the FDA/USDA/CDC *(1)* is underestimated for this susceptible population.

Key words: *Listeria monocytogenes*, guinea pigs, nonhuman primates, stillbirths and dose-response

2. Introduction

Listeriosis results from exposure to the food-borne pathogen *Listeria monocytogenes* (*L. monocytogenes*). In the United States in 2000, *L. monocytogenes* caused higher rates of hospitalization than any other food-borne pathogen and over one-third of the reported deaths [(1)]. Although rare among healthy individuals, listeriosis can lead to severe consequences, even death, in susceptible populations. The disease primarily affects persons of advanced age, fetuses or newborns and adults with compromised immune systems.

In a joint effort to reduce foodborne listeriosis, the Center for Food Safety and Applied Nutrition of the U.S. Food and Drug Administration (FDA), the Food Safety Inspection Service of the U.S. Department of Agriculture (USDA) and the Centers for Disease Control and Prevention (CDC) conducted a risk assessment for *L. monocytogenes* in selected ready-to-eat foods. The FDA/USDA/CDC *(1)* developed a dose-response curve based on mouse data adjusted to reflect human epidemiological data. Based on their dose-response graph (Figure IV-7) *(1)* the estimated human LD_{50} is approximately 10^{13} *L. monocytogenes* colony forming units (CFU). However, the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) *(2)* estimated an LD_{50} of 1.9×10^{6} *L. monocytogenes* CFU based on human outbreak data. Also, when compared to various animal models such as nonhuman primates ($LD_{50} = 4.0 \times 10^{7}$) *(3, 4)* and guinea pigs ($LD_{50} = 2.0 \times 10^{7}$) *(5)*, the human LD_{50} estimate of 10^{13} is high. Our objectives were to conduct a risk assessment of *L. monocytogenes* using dose-response data from nonhuman primate and guinea pig models and compare the results to previous *L. monocytogenes* risk assessments.

3. Hazard Identification

3.1. Listeriosis

The CDC has estimated that approximately 2,500 cases of listeriosis occur annually in the United States *(6)*. Approximately 500 cases result in death, making listeriosis an important public health concern *(6)*. One third of these annual cases in the United States occur during pregnancy *(7)*.

The most severe symptoms, including death, usually occur in people with predisposing conditions or susceptible populations including fetuses or the immune compromised. Following invasion of intestinal tissue, *L. monocytogenes* most often spreads to the blood, liver, placenta, or the central nervous system. The incubation period before individuals become ill can be anywhere from a few days for gastroenteritis, two to three weeks for meningitis and up to three months during pregnancy *(8)*. If the infection spreads to the nervous system, symptoms such as headache, stiff neck, confusion, loss of balance, or convulsions can also occur. Infected pregnant women may have no symptoms or experience only a mild, flu-like illness; however, infections during pregnancy can lead to miscarriage or stillbirth, premature delivery, or infection of the newborn *(9, 10, 11)*.

3.2. *Listeria monocytogenes*

L. monocytogenes is a ubiquitous and pathogenic bacterium that causes listeriosis in both animals and humans. Although *L. monocytogenes* is found in soil, water and foods, most exposure is attributable to food-borne transmission of the microorganism *(12)*. Foods most often associated with *L. monocytogenes* contamination possibly resulting in human listeriosis include industrially processed ready-to-eat foods

such as deli meats, pasteurized dairy products, smoked seafood and raw foods *(13)*. *L. monocytogenes* has been isolated from pasteurized and refrigerated foods such as pasteurized fluid milk and soft-ripened cheeses *(14, 15)*.

3.3. Susceptibility and High Risk Individuals

Healthy individuals may become infected with *L. monocytogenes*, but few become seriously ill. Persons with compromised immune systems, the elderly, fetuses and newborns are at increased risk of becoming fatally ill from *L. monocytogenes* exposure *(16, 17, 18)*. This risk assessment will focus on pregnant women and their fetuses.

Listeriosis has not been found to be life threatening to pregnant women and may be transferred to the fetus without the mother showing signs of illness *(8)*. A perinatal infection occurs primarily as the result of transplacental transmission to the fetus following maternal ingestion. Women may become infected with *L. monocytogenes* at any time during pregnancy, but most cases of listeriosis are reported in the third trimester *(19)*. In the first trimester, listeriosis may result in spontaneous abortion *(8, 15)*. In later stages of pregnancy, the result may be stillbirth, premature delivery or birth of a critically ill newborn *(8, 15)*.

3.4. Outbreaks

Many foodborne outbreaks of listeriosis have occurred within the last two decades. A total of 466 cases of listeriosis occurred during 12 severe listeriosis outbreaks in the United States between 1970 and 2002 *(7)*. Preliminary FoodNet data in the United States for the incidence of foodborne listeriosis indicated that occurrences of infection derived from *L. monocytogenes* decreased between 1996 and 2002; however, there was an increase in incidence from 2002 to 2003. Unfortunately since 2003, the number of

cases of listeriosis in the United States has not continually declined *(20)*, and a recent outbreak in Canada demonstrates that listeriosis is still a public health concern *(21)*.

L. monocytogenes was established as an important foodborne pathogen in 1985 after the Los Angeles outbreak that resulted in 142 cases of human listeriosis *(1)*. Of these 142 cases, 93 occurred in pregnant women or their offspring resulting in 20 fetal deaths and 10 neonatal deaths *(22)*.

Another listeriosis outbreak associated with Mexican-style soft cheese occurred in November, 2000 in Winston-Salem, North Carolina *(9)*. Among a total of 12 identified cases, one was an immunocompromised 70-year-old man and 11 patients were women between 18-38 years of age. Ten of the 11 women were pregnant and suffered fetal complications *(23)*.

Fresh soft cheese such as Mexican style cheese has been associated with outbreaks and sporadic cases of listeriosis in the United States *(22, 23, 24)*. It is assumed that fresh soft cheeses consumed during outbreaks in Los Angeles in 1985 and in North Carolina in 2001 were made from unpasteurized milk. This risk assessment focuses on consumption of contaminated Mexican-style soft cheese consumed by a 3rd trimester pregnant woman.

3.5. Animal Models

To use a dose-response curve based on experimental data from animals, the selection of the appropriate animal model should be based on mechanisms of action and adverse effects as seen in humans. A pregnant animal model that expresses similar mechanisms of action and adverse effects as humans is essential to accurately assess the risk of maternal exposure to *L. monocytogenes* on the resulting fetus and/or neonate.

Mice do not accurately estimate dose-response for human listeriosis because of molecular differences in their receptor protein (E-cadherin) *(25)* which is essential for transmigration of *L. monocytogenes* across the intestinal epithelial cell and because the adverse health effects are not the same (death in adult mice as opposed to stillbirths/meningitis in humans). A single amino acid shift located at position sixteen in murine E-cadherin (proline to glutamic acid) results in a loss of the ability for *L. monocytogenes* and the receptor to interact *(25)*. A transgenic mouse that expressed human E-cadherin solely in enterocytes was developed in order to demonstrate the interactions of internalin and E-cadherin *in vivo (26)*; however these receptors are localized within the intestine and are not associated with other tissues, more specifically, the placenta. The lack of expression of E-cadherin in other tissues limits the use of the transgenic model as a surrogate for humans.

Previous studies have shown that pregnant nonhuman primates are susceptible to listeriosis both naturally and experimentally *(3, 4, 27, 28)*. Furthermore, fetal infections in both humans and nonhuman primates can lead to similar conditions such as abortions, stillbirths, and/or neonatal deaths *(27, 29)*. For humans and nonhuman primates, the pathogenesis and morphological findings associated with stillbirths due to *L. monocytogenes* are essentially the same *(28, 29)*. Nonhuman primates exhibit dose-responses that are similar to humans. Yet the number of primates needed to thoroughly examine the low dose region of the dose-response curve is prohibitive.

Studies conducted in the 1970s used non-pregnant guinea pigs to investigate the interaction between *Listeria* and host cells. Using electron microscopy, the interaction between *L. monocytogenes* and intestinal epithelial cells was investigated after pre-

conditioned guinea pigs had been treated with 10^9 CFU through a stomach tube *(30)*. These studies showed that *L. monocytogenes* entered the small intestine epithelial cells and multiplied there before being phagocytosed by macrophages *(30)*. Pregnant guinea pigs were one of several animals used to characterize *Listeria* isolates *(31)*. Research has shown that following exposure to *L. monocytogenes*, pregnant guinea pigs exhibit similar side effects to humans including fetal abortion *(5, 31)* as well as gastrointestinal tract disturbances *(32)*. In a previous study, pregnant guinea pigs aborted on the 3rd and 7th day following infection with *L. monocytogenes* by intraperitoneal injection *(31)* but the experimental design did not include the treatment dose. Recently, it was discovered that E-cadherin has the same sequence in both humans and guinea pigs *(25, 33)*. Preliminary studies conducted by our laboratory *(5, 34, 35, 36)* as well as previous studies *(37)* support using the pregnant guinea pig as a surrogate for human listeriosis. Bakardjiev et al. *(37)* used the pregnant guinea pig to study feto- placental transmission of *L. monocytogenes* following invasion of the maternal system through intravenous inoculation. Due to the fact that humans are exposed to *L. monocytogenes* through ingestion, experimentally by-passing the gastrointestinal tract may lead to differences in dissemination and disease. By using an exposure regimen that is realistic to human exposures, we will be better equipped to estimate maternal and fetal health conditions following exposures to *L. monocytogenes*. Recently the guinea pig has been questioned as a model for human listeriosis because of a difference in the Met receptor between humans and guinea pigs *(38)*. However, the dose-response curves seen in guinea pigs and nonhuman primates are similar (Figure 3.1) and the number of predicted stillbirths in humans based on the guinea pig dose-response curve (data not shown) is very similar to

nonhuman primates (Table 3.3). This suggests that either the Met receptor is not essential for *L. monocytogenes* infection, or that guinea pigs have an alternate pathway by which *L. monocytogenes* invades and moves intracellularly. Some evidence for the latter has recently been published *(39)*.

More recently, gerbils have been proposed as an animal model to assess human listeriosis because their E-cadherin active site is the same as humans and their Met receptor contains key amino acids that are required for efficient interactions with the *L. monocytogenes* surface protein, InlB. Disson et al. *(40)* demonstrated that gerbils were susceptible to neonatal death following intravenous inoculation. However, currently no dose-response data exist on the effects of *L. monocytogenes* following oral inoculation in gerbils. Future studies will need to be done to evaluate whether gerbils are appropriate as surrogates for developing human dose-response data for listeriosis.

4. Exposure Assessment

The exposure assessment presents the prevalence of a pathogen in foods at the time of ingestion among certain populations. In reality, the actual extent of exposure is not known, especially the numbers of pathogens consumed and the frequency of food contamination. However, an assessment can be based on estimations of contamination levels *(2)*. Mexican-style soft cheese was selected for this risk assessment based on its association with *L. monocytogenes* outbreaks, high consumption among certain populations and potential to support the growth of *L. monocytogenes (1)*. The incidence of *L. monocytogenes* at different levels (1.6% - 43.3%) in fresh cheeses has been reported in different regions and countries *(41)*. This type of cheese is often imported and can come from unpasteurized sources *(9)*.

4.1. Food Consumption Data

For the purposes of this risk assessment, two U.S. food consumption data surveys were used. The USDA Agricultural Research Service's Continuing Survey of Food Intake by Individuals (CSFII, 1994-1996) *(42, 43)* is based on the recollections of 16,000 people about various foods eaten in two twenty-four hour periods. The Third National Health and Nutrition Examination Survey (NHANES III) used recall information of food eaten in one twenty-four hour period by 30,318 individuals during 1988 to 1994 *(44)*. Although different study designs were used, the two surveys correlate fairly well *(45)*. The CSFII and NHANES III contain data specific to this risk assessment's target population and food type. The CSFII collected data from 123 pregnant/lactating women, and the NHANES III collected data from 399 pregnant/lactating women. The surveys have consumption data for many foods, including fresh soft cheese (Mexican-style cheese) *(42, 43, 44)*.

4.2. Annual Number of Servings of Foods

In this assessment we used the estimate of the number of annual servings for fresh soft cheese published in the FDA/USDA/CDC *(1)* risk assessment. More particularly, we are focusing on the consumption of *L. monocytogenes* by pregnant women and the effect of the pathogen on fetuses. Information on consumption of food types and amounts during the third trimester of pregnancy is limited, and therefore the perinatal population was used in this risk assessment as was done in the FDA/USDA/CDC risk assessment. According to NHANES III and CSFII calculations, the U.S. perinatal population is estimated to consume 4.8×10^5 servings of fresh soft cheese annually *(42, 43)*. Perinatal death rates (the total number of deaths includes prenatal and stillbirth cases occurring in

the last trimester) per serving from listeriosis were estimated using an exposure period of ninety days and a pregnancy rate of 2.77% *(1)*.

4.3. Serving Size Distribution

In the FDA/USDA/CDC *(1)* risk assessment, the distribution of serving sizes (grams of food eaten per serving) is expressed as a series of population percentiles of the amount of a food type eaten per serving, the 50th (median), 75th, 95th and 99th percentiles *(1)*. The population percentiles for consumption of fresh soft cheese are 31, 85, 246 and 246 g/serving, respectively *(1)*. This distribution indicates that half of the servings were less than 31g and 95% of the servings were less than 246g.

4.4. Food Contamination Data

Food contamination data included an outbreak described above as a source but also information from the FDA/USDA/CDC *(1)*. The following components were used by the FDA/USDA/CDC *(1)* to calculate the contamination of food with *L. monocytogenes* at the time of consumption: prevalence and concentration (CFU/g) of *L. monocytogenes* in retail products, post retail growth, concentration of *L. monocytogenes* at the time of consumption (log CFU/g) and dose at consumption (log CFU/serving).

To represent the annual number of servings of Mexican-style soft cheese "fresh, soft cheese" categorical contamination data were used *(1)*. The estimated frequency of consumption of *L. monocytogenes* by pregnant women in the third trimester was derived by dividing the frequency of consumption of *L. monocytogenes* in the perinatal population by the estimated number of pregnant women in the last three months of pregnancy. To estimate the number of pregnant women in the last 3 months of pregnancy

(1.8×10^6), the annual pregnancy rate (2.77%) was multiplied by the U.S. population in 2003 (2.6×10^8) and 0.25 (0.25 = 3/12), to represent three months out of a year.

To estimate the number of servings consumed at a particular contamination level, the percentage of servings at a specific concentration was obtained by multiplying the annual number of perinatal servings (4.8×10^5) times the percent of servings at that contamination level *(1)*.

This risk assessment focuses on *L. monocytogenes* infection in pregnant women in the third trimester. Due to limited information for this specific population, limitations to this risk assessment include serving size distribution and frequency estimations using perinatal population data. Also, the fraction of servings of soft cheese contaminated at specific concentrations of *L. monocytogenes* is uncertain *(1)*.

5. Hazard Characterization

In this assessment, the purpose of the hazard characterization was to establish the relationship between the dose or concentration of *L. monocytogenes* ingested and adverse birth outcomes. Because there are no human clinical trials or outbreak data with adequate dose-response information for *L. monocytogenes*, animal studies were used to determine a dose-response relationship. Two animal models, nonhuman primates *(3, 4)* and guinea pigs *(5)* were used as surrogates for humans. The data collected in these studies, and summarized in Table 3.1, were fit to a logistic model. Median lethal dose (LD_{50}) comparisons were made between both animal models, and these LD_{50} values were also compared to both the FDA/USDA/CDC *(1)* and FAO/WHO *(2)* estimated human LD_{50} levels.

For the FDA/USDA/CDC *(1)* risk assessment, a mouse dose-response model *(46)* was used to predict the effect of *L. monocytogenes* in humans. Adjustment factors were required based on differences in exposure regimens as well as anatomical, physiological and immunological differences between mice and humans. Available quantitative data were used to adjust for host susceptibility and strain virulence in the FDA/USDA/CDC *(1)* dose-response model. However, limited data on other parameters such as host and food matrix differences between mice and humans was dealt with using a dose-response scaling factor. This scaling factor was developed to allow the mouse-derived model to apply to humans by adjusting the effective dose in mice to match the limited human surveillance data available. Without these adjustment factors, the FDA/USDA/CDC *(1)* risk assessment would have overestimated human mortality from listeriosis by a factor of over one million. Mice are not appropriate animal models to use as surrogates for humans in developing dose-response data because they are not susceptible to listeriosis following ingestion and due to differences in their response following alternative exposure routes.

Several dose-response models, such as exponential, logistic, Weibull gamma and log logistic, have been examined for use in risk assessment. Each of these models predicted a similar fit at the mid-range region of the dose-response curve *(5, 47)*. Thus we have chosen to compare studies at the LD_{50} because this point is generally independent of the model used. No specific dose-response model has been accepted for all foodborne pathogens, and it is unlikely that one model will be appropriate for all pathogens because of differences in mechanisms of pathogenicity (ie invasive, toxicoinfectious, preformed toxins, etc). The shape of the dose-response curve in the low

dose region is particularly difficult to predict due to the lack of data. However, understanding the mechanisms of pathogenicity should eventually provide the information needed.

Recently, data from a nonhuman primate model for listeriosis were published *(3, 4)*. In order to mimic human conditions, the pregnant rhesus monkeys were exposed to *L. monocytogenes* orally. Ten of the 33 pregnant rhesus monkeys that were exposed to a single dose of 10^2 to 10^{10} *L. monocytogenes* CFU had stillbirths *(3, 4)*. Using the primate data, the logistic model predicted 50% stillbirths at a dose of 10^7 *L. monocytogenes* CFU (Table 3.2), comparable to an estimated 10^6 *L. monocytogenes* CFU based on a listeriosis outbreak among pregnant women *(2)*. However, this LD_{50} is considerably less than that of the estimated 10^{13} *L. monocytogenes* CFU which was extrapolated from Figure IV-7 in the FDA/USDA/CDC *(1)* risk assessment that applied several adjustment factors and used an exponential model. Based on these studies, the dose of *L. monocytogenes* required for infection and the etiology of adverse effects during pregnancy are more similar between humans and primates than humans and mice *(3, 4)*.

Pregnant guinea pigs have also been used as a model for human listeriosis *(5, 34, 35, 36)*. In order to construct a dose-response curve, pregnant guinea pigs were orally challenged with doses ranging from 10^4 to 10^8 *L. monocytogenes* CFU and birth outcome was determined *(5)*. Williams et al. *(5)* used *L. monocytogenes* strain 12443, the same strain used in the rhesus monkey studies *(3, 4)*. Guinea pigs were inoculated with doses ranging from 10^4 to 10^8 *L. monocytogenes* CFU/5 ml of sterile whipping cream *(5)*.

The dose-response curves based on fetal mortality data were calculated using a logistic model represented with the following formula: $y = 1/1 + e^{-ax-b}$, where y represents

the % fetal mortality, x denotes *L. monocytogenes* dose, a represents the rate at which mortality increases at log dose a of *L. monocytogenes* and b denotes the y-intercept (Figure 3.1). The parameter estimates for the nonhuman primate logistic model are as follows: a = 0.65 and b = -5. For this model R^2 = 0.95. The parameter estimates for the guinea pig logistic model were as follows: a = 0.65 and b = -10.094. For this model R^2= 0.9853.

The LD_{50} estimated for the nonhuman primate data was 4.0 x 10^7 and for guinea pig data was 2.0 x 10^7. Based on the FAO/WHO *(2)* risk assessment, the LD_{50} is estimated at 1.9 x 10^6 for perinates or neonates after a population of pregnant women was exposed. This estimated LD_{50} level for humans was based on attack rates during a Mexican-style soft cheese outbreak in 1985 *(22)*. The difference between the estimated LD_{50} values when comparing humans to the two animal models (guinea pigs and nonhuman primates) is about one order of magnitude.

When comparing listeriosis in humans, nonhuman primates and guinea pigs, there are many similarities. As in humans, both guinea pigs *(5)* and nonhuman primates *(3, 4)* are susceptible during pregnancy to having stillbirths after oral exposure to *L. monocytogenes*. Also, guinea pigs *(5)* and nonhuman primates *(3, 4)* are susceptible to infection after oral exposure and reported pathological results are similar *(34, 35)*.

6. Risk Characterization

In this risk assessment, the risk characterization links the probability of exposure to *L. monocytogenes* in pregnant women in the United States from the consumption of Mexican-style soft cheese with adverse fetal outcomes. The primary focus is on the prediction of the relative probability of fetal mortality from a mother’s consumption of a

single serving of Mexican-style soft cheese contaminated with a virulent strain of *L. monocytogenes*. Fetal mortality was predicted using two animal models, guinea pigs and nonhuman primates.

The data collected from the guinea pig and nonhuman primate studies were fit to a logistic model. The logistic model was chosen on the basis of simplicity, its fit of the data at LD_{50}, and its frequent use in growth response studies. After plotting the data, the LD_{10}, LD_{50} and LD_{90} were obtained (Table 3.2).

The LD_{50} values of the guinea pig and nonhuman primate data were also compared to the dose-response curve in the FDA/USDA/CDC *(1)* risk assessment. The FDA/USDA/CDC *(1)* risk assessment included a scaling factor that adjusted the effective dose to match the dose-response mouse model with the surveillance outbreak data. Adjustment factors were used to compensate for a lack of knowledge or data. These included interspecies comparisons, variables such as age, body weights, surface area and/or any factors that affected absorption or distribution or the ultimate effect of a specific pathogen on target tissue. These uncertainties are usually accounted for using mathematical adjustments to compensate for safety. By applying adjustment factors, the dose-response curve will be shifted either to the right or left of the actual data and may over- or under- estimate the risk. For example, had the FDA/USDA/CDC not applied adjustment factors including exposure route differences, differences between humans and mice as well as immunological differences, they would have overestimated risk by a factor of over 1 million *(1)*. The perinatal LD_{50} level can be estimated as 10^{13} CFU from Figure IV-7 of the FDA/USDA/CDC *(1)* risk assessment, which is considerably more than the FAO/WHO's *(2)* estimate from human outbreak data of 1.9×10^{6}. Also, an

estimated human LD_{50} level of 10^{13} CFU is very high compared to the estimated LD_{50} values determined using the guinea pig and nonhuman primate data. Interestingly, adjustment factors were not necessary in order to achieve LD_{50} values similar to humans using the guinea pig and nonhuman primate models.

In order to predict the number of fetal deaths occurring in the United States annually due to *L. monocytogenes*, the fraction of servings contaminated with *L. monocytogenes* and the frequency of consumption was calculated for Mexican-style soft cheese. Fresh soft cheese contains high moisture and a favorable pH for *L. monocytogenes* to grow. Due to the favorable environment for *L. monocytogenes* growth, Mexican-style soft cheese has been found to be associated with outbreaks and sporadic cases of listeriosis in the United States *(22, 23, 24)*.

To estimate the number of servings of Mexican style soft cheese consumed by the pregnant population in their third trimester, perinatal population values were used. The estimated annual number of servings for fresh soft cheese for the perinatal population is 4.8×10^5 and the median amount consumed per serving is 31g *(1)*. As seen in Table V-3 of the FDA/USDA/CDC *(1)* risk assessment the estimated number of cases of perinatal listeriosis per year following consumption of fresh soft cheese is <0.1.

According to Table IV-11 of the FDA/USDA/CDC *(1)* risk assessment, the annual national estimated number of cases of listeriosis for the perinatal and neonatal subpopulations combined was 216. Of these 216 cases, 40 perinatal deaths were estimated. The FDA/USDA/CDC calculated the total number of perinatal deaths by multiplying the number of neonatal deaths (16) by 2.5 to account for stillbirths and

abortions that were omitted from the FoodNet surveillance reports. Assuming that stillbirths account for the remaining (40 minus 16), we can compare our predicted number of stillbirths (8) to their estimated number from surveillance (24). The similarity between these two numbers despite their different origins in predictive modeling versus surveillance is encouraging. Our estimate used a specific exposure scenario, a pregnant woman consuming Mexican style soft cheese, and was calculated by multiplying the probability of mortality with the annual number of servings consumed by the perinatal population at the specified contamination level. These numbers were then summed to give the total number of deaths (Table 3.3). That both the guinea pig and nonhuman primate models predict fewer perinatal deaths as a result of exposure to L. monocytogenes-contaminated soft cheese compared to the FDA/USDA/CDC risk assessment while at the same time having a lower LD_{50} could result from two possible sources, the use of adjustment factors in the risk assessment by the FDA/USDA/CDC and the overall shape of the dose-response curve. An adjustment factor developed from surveillance data was applied to the dose-response curve in the FDA/USDA/CDC risk assessment to predict the number of deaths per year. In other words, the surveillance based adjustment factor 'forced' the dose-response curve to predict the number of perinatal deaths seen in surveillance without regard to the shape of the dose-response curve. However, our dose-response curve was based solely on data from the guinea pig or primate studies without adjustment factors. This illustrates the importance of the shape of the dose-response curve in both predicting the overall number of deaths and in predicting the various points (example, LD_{10}, LD_{50}) along the dose-response curve. Using the model that provided the best fit for our data predicted 8 perinatal deaths, which

is a lower estimate when compared to the FDA/USDA/CDC risk assessment of 16 perinatal deaths. The advantage to using experimental data without surveillance-based adjustment factors is that the surveillance data can then be used to compare and validate the dose-response model.

Limitations include the assumption that serving size for the perinatal population is the same as the pregnant population in their third trimester. Combining the LD_{50}s estimated for the animal models into a range to predict the fraction of servings contaminated with 10^6-10^9 CFU/serving may not reflect actual exposures. Dose-response information based on human exposure would be the optimum method for predicting risk. However, due to the seriousness of the illness and the lack of dose information from outbreaks, this information is not available.

In summary, the LD_{50} values of the guinea pig and nonhuman primate data were compared to the FDA/USDA/CDC *(1)* as well as the FAO/WHO *(2)* estimated human LD_{50} levels. The FDA/USDA/CDC *(1)* estimated the human LD_{50} level to be 10^{13} *L. monocytogenes* CFU which is considerably more than the FAO/WHO's estimate of 1.9 x 10^6 based on outbreak data and when compared to the LD_{50} values computed using the guinea pig and nonhuman primate data. Adjustment factors were not necessary in order to achieve LD_{50} values similar to humans in the guinea pig and nonhuman primate models. The risk assessment predicts 8 stillbirths per year from consumption of contaminated soft cheese by pregnant women. Because this is based on an estimate of the number of servings of soft cheese contaminated with *L. monocytogenes* in an average year, it will underestimate deaths from outbreaks associated with a higher contamination

levels. In those cases, the exposure assessment would need to be adjusted to reflect a greater percentage of contaminated servings at higher concentrations.

When selecting dose-response data from animal models for use in human risk assessment, consideration needs to be given to the mechanism of action resulting in the adverse effect and to the selection of the most appropriate animal model. For *L. monocytogenes,* the data from studies in mice were originally used due to the lack of dose-response data from other animal models. Recent studies showing the different mechanisms by which *L. monocytogenes* gains entry into the intestinal epithelium illustrates the importance of selecting models similar to humans. In addition, new information provides the opportunity and demonstrates the importance of the iterative process in risk assessment. Whenever new mechanistic information is available, it should be used to revise risk assessments providing more realistic risk estimates for humans.

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Table 3.1 ***Dose- response of fetal mortality after maternal challenge with L. monocytogenes***

Guinea Pig[a]		Nonhuman Primates[b,c]	
Maternal Dose (log_{10} CFU)	**% litters with ≥ 1 fetal deaths**	**Maternal Dose (log_{10} CFU)**	**% stillbirths**
Control	0	---	**---**
---	---	2.5	0
---	---	3.2	25
4	0	4.3	0
5	0	5.1	20
6	22	6.2	33
7	33	7.1	40
8	75	8.1	50
---	**---**	10.6	100

Adapted from [a]Williams et al., 2007, [b]Smith et al., 2003 and [c]Smith et al., 2008.

Table 3.2 ***Estimated LD_{10}, LD_{50}, and LD_{90} dose (CFU/serving) for*** **L.monocytogenes** ***extrapolated from the logistic model***

Logistic Model			
Animal Model	**LD_{10}**	**LD_{50}**	**LD_{90}**
Guinea Pig	$5.0x10^{5}$	$2.0x10^{7}$	$7.9x10^{8}$
Nonhuman Primate	$1.6x10^{4}$	$4.0x10^{7}$	$7.9x10^{10}$

Table 3.3 ***Predicted number of fetal deaths per year for the perinatal population in the United States based on the consumption of*** **L. monocytogenes** ***contaminated soft cheese***.

Annual number of perinatal servings	Log dose *L.monocytogenes*	Servings with contamination levels (%)[a]	Number of servings	Probability of mortality per serving[b]	Predicted number of deaths
4.8×10^5	-4	97.86	4.70E+05	0.000005	2.35E+00
	-3	0.0337	1.62E+02	0.000010	1.55E-03
	-2	0.027	1.30E+02	0.000018	2.38E-03
	-1	0.0414	1.99E+02	0.000035	6.97E-03
	0	0.3138	1.51E+03	0.000067	1.01E-01
	1	0.7075	3.40E+03	0.000127	4.33E-01
	2	0.4402	2.11E+03	0.000241	5.10E-01
	3	0.2516	1.21E+03	0.000452	5.46E-01
	4	0.1319	6.33E+02	0.000832	5.27E-01
	5	0.0741	3.56E+02	0.001480	5.27E-01
	6	0.0425	2.04E+02	0.002497	5.09E-01
	7	0.0278	1.33E+02	0.003894	5.20E-01
	8	0.0165	7.92E+01	0.005498	4.35E-01
	9	0.0108	5.18E+01	0.007006	3.63E-01
	9.5	0.0138	6.62E+01	0.007640	5.06E-01
	10	0.0081	3.89E+01	0.008176	3.18E-01
	10.5	0.0016	7.68E+00	0.008612	6.61E-02
	11	0.0002	9.60E-01	0.008957	8.60E-03
	12	0	0.00E+00	0.009427	0.00E+00
	Total	**100.0025**		**Total**	**7.73E+00**

[a] The numbers represent the percentage of servings that are contaminated with the log dose of *L. monocytogenes* given in the first column.

[b] These values were calculated based on data from our primate model.

Figure legend

Figure 3.1 ***Dose-response of* L. monocytogenes *induced fetal mortality in guinea pigs and nonhuman primates.*** A logistic model was used to fit the dose-response data on the basis of the dose resulting in fetal deaths.

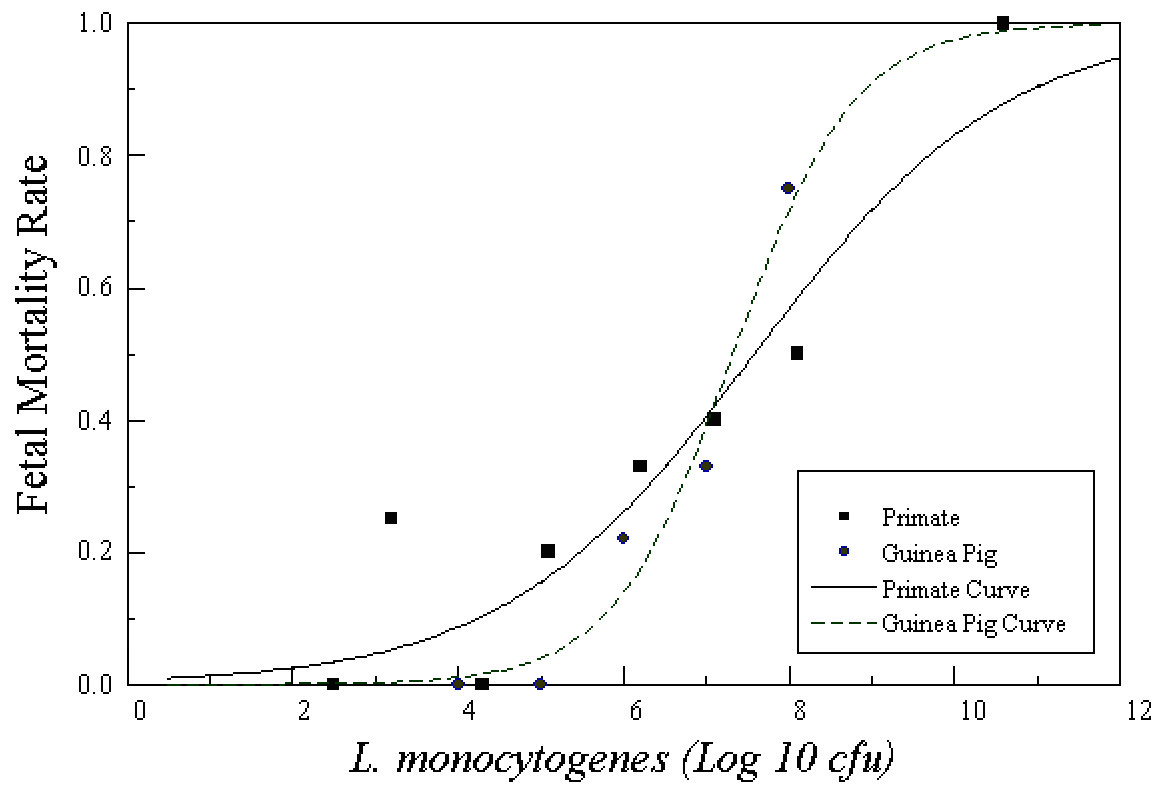

Figure 3.1

9. Erratum

"Risk of Fetal Mortality After Exposure to Listeria monocytogenes Based on Dose-Response Data from Pregnant Guinea Pigs and Primates" by Denita Williams, Jennifer Castleman, Chi-Ching Lee, Beth Mote, and Mary Alice Smith. *Risk Analysis*, 29(11):1495-1505[1]

Inadvertent errors reported by authors:

On page 1501, the third line lists the parameter estimates for the guinea pig logistic model with alpha as 0.65 when it should be 1.38. This number given in the manuscript was inadvertently copied from the number for the primate parameter.

In Table 3, column 5, the formula used to calculate the probability of mortality per serving should use 1 as the numerator (probability of mortality) rather than 0.01 (representing percentage of mortality). In producing this table, it was the authors' desire to reproduce, as nearly as possible, the calculations presented in the FDA/USDA/CDC (2003) risk assessment. However, a similar table is not published in the risk assessment (FDA/USDA/CDC 2003). The result of this error is that the predicted number of deaths is underestimated by a factor of 100.

Although the predicted number of deaths increased from 7.73 to 773, the important point of the manuscript remains intact—that primates and guinea pigs represent much better models for estimating human risk for exposure to *Listeria monocytogenes* than do mice. This is supported by the comparisons of LD50s as stated in the manuscript: the LD50 values calculated using the primate (10^7 cfu) and guinea pig (10^7 cfu) data are comparable to the estimated LD50 of 10^6 cfu from a human outbreak (FAO/WHO, 2004). In addition,

the larger number of predicted deaths at 773 is much lower than the predicted deaths using the mouse data without correction factors (with an LD50 of ~10^{13} cfu).

CHAPTER 4

THE APPEARANCE OF *LISTERIA MONOCYTOGENES* IN TISSUES IN PREGNANT GUINEA PIGS 2, 6, 9 AND 21 DAYS POST TREATMENT[2]

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[2] Williams, D., Dunn, S., Richardson, A., Frank, J.F., and Smith, M.A. Submitted to *Journal of Food Protection*, 04/14/2010. (in revision)

1. Abstract

Pregnant women are twenty times more likely to develop listeriosis than the general population, and infection can result in abortion, stillbirth or neonatal illness. The objective of this study was to orally-challenge pregnant guinea pigs with *L. monocytogenes* to assess maternal and fetal effects at post treatment days 2, 6, 9 and 21 using fluorescent microscopy and a traditional culturing method. Guinea pigs were treated on gestation day 35 with doses ranging from 10^4- 10^8 *L. monocytogenes* CFU. *L. monocytogenes* was isolated and viewed in maternal and fetal tissues as early as 2 days post treatment. Isolation of *L. monocytogenes* from placentas, fetal livers and brains were similar to that seen in maternal spleens indicating that invasion of the spleen could be indicative of fetal invasion. When comparing fecal shedding, all animals treated with10^4 CFU were shedding by post treatment day 7; whereas, 100% of animals treated with the higher doses of 10^6 or 10^8 CFU were shedding *L. monocytogenes* by post treatment day 5. These data suggest that *L. monocytogenes* crosses the fetoplacental barrier and invades the fetus by 2 days following maternal ingestion. When comparing sensitivity between the two detection methods, microscopy and culturing, at the highest dose of 10^8 CFU microscopy was more sensitive than culturing in fetal livers and brains. However, at the lowest dose, 10^4 CFU, culturing was more sensitive in confirming the presence of *L. monocytogenes* in maternal spleens suggesting that used alone, each method could underestimate the number of samples positive for *Listeria*.

2. Introduction

Since 2002, the number of cases of listeriosis in the United States has not shown a continued decrease *(4)* suggesting that *Listeria monocytogenes* (*L. monocytogenes*) remains a public health concern. Listeriosis is most commonly associated with the elderly, immunocompromised individuals and pregnant women *(8)*. When listeriosis is contracted during pregnancy, the fetus/newborn suffers adverse effects which may include death. Although previous studies indicate that fetal effects following maternal ingestion of *L. monocytogenes* depend on the trimester in which the exposure occurs *(7, 11)*, a time course of fetal invasion is yet to be addressed.

Due to the high mortality rate and severity of health effects following exposure to *L. monocytogenes*, human clinical trials are prohibited. Previously we have shown that following an oral challenge with *L. monocytogenes*, pregnant guinea pigs *(13, 14, 15, 23, 24)* and pregnant nonhuman primates *(20, 21)* have adverse effects similar to humans including delivery of nonviable fetuses. The similarities among guinea pigs, nonhuman primates and humans can most likely be attributed to molecular similarities in their E-cadherin receptors. Our main objective of this study was to determine the time course of invasion of *L. monocytogenes* in pregnant guinea pigs and their fetuses. Because we were investigating *L. monocytogenes* invasion as early as two days post-treatment, a sensitive method to detect *L. monocytogenes* in tissues was needed. Using a fluorescent-tagged *L. monocytogenes* strain, we compared invasion of tissues using a standard culture method to visualizing fluorescent-labeled *L. monocytogenes.*

Detection methods for *Listeria* spp. generally involve selective enrichment, followed by plating on selective and differential media. Two commonly used methods,

the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA), use selective enrichment; however, concentrations of selective agents differ in these formulations because of the background microflora that must be suppressed in different foods and raw products. The CDC reported that utilizing the USDA method resulted in 90% sensitivity for isolating *L. monocytogenes* from food and fecal samples.

Several studies conducted between 1999 and 2000 described the construction of green fluorescence protein (gfp) expression vectors allowing visualization of *L. monocytogenes* in cultured cells by fluorescence microscopy *(2, 10, 12)*. In a more recent study by Fortineau et al. *(9)*, three gfp plasmids were constructed and evaluated for efficiency of expression, pNF6, pNF7 and pNF8. Of the three gfp plasmids, pNF8 was found to be the most useful as evident by detection in tissue cultures, fluorescence in cell cultures as well as tissue samples *(9)*. The objective of this study was to investigate the invasion of *L. monocytogenes* in maternal and fetal tissues at various time points following maternal ingestion using two different methods, culturing and microscopic analysis.

3. Materials and Methods

3.1 Inoculum Preparation. The *L. monocytogenes* strain used in this study (12443) was isolated from a stillbirth from a rhesus monkey, and the strain was subsequently used to induce stillbirths in primate *(21)* and guinea pig *(24)* studies. Strain 12443 was labeled with the gfp-plasmid pNF8 using methodology previously described by Fortineau et al. *(9)* by Dr. Li Min at the Center for Food Safety University of Georgia, Griffin, Ga. Three days prior to treatment, *L. monocytogenes* cells were activated by three successive

transfers in 10 ml aliquots of Tryptic Soy Broth (BD Diagnostics, Sparks, MD) supplemented with erythromycin for a final concentration of 8µg/ml and incubated at 35°C for 24 hours. Cultures were then harvested by centrifugation (9,000 x g at 4°C for 30 min), washed twice and resuspended in sterile phosphate buffered saline (PBS). Cultures were diluted to give a final concentration of the appropriate dose 10^4, 10^6 or 10^8 colony forming units (CFU) per 5 ml. Commercial heavy whipping cream that contained 36% milk fat, was used as the vehicle. The inoculum was sweetened with 0.5 grams of Splenda® to make it more palatable. Whipping cream was sterilized by autoclaving at 121°C for 11 minutes. The appropriate number of *L. monocytogenes* cells was added to the sterilized whipping cream. Sterile PBS (1.0 ml) was added to the whipping cream and administered to control animals.

The number of *L. monocytogenes* cells in the inoculated sample was determined by serially diluting the cell suspension in PBS (0.01M) and plating onto Listeria Selective Agar (LSA) (Oxoid, Ogdenburg, NY) and Tryptic Soy Agar (TSA) (BD Diagnostics, Sparks, Maryland). The plates were incubated at 35°C for 24 h before colony enumeration. Following incubation, the cells were viewed using an epifluorescent microscope to determine the efficiency of gfp expression in the inoculum. The cell populations obtained were used to confirm the dose of *L. monocytogenes* administered to the guinea pigs.

3.2 Animals and Treatment. Animal husbandry and treatments were previously described by Williams et al. *(24)*. Briefly, timed-pregnant guinea pigs were obtained from Elm Hill Breeding Laboratories on gestation day (gd) 29, housed in cages containing air-filters, maintained on a 12 hour light/dark cycle, and temperature and

humidity were 70ºC ± 2ºC and 55% ± 15%, respectively. Following a one-week acclimation period, guinea pigs were orally treated on gd 35 with doses ranging from 10^4 to 10^8 *L. monocytogenes* CFU per 5 ml of sterile whipping cream, and pregnancy allowed to continue normally. All guinea pigs were observed daily for changes in fecal output or appearance, as well as the degree of activity or appearance. Guinea pigs were sacrificed on gd 38, 42, 45 or 56 and tissue samples were collected for further analysis.

3.3 Tissue Collection and Fetal Viability. Following necropsy, maternal and fetal tissues were collected and weighed, transferred to a primary enrichment broth and used for further analysis. Tissues analyzed include: maternal blood, gallbladder, liver, and spleen, along with placenta and fetal liver and brain. Fetal viability at the time of sacrifice was assessed as described by Williams et al. *(24)*.

3.4. *L. monocytogenes* Confirmation. Tissues were confirmed positive for *L. monocytogenes* according to USDA's qualitative methodology described by Cook *(5)*. The qualitative method includes enrichment in both nonselective and selective media followed by plating onto LSA. Immediately following the necropsy, tissue samples were placed in a nonselective enrichment medium, UVM broth (BD Diagnostics, Sparks, Maryland). After 24 hours, 0.1 ml of UVM-enriched sample was transferred to 9.9 ml of the secondary enrichment, Fraser Broth (Remel, Lenexa, KS). Positive Fraser samples were streaked onto Listeria Selective Agar (Fisher Scientific, Pittsburg, PA) and incubated for 24 h at 35°C. Selected isolated colonies were streaked onto Rapid L'mono plates (Bio-Rad, Hercules, CA) for confirmation as *L. monocytogenes*.

3.5 Fecal collections and *L. monocytogenes* confirmation. To determine if guinea pigs were exposed to *L. monocytogenes* prior to arriving to the UGA Animal Facility, fecal

samples were collected from each dam before treatment. Following treatment with *L. monocytogenes*, fecal samples were collected from each dam approximately three times per week. Pre- and post treated samples were analyzed using the qualitative methodology previously described for tissue analysis.

3.6 Microscopic Analysis. Maternal and fetal tissue samples for microscopic analysis were stored on dry ice immediately after necropsy. These samples were mounted onto a slide and counterstained with ProLong ® Gold antifade reagent with DAPI (Invitrogen, Ltd, Eugene, OR). The samples were viewed using a Nikon Eclipse E600 epifluorescent microscope equipped with a 100X oil immersion lens and appropriate filters for viewing gfp (excitation 488 nm; emission 509 nm). Fifteen fields per sample were viewed and the number of fluorescent cells counted.

3.7 Statistical analyses. All statistical analyses were done using SAS version 9.1 (SAS Institute, Cary, N.C.). Tukey's tests were used to determine significant differences ($P \leq 0.05$) in values among the three treatment groups used in this study (10^4, 10^6, and 10^8 *L. monocytogenes* CFU) as well as to compare detection methodologies (microscopy and culturing). Following one-way analysis of variance (ANOVA), Dunnett's t-test was used to identify treatment groups that were significantly ($P \leq 0.05$) different from the control group.

4. Results

To determine the amount of time it takes for *L. monocytogenes* to colonize fetal tissues following maternal exposure, we examined the invasion of the pathogen in maternal livers and spleens, placentas, fetal livers and brains at post-treatment days 2, 6, 9 and 21. We were also interested in whether colonization of these maternal and fetal

tissues followed a pattern regardless of the initial concentration of *L. monocytogenes* at the time of ingestion; therefore, we exposed pregnant guinea pigs to 10^4, 10^6 or 10^8 *L. monocytogenes* CFU. Also, we wanted to determine if invasion of maternal tissues by *L. monocytogenes* correlated with fetal tissue invasion.

4.1 Invasion of maternal and fetal tissues at post-treatment days 2, 6, 9 and 21. *L. monocytogenes* was isolated from maternal livers and spleens as early as 2 days post treatment (Table 4.1). In dams exposed to 10^4 CFU, *L. monocytogenes* was isolated from fewer maternal livers as the days from treatment increased. However, there was a biphasic pattern observed in the spleens where the number of positive samples declined by post treatment day 6, increased on day 9 and was not detected by day 21. Patterns of isolation seen in placentas, fetal livers and brains were similar to that seen in the maternal spleens (Table 4.1).

In the group receiving 10^6 *L. monocytogenes* CFU, all maternal spleens were invaded by post treatment day 6 (Table 4.1). In this group, isolation of *L. monocytogenes* from fetal liver or brain tissues was less than that of the maternal livers or spleens at each treatment day (Table 4.1).

At the highest dose of 10^8 CFU on post treatment day 21, a different pattern was observed. Isolation of *L. monocytogenes* from maternal livers and spleens, placentas, fetal livers and brains was equal to or greater than earlier time points (Table 4.1).

When assessing all post treatment days and dose groups, in all but three cases, *L. monocytogenes* was isolated from more maternal livers than spleens. In two of the three cases, *L. monocytogenes* was isolated from the same percentage of maternal livers and

spleens (Table 4.1). Throughout the study, *L. monocytogenes* was not isolated from any of the maternal or fetal tissues from control animals.

4.2 Fecal shedding of gfp-*L. monocytogenes* 12443. Fecal samples were collected from each dam to determine how shedding patterns correlated with maternal and fetal tissue invasion and birth outcome. *L. monocytogenes* was isolated from fecal samples of dams exposed to doses ranging from 10^4 to 10^8 *L. monocytogenes* CFU (Figure 4.1). Interestingly, dams exposed to 10^6 and 10^8 *L. monocytogenes* CFU shed the pathogen throughout the entire study after their initial exposure. Contrastingly, those exposed to 10^4 CFU stop shedding *L. monocytogenes* around post treatment day 19. In the dams exposed to 10^4 *L. monocytogenes* CFU, the pathogen was isolated from 100% of fecal samples by post treatment day 7, whereas in animals treated with 10^6 or 10^8 CFU all animals were shedding by post treatment day 5. Of guinea pigs exposed to 10^6 CFU, there was only one day (post treatment day 21) in which *L. monocytogenes* was isolated from fewer than 50% of the dams. Of dams exposed to 10^8 CFU, *L. monocytogenes* was isolated from all fecal samples on five of nine collection days (post treatment days 5, 7, 9, 12 and 19). There was a dose dependent trend in the fecal samples positive for *L. monocytogenes*. Significantly fewer animals shed *Listeria* in the 10^4 CFU group compared to the 10^8 CFU on post treatment days 2, 5, 9, 12, 19 and 21. When comparing those that received 10^6 CFU to those that received 10^8 CFU, a significant difference in fecal shedding was only seen on post treatment day 21 (Figure 4.1). All treatment days and doses were significantly different from controls except on post treatment days 19 and 21, when *Listeria* could not be isolated from dams exposed to 10^4 CFU.

4.3 Comparison of culture and microscopic analysis of maternal and fetal tissues. To detect the presence of *L. monocytogenes,* maternal and fetal samples were processed using two different detection methods, culturing after enrichment and viewing using fluorescent microscopy. For this study, data from post treatment days were combined and analyzed by dose. At each dose (10^4, 10^6 and 10^8), numbers of positive samples by culturing were compared to numbers of positive samples viewed by microscopy for all tissues. The total positive was calculated by combining the number of positives by culture and/or microscopy. Samples that were positive using both methods were counted only once. Gfp-labeled *L. monocytogenes* could be viewed in maternal and fetal tissues as early as 2 days post treatment at doses ranging from 10^4 to 10^8 CFU. Regardless of dose, there were no significant differences when comparing positive maternal livers using both methods. However, at the lowest dose of 10^4 CFU, culturing was more sensitive than microscopy when analyzing maternal spleens (Figure 4.2A). By culturing, we isolated *L. monocytogenes* from 50% of the total number of maternal spleens compared to viewing 17% by microscopy (Figure 4.2A). Maternal gallbladders were also collected for microscopic analysis; however, we could only visualize gfp-*L. monocytogenes* in gallbladders of dams exposed to 10^8 CFU.

In placental tissues, there were no significant differences between microscopy and culturing regardless of the dose administered to the dams (Figures 4.2A-4.2C). When comparing fetal livers and brains a different statistical trend was observed. At the highest dose of 10^8 CFU, microscopy was the most sensitive method for *L. monocytogenes* detection. When analyzing fetal livers by culture, 33% of the total were positive for *L.*

monocytogenes compared to 79% using microscopy. When analyzing fetal brains by culture, 28% were positive compared to 77% using microscopy (Figure 4.2C).

5. Discussion

Case reports of listeriosis indicate that the time from exposure to *L. monocytogenes* until stillbirth or birth of an infected infant can be several weeks. Little is known about the time course of invasion and whether an earlier identification of exposure and therapeutic intervention would result in more favorable outcomes for the fetus or infant. To understand the time course of invasion, pregnant guinea pigs were exposed to gfp-labeled *L. monocytogenes* and maternal and fetal tissues collected at various time points after exposure.

As reported in our previous study, Williams et al. *(24)*, maternal liver invasion was not a good predictor of fetal tissue invasion or mortality rate. In the current study, regardless of dose or day of sacrifice as in all but one case, we isolated *L. monocytogenes* from the liver of at least 50% of the dams. Fetuses from these dams were viable and not all had positive livers and/or brains despite maternal liver invasion. Interestingly, at all doses (10^4-10^8 *L. monocytogenes* CFU) and post treatment days (2, 6, 9 and 21), isolation from maternal spleens were similar to isolation from fetal tissues suggesting that invasion of the maternal spleen is indicative of fetal tissue invasion by *Listeria*. In contrast to the earlier post-treatment days (2, 6 and 9), in previous studies stillbirths occurred in 22% and 75% of dams exposed to 10^6 and10^8 CFU, respectively, that were scheduled for sacrifice on post-treatment day 21 *(24)*. These results indicate that it takes longer than 9 days for the disease to progress and kill the fetus.

All dams exposed to *L. monocytogenes* (regardless of dose) shed the pathogen at some point throughout the study. However those exposed to 10^6 or 10^8 CFU shed for the duration of the study. Interestingly, our current fecal shedding results did not predict fetal outcome as well as our previous primate *(21)* and guinea pig *(24)* studies. In those studies, pregnant rhesus monkeys and guinea pigs that delivered stillborn infants shed *L. monocytogenes* at higher rates and for longer periods of time compared to normal pregnancies. Williams et al. *(24)* reported that animals with greater than 60% positive fecal samples had pregnancies ending in stillbirths. In this study, there were no fetal deaths at the lowest dose of 10^4 CFU, yet *L. monocytogenes* was isolated from more than 60% of fecal samples from two, dams exposed to 10^4 CFU. These data suggest that fecal shedding alone may not be a good predictor of fetal outcome.

The use of green fluorescent protein was introduced approximately 14 years ago as a tool for studying the pathogenicity of *Salmonella typhimurium* and *Yersenia paratuberculosis (22)*. More recently, gfp has been applied in studies of various other bacteria including *Steptococcus suis* and *Listeria monocytogenes (1, 9, 16)*. The advantage of using fluorescent markers is that they allow direct visualization and real-time studies of tagged bacteria.

Utilizing fluorescent microscopy, in the current study we show that *L. monocytogenes* cells appeared in not only maternal tissues but placentas and fetal livers and brains as early as 2 days post treatment. Overall, neither method, microscopy nor culturing, was more sensitive in confirming the presence of *L. monocytogenes* in maternal and fetal tissues. Although in all but 4 cases, culturing confirmed the presence of *L. monocytogenes* in an equal or higher number of samples when compared to microscopy

(Figures 4.2A-4.2C). At the highest dose given, 10^8 *L. monocytogenes* CFU, microscopy was more sensitive than culturing in confirming presence of the pathogen in fetal liver and brain. Our data suggest that microscopy or culturing alone may underestimate the number of samples positive for *L. monocytogenes* present in tissues; however, combined they could be useful tools in confirming bacterial load and dissemination.

The guinea pig model was used as a surrogate for human listeriosis in this study. To date, guinea pigs have been used in multiple studies that assessed various effects of *L. monocytogenes* following an oral challenge *(1, 13, 14, 15, 18, 19, 23, 24)*. Despite scientific advances made as a result of the establishment of the guinea pig model, recently the guinea pig has been questioned as an animal model of choice for elucidating human listeriosis. The reluctance is based on molecular differences in guinea pig Met receptors compared to human Met receptors *(3)*. The Met receptor is believed to increase the ability of the pathogen to invade nonphagocytic cells including epithelial and endothelial cells and hepatocytes. Despite these molecular differences, we have shown that following maternal ingestion of *L. monocytogenes,* guinea pigs exhibit similar effects as human including maternal and fetal tissue invasion as well as delivery of nonviable fetuses *(13, 14, 23, 24)*. The dose-response curves developed using this guinea pig model predict an LD_{50} of approximately 10^7 *(23)* which is comparable to an LD_{50} of 10^6 that was based on a human outbreak involving pregnant women *(8)*. This data suggest that either the Met receptor is not essential for *L. monocytogenes* invasion, or that *L. monocytogenes* utilizes an alternate pathway for invasion of maternal and fetal tissues in the guinea pig. Some evidence for the latter has recently been published *(17)*.

More recently, gerbils have been proposed as an animal model to assess human listeriosis because their E-cadherin active site is the same as humans and their Met receptor contains key amino acids that are required for efficient interactions with the *L. monocytogenes* surface protein, InlB. Disson et al. *(6)* demonstrated that gerbils were susceptible to neonatal death following intravenous inoculation. However, there is no dose-response data on the effects of *L. monocytogenes* following oral inoculation in gerbils. Future studies are needed to evaluate whether gerbils are appropriate surrogates for human listeriosis.

In conclusion, we show that in pregnant guinea pigs *L. monocytogenes* crosses the fetoplacental barrier and invades the fetus as early as 2 days post-treatment. Additional studies are needed to determine if the pathogen invades fetal tissues earlier than 2 days post-treatment. The ability to determine the exact time course for *L. monocytogenes* invasion of maternal and fetal tissues may allow for therapeutic options or preventive strategies for adverse effects including death.

6. Acknowledgments

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Table 4.1 *Isolation of* **L. monocytogenes** *from Maternal and Fetal Tissues on Post-treatment days 2, 6, 9 and 21*

Post-treatment Day	Total # of Dams	Maternal Livers (% Positive[a])	Maternal Spleens (% Positive[a])	Average Placentas (% Positive[b])	Average Fetal Livers (% Positive[b])	Average Fetal Brains (% Positive[b])
Controls						
Day 2	2	0	0	0	0	0
Day 6	3	0	0	0	0	0
Day 9	3	0	0	0	0	0
Day 21	9	0	0	0	0	0
Dams treated with 10^4 *L. monocytogenes* CFU						
Day 2	5	80	40	48	48	48
Day 6	3	67	33	28	28	28
Day 9	2	50	50	59	59	59
Day 21	4	25	0	0	0	0
Dams treated with 10^6 *L. monocytogenes* CFU						
Day 2	3	67	67	28	28	0
Day 6	3	67	100	56	26	26
Day 9	3	100	67	67	50	50
Day 21	9	89	22	13	13	13
Dams treated with 10^8 *L. monocytogenes* CFU						
Day 2	4	100	75	49	17	13
Day 6	4	100	50	38	38	29
Day 9	4	75	50	38	31	31
Day 21	4	100	75	75	75	75

[a] Percent of guinea pigs with maternal tissues positive for *L. monocytogenes*

[b] Percentages (total positive/total number) of positive fetal tissue samples were calculated for each dam. These percentages were then averaged for dams for each post treatment day and dose.

Figure legends

Figure 4.1 Fecal shedding of* L. monocytogenes *by pregnant guinea pigs on pre- and post treatment days. For each post treatment day, groups sharing different uppercase letters are significantly different ($P \leq 0.05$). Fecal samples were collected from the following number of dams on each day: $10^0 \geq 9$, $10^4 \geq 4$, $10^6 \geq 9$ and $10^8 \geq 4$.

Figures 4.2A-4.2C Comparison of culture and microscopy data for maternal and fetal tissues following maternal exposure to 10^4 (4.2A), 10^6 (4.2B), and 10^8 (4.2C)* L. monocytogenes *CFU. For each maternal and fetal tissue, bars with different uppercase letters are significantly different when comparing microscopy and culturing ($P \leq 0.05$).

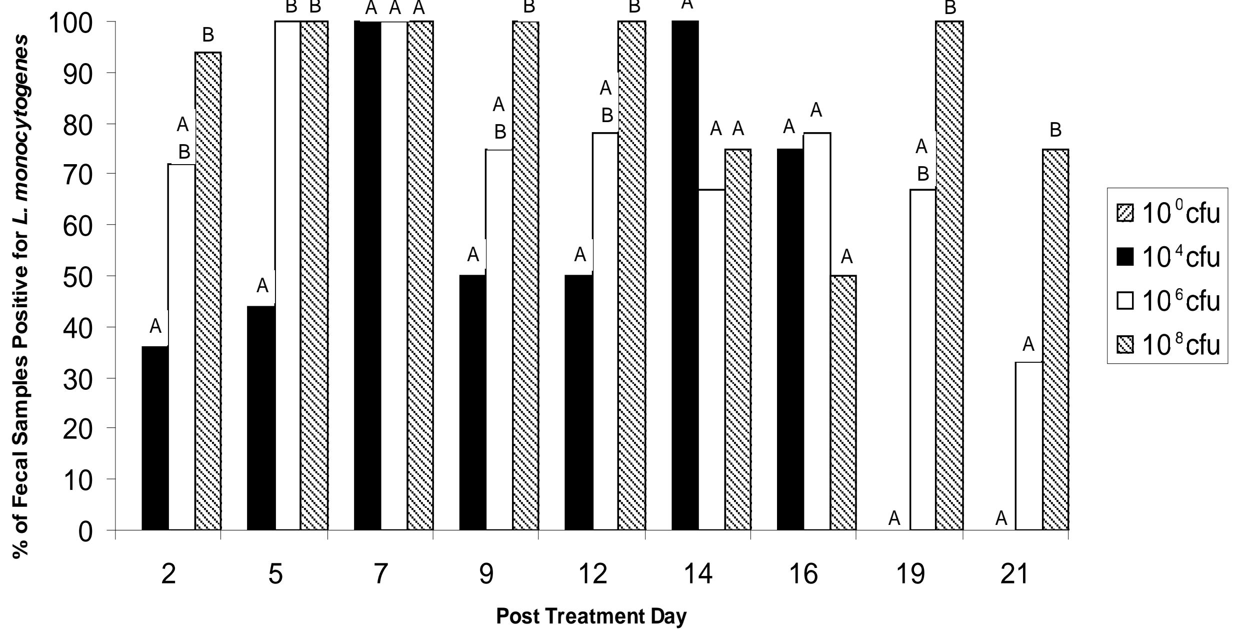

Figure 4.1

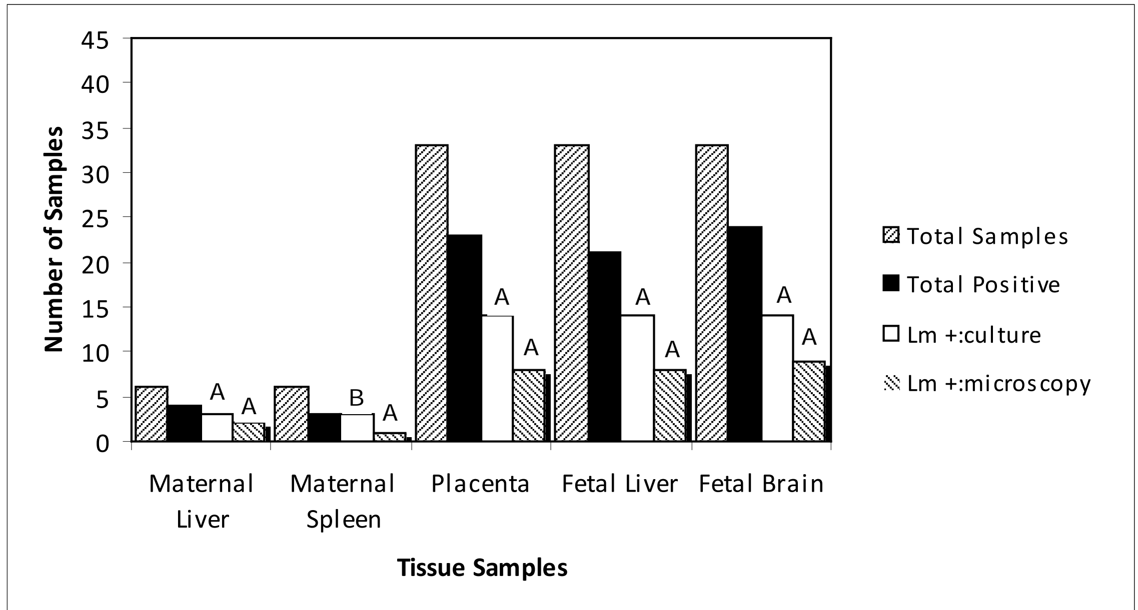

Figure 4.2A

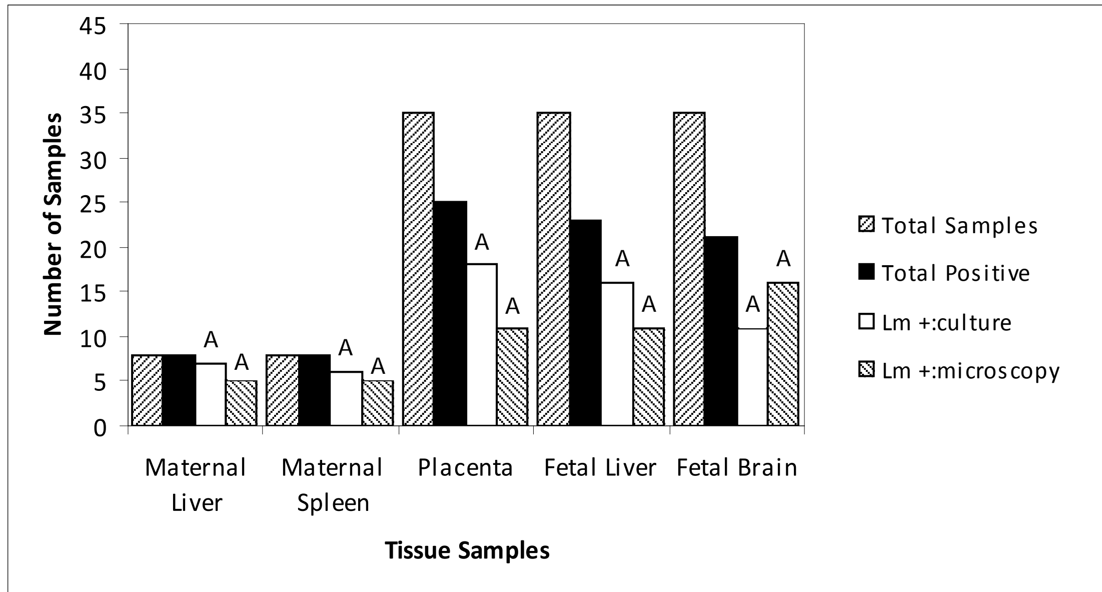

Figure 4.2 B

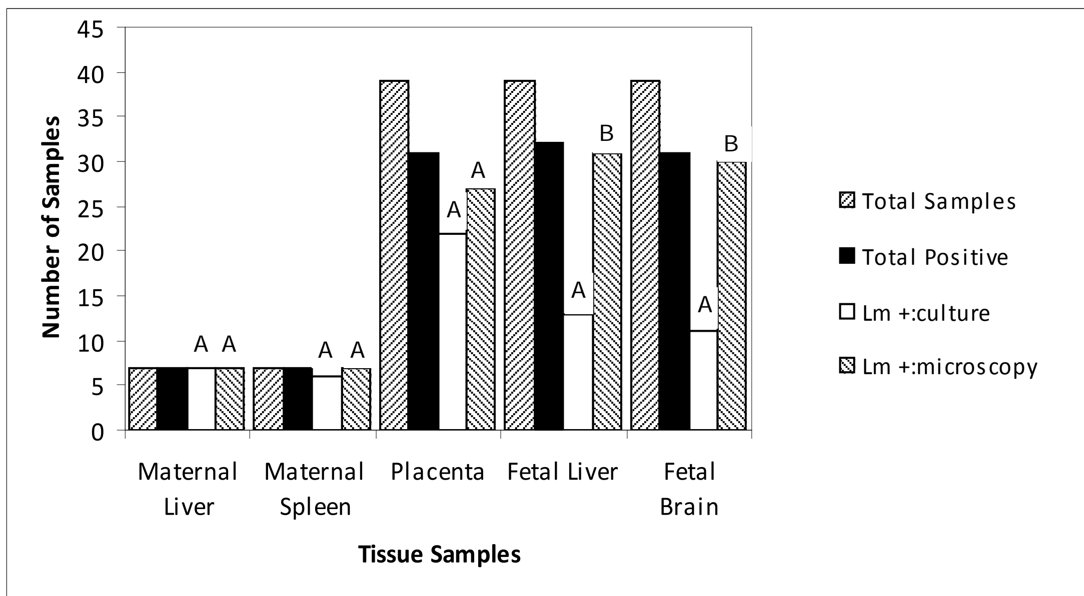

Figure 4.2C

CHAPTER 5

CHARACTERIZATION OF SUSCEPTIBILITEIS TO *LISTERIA MONOCYTOGENES* EXPOSURE IN PREGNANT AND NON-PREGNANT GUINEA PIGS[3]

[3] Williams, D., Richardson, A., Frank, J.F., and Smith, M.A. To be submitted to *Infection and Immunity*.

1. Abstract

Listeria monocytogenes has a predilection for the placenta. Placental invasion can lead to dissemination of *Listeria* to the fetus resulting in adverse effects or death. The objectives of the current study were to use guinea pigs to determine 1) whether gestation and dose impact the risk of tissue invasion with *L. monocytogenes,* fecal shedding and birth outcome and 2) whether maternal treatment with *L. monocytogenes* induces changes in select pro-inflammatory and anti-inflammatory cytokine mRNA expression in fetal liver and brain. *L. monocytogenes*, even in low numbers, can cross the intestine to invade internal organs when exposed on gestation day 35, as it was isolated from 29% and 14% of the dams' livers and spleens, respectively. Exposure during an earlier gestation (gd 22) resulted in maternal and fetal tissue invasion along with fetal death in which *L. monocytogenes* was isolated from all of the placentas, fetal livers and brains. Of the two dams with nonviable fetuses, *L. monocytogenes* was isolated from 100% and 33% of their fecal samples. Adult female guinea pig tissues can be invaded after *L. monocytogenes* exposure and the likelihood of invasion and fecal shedding are dose dependent. Of the dams that received 10^7 *L. monocytogenes* CFU on gd 35, all four cytokines, IL-5, IL-2, IFN-γ and TNF-α, were detected in fetal livers with IL-5 and TNF-α being significantly increased while IL-2 was significantly decreased when compared to fetal livers from controls. Of the fetal brains, IL-5, IL-2, IFN-γ and TNF-α, were detected at lower concentrations with IL-5 and TNF-α being significantly decreased.

2. Introduction

According to the Centers for Disease Control (CDC), *Listeria monocytogenes* (*L. monocytogenes*) is the fourth most common foodborne pathogen in the United States. However of these foodborne pathogens, *L. monocytogenes* has the highest case fatality rate with 20% of all its cases resulting in death. One-third of these cases are pregnancy-related as the fetus/neonate is 20 times more likely to develop listeriosis than the general population (15).

Following ingestion, the mother is usually asymptomatic which results in an infected fetus unexpectedly delivering prematurely with complexities such as meningitis or septicemia or even worse stillborn. Fetal effects are dependent on the point during gestation when exposed. Early onset or *in utero* exposures can result in miscarriages or pre-term deliveries of nonviable fetuses or complications such as encephalitis, meningitis or septicemia. Second- and third-trimester infections can result in premature delivery followed by neonatal illness or pre-term delivery of a stillborn (5, 6). Currently there are no published data that address the gestational effects of *L. monocytogenes* using an animal model.

To prevent fetal loss, pregnant women experience a shift in their normal pro-inflamatory:anti-inflamatory cytokine ratio. Without this shift, the paternal genes would provoke cell-mediated immune responses from the mother potentially leading to fetal loss. There is some evidence that anti-inflammatory cytokines are upregulated during pregnancy which results in suppressed cellular mediated immunity (20, 25). This suppression of cell mediated immunity allows intracellular pathogens such as *L. monocytogenes* to invade host cells and spend most of their existence in the host cell

intracellular environment which ultimately protects the pathogens from the host's humoral immune system. The mechanism by which *L. monocytogenes* causes stillbirths is unknown. Recently, a study addressed placental profiles of select cytokines following maternal ingestion of *L. monocytogenes* (7); however, there are no published data on cytokine expression in fetal liver and brain following a maternal exposure to *L. monocytogenes*.

The guinea pig model was used as a surrogate for human listeriosis in this study. To date, guinea pigs have been used in multiple studies that assessed various effects of *L. monocytogenes* following oral challenges (2, 7, 8, 9, 17, 21, 26, 27). *L. monocytogenes* efficiently interacts with guinea pig E-cadherin receptors (13) which enables the pathogen to cross the intestinal lining and gain entry to systemic circulation following an oral inoculation. Although studies have shown that guinea pigs are vulnerable to *L. monocytogenes* infection following ingestion, there is still a need to further characterize the susceptibilities of this animal model. The objectives of the current study are to use guinea pigs to determine 1) whether gestation and dose impact the risk of tissue invasion with *L. monocytogenes,* fecal shedding and birth outcome and 2) whether maternal treatment with *L. monocytogenes* induces changes in select pro-inflammatory and anti-inflammatory cytokine mRNA expression in fetal liver and brain.

3. Materials and Methods

3.1 Inoculum Preparation. Inoculum preparation was previously described in detail by Williams et al. (27). Briefly, three days prior to treatment *L. monocytogenes* strain 12443 was activated by three successive transfers in Tryptic Soy Broth (TSB) (BD Diagnostics, Sparks, MD). Cultures were then harvested by centrifugation (9,000 x g at 4°C for 30

min), washed twice and resuspended in sterile phosphate buffered saline (PBS) (0.01M). Cultures were diluted in PBS to give final concentrations of 10^2 to 10^8 colony forming units (CFU) in 1 ml and was added to 4 ml of sterilized whipping cream sweetened with Splenda®. The number of *L. monocytogenes* cells in the inoculated sample was determined by plating serial dilutions of the cell suspension onto Listeria Selective Agar (LSA) (Oxoid, Ogdenburg, NY). Sterile whipping cream containing PBS (1.0 ml) was administered to control animals.

3.2 Animals and Treatment. Guinea pig husbandry and treatment methods were previously described by Williams et al. (27). Briefly, timed-pregnant and adult, non-pregnant guinea pigs were obtained from Elm Hill Breeding Laboratories. Timed-pregnant guinea pigs arrived on gestation day (gd) 15 or 29. Animals were housed in individual cages with control and treated animals in separate rooms with 12 h: 12 h light/dark cycles. Rodent chow and drinking water were available *ad libitum* except for the day of treatment when food was removed for four hours prior to feeding *L. monocytogenes*. Guinea pigs were orally treated on gd 22 or 35 with doses ranging from 10^2 to 10^8 *L. monocytogenes* CFU per 5 ml of sterile whipping cream. Guinea pigs were observed daily for changes in activity, appearance or fecal output. Guinea pigs were sacrificed on gd 22 or 56, and maternal and fetal tissue samples were collected for further analysis. The normal gestation for guinea pigs is approximately 65 days.

3.3 Fecal and Tissue Collection and Fetal Viability. Fecal pellets were collected from each non-pregnant and pregnant guinea pig once prior to treatment with *L. monocytogenes* and at least 3 times per week following ingestion of *L. monocytogenes*. Following necropsy, tissues from pregnant and non-pregnant guinea pigs were sectioned

and samples from each tissue were transferred to a primary enrichment broth for culture analyses and liquid nitrogen and RNA*later*® (Sigma-Aldrich, St. Louis, MO) for PCR analyses. Tissues collected were: maternal blood, liver, and spleen, along with placenta and fetal liver and brain. Fetal viability was determined as described previously by Williams et al. (27).

3.4 RNA Extraction. RNA was extracted using an RNeasy® Mini Kit (Qiagen Inc., Valencia, California) according to the manufacturer's protocol for the purification of total RNA from animal tissues. Briefly, a 30 mg sample of fetal liver or brain was placed into a guanidine-thiocyanate-containing buffer allowing for RNAse inactivation and purification of intact RNA. Tissues were homogenized using a Sonic Dismembrator and added to a spin column. Following a series of washes, total RNA was eluted from the spin columns with 40µl of RNAse free water. The concentration of extracted RNA was quantified by measuring the absorbance at 260nm using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

3.5 Cytokine Analyses. Primers and probes used in this study were synthesized by Applied Biosystems (Foster City, CA). Specific primers and probes targeted: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), tumor necrosis factor (TNF)-α, interleukin (IL)-2, IL-5 (12) and interferon (IFN)-γ (10). Following RNA extractions, real-time RT-PCR was performed using a One Step QRT-PCR Kit (Verso™ One Step QRT-PCR kit, ABGene, Rochester, NY) and an Eppendorf Mastercycler EP Sequence Detection System (Eppendorf, Hamberg, Germany) following the manufacturer's protocol. The thermal cycling program was optimized in our laboratory based on the manufacturer's protocol, 46.4°C for 30 min, 95°C for 15 min followed with 40 cycles of

95°C for 30s, 56°C for 30s and 72°C for 30s for all targets. In each cytokine analysis, the threshold cycle (Ct) values from the gene of interest (IFN-γ, IL-2, TNF-α or IL-5) were normalized to the Ct values for the housekeeping gene, GAPDH. The $2^{-\Delta\Delta Ct}$ method of comparative analysis described by Livak and Schmittgen (14) was used to express the difference in cytokine mRNA expression in fetal liver and brain from treated animals compared to mRNA expression in fetal liver and brain from gestation day matched control animals.

3.6 *L. monocytogenes* Confirmation. Methodology used to confirm the presence of *L. monocytogenes* in fecal samples as well as maternal and fetal tissues was published in Williams et al. (27). We relied on USDA's qualitative methodology previously described by Cook (4). Briefly, fecal and tissue samples were enriched in nonselective and selective media followed by plating onto LSA. Selected isolated colonies were streaked onto Rapid L'mono plates (Bio-Rad, Hercules, CA) for confirmation as *L. monocytogenes*.

3.7 Statistical analyses. Statistical analyses were done using SAS version 9.1 (SAS Institute, Cary, N.C.) and Microsoft Excel (Microsoft Corporation, Redmond, W.A.). One-way analysis of variance (ANOVA) tests were done using Dunnett's t-tests to identify treatment groups that were significantly ($P \leq 0.05$) different from the control group. Tukey's tests and Excel *t*-Test were used to determine significant differences ($P \leq 0.05$) in values among all experimental groups. To determine significant alterations in cytokine mRNA expression, the Ct values from fetal liver and brain from treated animals were compared to the Ct values for fetal liver and brain from gestation day matched control animals using Tukey's test ($P \leq 0.05$).

4. Results

We examined the invasion of the pathogen in livers and spleens to characterize susceptibilities of pregnant and non-pregnant guinea pigs following exposure to 10^2 *L. monocytogenes* CFU. Using pregnant guinea pigs, we determined whether exposure at an earlier gestation (gd 22) affected birth outcome and tissue colonization compared to data gathered from exposures on gestation day 35 (27). Also, select cytokines were measured to determine whether a maternal exposure to *L. monocytogenes* resulted in changes in levels of pro-inflammatory and anti-inflammatory cytokines in fetal livers and brains. The cytokines analyzed in this study are present in fetal tissues throughout human gestation (3, 18, 19) and have been linked to adverse pregnancy outcomes when altered. In a mouse model of *Brucella*- induced spontaneous abortions, IFN-γ was significantly increased. This increase contributed to abortion (11). In pregnant mice, *L. monocytogenes* infection resulted in secretion of inflammatory cytokines in the placenta after intravenous exposure (1).

4.1 Isolation of *L. monocytogenes* from pregnant guinea pigs following ingestion of 10^2 CFU. Following an oral inoculation of 10^2 CFU, *L. monocytogenes* cells were isolated from 29% and 14% of the dams' livers and spleens, respectively (Table 5.1). Although these values were not statistically significant ($P = 0.086$ for liver and $P = 0.178$ for spleen) when compared to controls this is likely a result of the small sample size. It is also important to note that we have never isolated *L. monocytogenes* from control animals. There were no premature deliveries of nonviable fetuses following this low dose maternal exposure. *L. monocytogenes* cells could not be isolated from placentas, fetal livers or fetal brains from exposed dams. Dams exposed to 10^2 *L. monocytogenes*

CFU shed the pathogen throughout the study (Table 5.2). Fecal shedding in the treated animals (10%) was significantly higher ($P = 0.003$) than that of the control animals (0%) (Table 5.2). In four of the seven treated animals that shed *L. monocytogenes* in their feces also had tissue invasion. However, *L. monocytogenes* could not be isolated from maternal tissues of two animals that shed the pathogen in their feces. Also, *L. monocytogenes* invaded both the maternal liver and spleen in one dam, yet the pathogen was not isolated from any of the dam's fecal samples.

4.2 Invasion of tissues from pregnant guinea pigs exposed to 10^7 CFU on gd 22.

Exposing pregnant guinea pigs to *L. monocytogenes* at an earlier gestation day (gd 22) to a relatively high dose resulted in maternal and fetal tissue invasion as well as deliveries of nonviable fetuses. *L. monocytogenes* cells were isolated from 75% of maternal livers (Table 5.3). Those with infected livers also had *Listeria*-invaded spleens. Placenta, fetal liver and brain from dams in the experimental group had a significantly higher percentage of *Listeria*-invaded samples when compared to fetal tissues from controls. It must be noted that fetal samples were all obtained from one dam due to the high false pregnancy rate among the controls. Of the four treated guinea pigs, two prematurely delivered nonviable fetuses. Of the nine stillbirths, *L. monocytogenes* cells were isolated from all of the placentas, fetal livers and brains.

All treated guinea pigs shed *L. monocytogenes* at a significantly higher percentage when compared to control animals (Table 5.4). However the guinea pigs with nonviable fetuses shed at a higher percentage when compared to those with viable fetuses. Of the two that prematurely delivered stillborn fetuses, *L. monocytogenes* was isolated from 100% and 33% of their fecal samples. These results are consistent with previous guinea

pig (27) and nonhuman primate (24, 23) studies where dams delivering stillbirths shed *L. monocytogenes* at higher rates for longer periods of time.

4.3 Invasion of non-pregnant guinea pig tissues. Adult female (non-pregnant) guinea pigs were orally exposed to doses ranging from 10^2 to 10^8 *L. monocytogenes* CFU, and liver, spleen and gallbladder samples were analyzed for culturable *L. monocytogenes* cells. Of these treated non-pregnant guinea pigs, *L. monocytogenes* cells could not be cultured from tissues of those exposed to $\leq 10^4$ *L. monocytogenes* CFU (Table 5.5). *L. monocytogenes* cells were isolated from 40% and 75% of livers of those exposed to 10^6 and 10^8 CFU, respectively. Of the animals with infected livers, one from the 10^6 CFU treatment group and three from the 10^8 CFU treatment group had infected spleens. None of the non-pregnant animals had infected gallbladders at the time of sacrifice. A similar trend was observed in livers and spleens of animals exposed to 10^6 and 10^8 CFU where isolation of *L. monocytogenes* was significantly increased when compared to controls and animals treated with 10^2 or 10^4 CFU. Interestingly, in pregnant guinea pigs, *L. monocytogenes* could be isolated from tissues of those exposed to doses as low as 10^2 CFU suggesting that like humans, pregnant guinea pigs are more susceptible to infection from *L. monocytogenes* exposure than reproductive age non-pregnant females.

In non-pregnant animals, *L. monocytogenes* was isolated from feces of non-pregnant animals at all doses administered with 57% (Table 5.6). There was not a significant difference when comparing shedding from pregnant animals receiving 10^2 CFU to non-pregnant animals receiving the same dose.

4.4 Fetal livers and brains cytokine analyses. Fetal livers and brains were analyzed and compared with gestation-matched controls to determine if treatment with *L.*

monocytogenes disrupted mRNA expression in select inflammatory cytokines (IFN-γ, IL-2 and TNF-α) and an anti-inflammatory cytokine, IL-5 (Figures 5.1A and 5.1B). In the present study, we compared cytokine levels in fetal liver and fetal brain of dams exposed to 10^7 *L. monocytogenes* CFU on gd 35 to mRNA expression in 1) fetal tissues of dams exposed to 10^2 *L. monocytogenes* CFU on gd 35 and 2) fetal tissues of dams exposed to 10^7 *L. monocytogenes* CFU on gd 22. Of the fetal brains from dams exposed on gd 35 to 10^2 CFU, 2 cytokines, IL-2 and TNF-α, were detected; whereas, all cytokines were detected in fetal brains of dams exposed on gd 35 to 10^7 CFU (Figure 5.1B). Despite the dose given, these cytokines were all expressed at lower levels than that of the fetal brains of the controls.

A similar pattern was observed when analyzing fetal liver of dams exposed to 10^2 CFU on gd 35 where only 2 of the select cytokines, IL-2 and TNF-α were detected (Figure 5.1A). In fetal liver of dams exposed to 10^7 CFU on gd 35, IL-2 was the only cytokine detected at lower levels (0.46 fold) than controls. In contrast, TNF-α, IFN-γ and IL-5 were increased by 1.84, 1.597 and 2.632 fold, respectively, when compared to controls. Treatment with 10^7 *L. monocytogenes* CFU on gd 22 resulted in non-detectable amounts of IL-2, TNF-α, IFN-γ and IL-5 in fetal liver and brain.

5. Discussion

Previously we have shown that exposing pregnant guinea pigs during the second half of pregnancy to *L. monocytogenes* resulted in maternal and fetal tissue invasion and deliveries of stillborn fetuses or nonviable fetuses at the time of sacrifice (27, 9, 26). Previous studies using both pregnant guinea pigs and nonhuman primates have also addressed the correlation of fecal shedding of *L. monocytogenes* with maternal and fetal

tissue invasion as well as birth outcomes following maternal exposure to the pathogen (24, 27, 9, 23). Because listeriosis most commonly manifests in humans during the third trimester (16, 22) our previous research in nonhuman primates and guinea pigs involved exposures during the second half of pregnancy. In humans, cell-mediated immunity is the lowest during the third trimester (16, 22) which ultimately aids in the survival of the pathogen within the host cells while also increasing its ability to reach the fetus.

Using guinea pigs, we determined if the risk of fetal tissue invasion with *L. monocytogenes*, as well as the delivery of stillbirths or occurrence of nonviable fetuses changed with gestational age and dose. Also, we further characterized guinea pig susceptibility to infection with *L. monocytogenes* by assessing whether fecal shedding and tissue invasion increased/decreased in gestating animals when compared to non-gestating animals. We also assessed the impact of gestation and dose on select pro-inflammatory and anti-inflammatory cytokines found in fetal livers and brains following maternal ingestion.

In the current study, exposure of guinea pigs to the low dose of 10^2 *L. monocytogenes* CFU resulted in maternal tissue invasion; however, the pathogen was not isolated from placentas, fetal livers or brains (Table 5.1). In our previous guinea pig study we isolated *L. monocytogenes* from 25% of maternal livers and none of the maternal spleens following exposure to 10^4 CFU (27). Also there were no fetal deaths in dams exposed to $\leq 10^5$ CFU (27). That we could not isolate *L. monocytogenes* from the maternal spleen in the previous study may be attributed to the small sample size (n = 4). The biological significance of isolating *L. monocytogenes* from dams receiving 10^2 CFU suggests that *L. monocytogenes*, even in low numbers, can cross the intestine to invade

internal organs. The absence of *L. monocytogenes* in these fetal tissues suggests that the initial concentration of cells at the time of maternal ingestion may impact whether fetal tissues are invaded, and ultimately, birth outcomes.

It has been documented that the clinical manifestation of listeriosis in the fetus appears to be dependent on the point of gestation when exposure occurs. Second and third trimester exposures can result in premature delivery that is followed by neonatal illness or preterm delivery of a stillborn fetus (5, 6). First trimester exposures generally lead to spontaneous abortions (5, 6). The present research focused on further characterizing the susceptibility of the guinea pig to listeriosis by assessing how maternal exposure to a relatively high dose of *L. monocytognes* (10^7 CFU) during an earlier gestation (gd 22) impacts maternal and fetal tissue invasion as well as birth outcome. Upon completion of this study, we found that maternal liver and spleen invasion in dams exposed to 10^7 CFU on gd 22 was not significantly different from dams exposed to 10^7 CFU on gd 35 (27). However when comparing placentas, fetal livers and brains from dams exposed to 10^7 *L. monocytogenes* CFU on either gd 22 or 35 (27) a different trend was observed. Isolation of *L. monocytogenes* from placentas and fetal brains from dams exposed to the pathogen on gd 22 was significantly higher than isolation from those exposed on gd 35. However, isolation of the pathogen from fetal livers of dams exposed to 10^7 *L. monocytogenes* CFU on either gd 22 or 35 was not significantly different. Dams exposed on gd 22 (50%) and gd 35 (33%) (2) prematurely delivered stillborn fetuses; although there was not a significant difference in the occurrence of nonviable fetuses among these experimental groups. Data gathered from these experimental groups suggest

that maternal tissue invasion may not be dependent on gestation. Interestingly, in guinea pigs, early gestation exposure appeared to increase fetal susceptibility to *Listeria*.

It is estimated that healthy individuals are exposed to *L. monocytogenes* approximately five times per year, yet they rarely become seriously ill (15). Pregnant women are twenty times more likely to develop listeriosis compared to the general public (13). Using guinea pigs we assessed how risk of infection with *L. monocytogenes* increased/decreased with pregnancy by comparing tissue invasion in both pregnant and non-pregnant animals. Data showing isolation of *L. monocytogenes* from pregnant animals exposed to $\geq 10^4$ CFU were obtained from a previous guinea pig study (27). Our results indicate that pregnancy does not increase susceptibility of the adult guinea pig to *L. monocytogenes* infection as there were no statistical differences when comparing isolation in pregnant animals to non-pregnant animals dosed with 10^2 to 10^8 CFU (Tables 5.1 and 5.5; Williams et al., 2007). However, the presence of the placenta and fetus make the pregnancy vulnerable to adverse effects. This is somewhat supported by human literature because the adverse effects occur in fetuses with no long-term adverse effects to the mother.

Results of investigations of cytokine mRNA expression in fetal livers and brains of guinea pigs exposed to *L. monocytogenes* have not been previously reported. However a previous study conducted by our laboratory assessed mRNA expression in placentas following exposure to *L. monocytogenes* in pregnant guinea pigs (7). In this study we found that by gd 55, IFN-γ (a pro-inflammatory cytokine) was significantly increased, however there were no significant changes in IL-5 (an anti-inflammatory cytokine) concentrations. The increase in IFN-γ resulted in a shift from a favorable condition to

one that has been associated with premature delivery. To further elucidate the pregnant guinea pig cytokine profile fetal livers and brains from dams exposed to 1) 10^2 *L. monocytogenes* CFU on gd 35 2) 10^7 *L. monocytogenes* CFU on gd 35 and 3) 10^7 *L. monocytogenes* CFU on gd 22 were analyzed to determine if treatment dose and gestational age affected cytokine mRNA expression following maternal ingestion.

In fetal livers from dams exposed to 10^2 *L. monocytogenes* CFU on gd 35, two of the four cytokines (IL-2 and TNF-α) were detected at significantly lower levels than fetal livers of gestation-matched controls. However, IL-5 and IFN- γ were not detected. Of the dams that received 10^7 *L. monocytogenes* CFU on gd 35, all four cytokines, IL-5, IL-2, IFN-γ and TNF-α, were detected in fetal livers with IL-5 and TNF-α being significantly increased while IL-2 was significantly decreased when compared to fetal livers from control animals. It appears that the low dose of 10^2 *L. monocytogenes* CFU affected cytokine concentrations differently when compared to the higher dose of 10^7 *L. monocytogenes* CFU. The low dose exposure (10^2 CFU) resulted in only two of the select cytokines being expressed and reductions in the concentrations of IL-2 and TNF-α, whereas an increase in dose from 10^2 to 10^7 CFU elicited secretion of all four selected cytokines.

The same trend was observed in fetal brains where dams exposed to 10^2 *L. monocytogenes* CFU on gd 35, only expressed two of the four cytokines, IL-2 and TNF-α. Both were detected at lower levels when compared to fetal livers of gestation-matched controls. Of the dams that received 10^7 *L. monocytogenes* CFU on gd 35, all four cytokines, IL-5, IL-2, IFN-γ and TNF-α, were detected in fetal brains at lower concentrations with IL-5 and TNF-α being significantly decreased. It appears that the

low dose of 10^2 *L. monocytogenes* CFU affected cytokine concentrations similarly when compared to the higher dose of 10^7 *L. monocytogenes* CFU. Despite the dose, all select cytokines were expressed at lower levels in fetal brain of experimental groups when compared to fetal brains from controls.

In this study, gestation also appeared to affect the levels of select cytokines. IL-5, IL-2, IFN-γ or TNF-α was not detectable in fetal livers or brains from dams exposed to 10^7 *L. monocytogenes* CFU on gd 22. However as seen in Figures 5.1A and 5.1B dams exposed to *L. monocytogenes* on gd 35 resulted in cytokine expression in both fetal liver and brain. A previous study conducted in our lab (7) showed that IL-5, IL-2, IFN-γ and TNF-α were detected in placentas of dams exposed to *L. monocytogenes* on gd 35. Our inability to detect IL-5, IL-2, IFN-γ and TNF-α in any of the fetal livers and brains of the experimental or control animals suggests that they may not be expressed at such an early gestation.

In conclusion, we show that dose of *L. monocytogenes*, gestational age at time of exposure to *L. monocytogenes* as well as pregnancy status of the host all affect the susceptibility of pregnant guinea pigs to infection with the pathogen. These results add to the growing body of knowledge that support the use of guinea pigs to elucidate effects associated with *L. monocytogenes* infection.

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Table 5.1 Isolation of* Listeria monocytogenes *from maternal and fetal tissues following maternal ingestion of 10^2 CFU

Dose *L. monocytogenes* (CFU)	Total Number of Dams	Maternal Livers Positive (%)	Maternal Spleens Positive (%)	Total Number of Fetuses	Placentas Positive (%)*	Fetal Livers Positive (%)*	Fetal Brains Positive (%)*
Control	9	0/9 (0%)^A	0/9 (0 %)^A	33	0/33 (0%)^A	0/33 (0%)^A	0/33 (0%)^A
10^2	7	2/7 (29%)^A	1/7 (14%)^A	37	0/37 (0%)^A	0/37 (0%)^A	0/37 (0%)^A

^{A} Groups with different uppercase letters are significantly different ($P \leq 0.05$).

* Denominators represent the total number of fetuses per treatment group.

Table 5.2 Isolation of* Listeria monocytogenes *from fecal samples of pregnant guinea pigs exposed to 10^2 CFU

Dose *L. monocytogenes* (CFU)	Total Number of Guinea pigs	Fecal Samples Positive (%)
Control	9	0/90 (0%)[A]
10^2	7	7/70 (10%)[B]

Groups with different uppercase letters are significantly different ($P \leq 0.05$).

***Table 5.3 Isolation of* Listeria monocytogenes *from fetal tissues of dams exposed orally at gestation day 22 to 10^7 CFU* L. monocytogenes**

Dose *L.monocytogenes* (CFU)	Total Number of Dams	Maternal Livers Positive (%)	Maternal Spleens Positive (%)	Total Number of Fetuses	Number of Nonviable Fetuses	Placenta Positive (%)*	Fetal Liver Positive (%)*
Controls	10	0/10 (0%)	0/10 (0%)	37	0/37 (0%)^A	0/37 (0%)^A	0/37 (0%)^A
10^7	4	3/4 (75%)	3/4 (75%)	16	9/16 (56%)^B	14/16 (88%)^B	10/16 (63%)^B

Groups with different uppercase letters are significantly different ($P \leq 0.05$).

* Denominators represent the total number of fetuses per treatment group.

Table 5.4 Isolation of* Listeria monocytogenes *from fecal samples of pregnant guinea pigs exposed orally on gestation day 22

Dose *L. monocytogenes* (CFU)	Total Number of Guinea Pigs	Fecal Samples Positive (%)
Control	10	0/112 (0%)[A]
10^7	4	13/38 (34%)[B]

Groups with different uppercase letters are significantly different ($P \leq 0.05$).

Table 5.5 ***Isolation of* Listeria monocytogenes *from non-pregnant guinea pig tissues following an oral challenge with doses ranging from 10^2 to10^8 CFU***

Dose *L. monocytogenes* (CFU)	Total Number of Guinea Pigs	Liver Positive (%)	Spleen Positive (%)
Control	14	0/14 (0%)^{A}	0/14 (0%)^{A}
10^2	5	0/5 (0%)^{A}	0/5 (0%)^{A}
10^4	3	0/3 (0%) ^{A}	0/3 (0%) ^{A}
10^6	5	2/5 (40%)^{A,B}	1/5 (20%)^{A,B}
10^8	8	6/8 (75%)^{B}	3/8 (38%)^{B}

Groups with different uppercase letters are significantly different ($P \leq 0.05$).

Table 5.6 ***Isolation of*** **Listeria monocytogenes** ***from fecal samples of non-pregnant guinea pigs following an oral challenge with doses ranging from 10^2 to 10^8 CFU***

Dose *L. monocytogenes* (CFU)	Total Number of Guinea Pigs	Fecal Samples Positive (%)
Control	14	0/116 (0%)^{A}
10^2	5	2/50 (4%)^{A}
10^4	3	6/22 (27%)^{B}
10^6	5	14/31 (45%)^{B,C}
10^8	8	25/44 (57%)^{B,C}

Groups with different uppercase letters are significantly different ($P \leq 0.05$).

Figure Legends

Figures 5.1A and 5.1B. Select inflammatory and anti-inflammatory cytokine mRNA expression in fetal liver and brain following maternal ingestion of 10^2 or* 10^7 L. monocytogenes *CFU on gestation day 35. Cytokine mRNA expression is reported as a fold-change relative to mRNA expression in fetal liver (A) and brain (B) of gestation day matched control animals. For all dams ≥ 2 fetal livers and brains per litter were analyzed. For control groups $n \geq 2$ dams, and for treated groups $n \geq 3$ dams. * denotes statistical difference ($P \leq 0.05$).

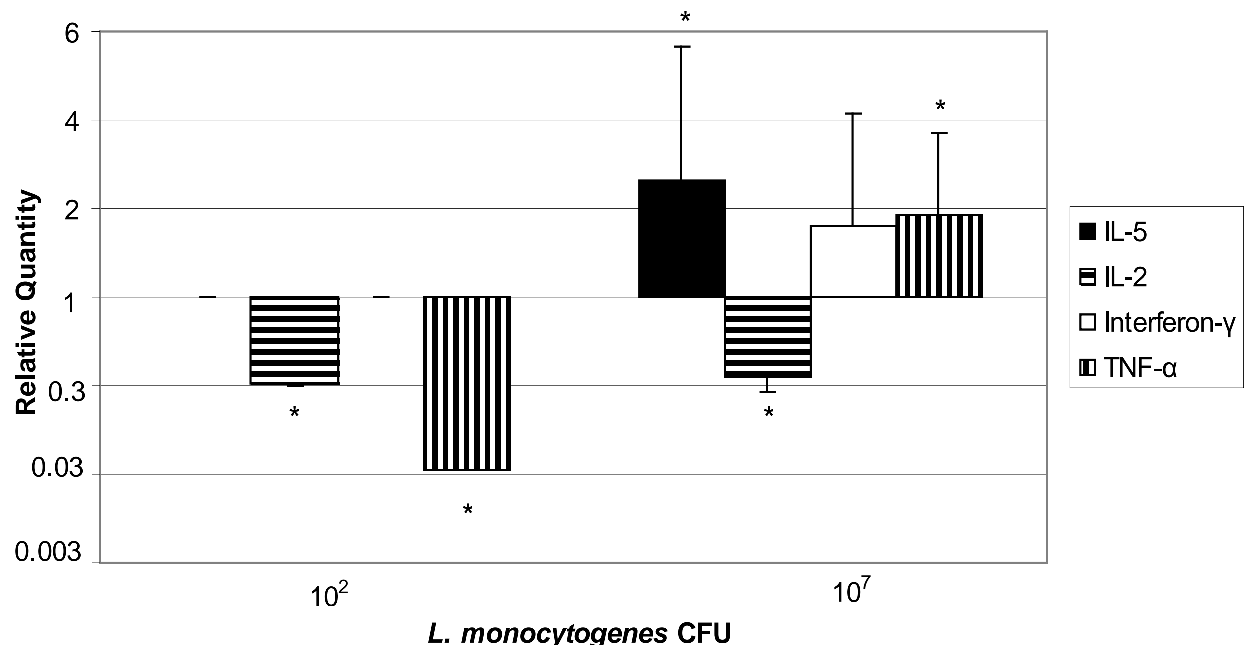

Figure 5.1A

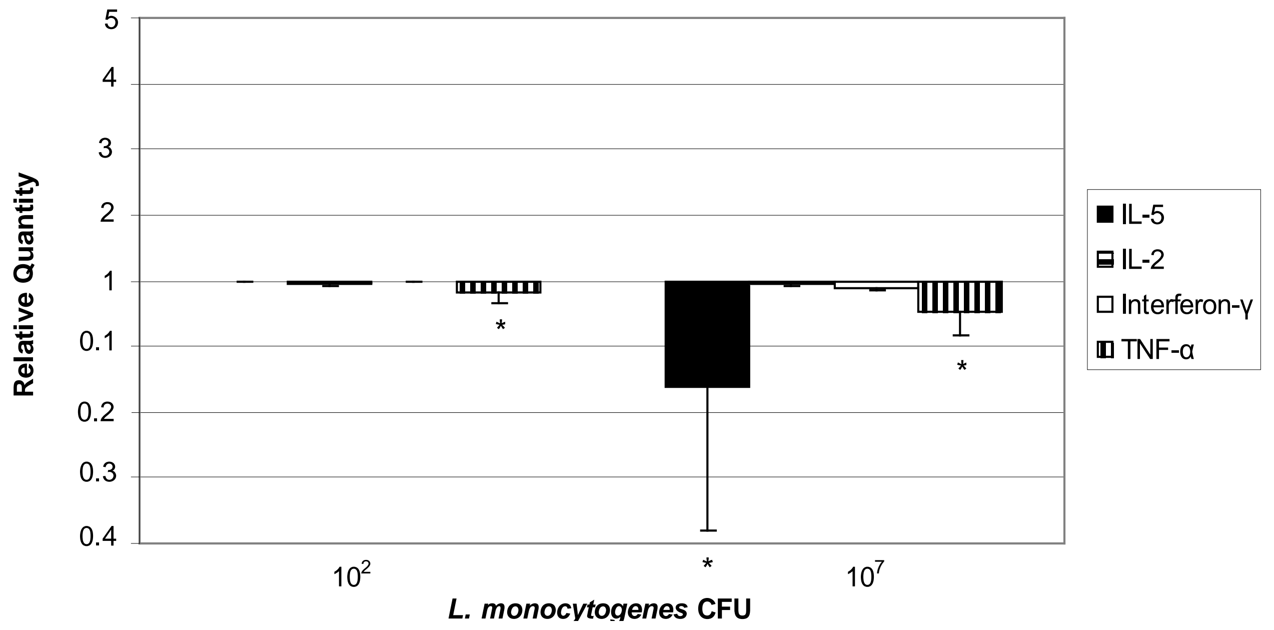

Figure 5.1B

CHAPTER 6

SUMMARY AND CONCLUSIONS

Listeria monocytogenes was first described in 1926 as a pathogenic species to guinea pigs and rabbits (Murray et al., 1926). *Listeria* was not considered a human pathogen until 1981 when it was directly linked to consumption of contaminated coleslaw (Schlech et al., 1983). To date, the prevalence of *L. monocytogenes* within the food supply continues to be a public health concern. Although rare among healthy individuals, listeriosis can lead to severe consequences, even death, in susceptible populations. Approximately 500 people die annually from listeriosis (Mead et al., 1999; Southwick et al., 1996). The disease primarily affects persons of advanced age, fetuses or newborns and adults with compromised immune systems. Pregnancy-related cases account for one-third of the total number of cases. The mechanisms of fetal invasion and death are unknown. The overall goal of our research is to better understand and predict how *L. monocytogenes* cause adverse effects in humans. To address this overall goal we completed the following: conducted a risk assessment of *L. monocytogenes* using dose-response data from nonhuman primate and guinea pig models and compared the results to published *L. monocytogenes* risk assessments, investigated the invasion of *L. monocytogenes* in maternal and fetal tissues at various time points following maternal ingestion using two different methods, culturing and microscopic analysis, investigated whether gestational age of the fetus impacts the risk of tissue invasion with *L. monocytogenes,* fecal shedding and birth outcome, determined whether exposure to a low

dose of 10^2 CFU *L. monocytogenes* could result in tissue invasion and adverse pregnancy outcome and determined whether maternal treatment with *L. monocytogenes* induces changes in select pro-inflammatory and anti-inflammatory cytokine mRNA expression in fetal liver and brain.

We revised the FDA/USDA/CDC (2003) risk assessment of *L. monocytogens* using dose-response data gathered from our guinea pig and nonhuman primate models. Based on non-human primate (Smith et al., 2003; Smith et al., 2008) and guinea pig data (Williams et al., 2007) the human LD_{50} values are 4.0 x 10^7 and 2.0 x 10^7, respectively. Both estimates are considerably lower than the estimated LD_{50} of 10^{13} CFU based on murine data (FDA/USDA/CDC, 2003). The high LD_{50} estimate from mice is more than likely attributed to the adjustment factors that were required since mice are not susceptible to listeriosis following ingestion. Interestingly, without adjustment factors, guinea pig and nonhuman primate LD_{50}s are comparable to the LD_{50} of 1.9 x 10^6 based on a human outbreak (FAO/WHO, 2004). These data suggest that guinea pigs and nonhuman primates more accurately assess risk of listeriosis in humans than mice.

Case reports of listeriosis indicate that the time from exposure to *L. monocytogenes* until stillbirth or birth of an infected infant can be several weeks. Little is known about the time course of invasion and whether an earlier identification of exposure and therapeutic intervention would result in more favorable outcomes for the fetus or infant. At all doses (10^4-10^8 *L. monocytogenes* CFU) and post treatment days (2, 6, 9 and 21), isolation from maternal spleens were similar to isolation from fetal tissues suggesting that invasion of the maternal spleen may be correlated with fetal tissue invasion by *Listeria*. There were no fetal deaths in dams before post-treatment day 9.

However, some stillbirths occurred before ptd 21 in the highest dose groups, 10^6 and 10^8 CFU. These results indicate that it takes longer than 9 days for the disease to progress and kill the fetus. Utilizing fluorescent microscopy, in the current study we show that *L. monocytogenes* cells appeared in not only maternal tissues but placentas and fetal livers and brains as early as 2 days post treatment. Overall, neither method, microscopy nor culturing, was more sensitive in confirming the presence of *L. monocytogenes* in maternal and fetal tissues. Although in all but 4 cases, culturing confirmed the presence of *L. monocytogenes* in an equal or higher number of samples when compared to microscopy. Our data suggest that microscopy or culturing alone may underestimate the number of samples positive for *L. monocytogenes* present in tissues; however, combined they could be useful tools in confirming bacterial load and dissemination.

Exposing pregnant guinea pigs to *L. monocytogenes* at an earlier gestation day (gd 22) to a relatively high dose resulted in maternal and fetal tissue invasion as well as deliveries of nonviable fetuses. We found that maternal liver and spleen invasion in dams exposed to 10^7 CFU on gd 22 was not significantly different from dams exposed to 10^7 CFU on gd 35 (Williams et al., 2007). However when comparing placentas, fetal livers and brains from dams exposed to 10^7 *L. monocytogenes* CFU on either gd 22 or 35 (Williams et al., 2007) a different trend was observed. Isolation of *L. monocytogenes* from placentas and fetal brains from dams exposed to the pathogen on gd 22 was significantly higher than isolation from those exposed on gd 35. However, isolation of the pathogen from fetal livers of dams exposed to 10^7 *L. monocytogenes* CFU on either gd 22 or 35 was not significantly different. Dams exposed on gd 22 (50%) and gd 35 (33%) (Williams et al., 2007) prematurely delivered stillborn fetuses; although there was

not a significant difference in the occurrence of nonviable fetuses among these two experimental groups. Guinea pigs with nonviable fetuses shed at a higher percentage when compared to those with viable fetuses. Data gathered from these experimental groups suggest that maternal tissue invasion may not be dependent on gestation. Interestingly, in guinea pigs, early gestation exposure appeared to increase fetal susceptibility to *Listeria*.

Following an oral inoculation of 10^2 CFU, *L. monocytogenes* cells were isolated from 29% and 14% of the dams' livers and spleens, respectively. There were no premature deliveries of nonviable fetuses following this low dose maternal exposure. The biological significance of isolating *L. monocytogenes* from dams receiving 10^2 CFU suggests that *L. monocytogenes*, even in low numbers, can cross the intestine to invade internal organs. The absence of *L. monocytogenes* in these fetal tissues suggests that the initial concentration of cells at the time of maternal ingestion may impact whether fetal tissues are invaded, and ultimately, birth outcomes.

In fetal livers from dams exposed to 10^2 *L. monocytogenes* CFU on gd 35, two of the four cytokines (IL-2 and TNF-α) were detected at significantly lower levels than fetal livers of gestation-matched controls. However, IL-5 and IFN- γ were not detected. Of the dams that received 10^7 *L. monocytogenes* CFU on gd 35, all four cytokines, IL-5, IL-2, IFN-γ and TNF-α, were detected in fetal livers with IL-5 and TNF-α being significantly increased while IL-2 was significantly decreased when compared to fetal livers from control animals. It appears that the low dose of 10^2 *L. monocytogenes* CFU affected cytokine concentrations differently when compared to the higher dose of 10^7 *L. monocytogenes* CFU. The low dose exposure (10^2 CFU) resulted in only two of the

select cytokines being expressed and reductions in the concentrations of IL-2 and TNF-α, whereas an increase in dose from 10^2 to 10^7 CFU elicited secretion of all four selected cytokines.

The same trend was observed in fetal brains where dams exposed to 10^2 *L. monocytogenes* CFU on gd 35, only expressed two of the four cytokines, IL-2 and TNF-α. Both were detected at lower levels when compared to fetal livers of gestation-matched controls. Of the dams that received 10^7 *L. monocytogenes* CFU on gd 35, all four cytokines, IL-5, IL-2, IFN-γ and TNF-α, were detected in fetal brains at lower concentrations with IL-5 and TNF-α being significantly decreased. It appears that the low dose of 10^2 *L. monocytogenes* CFU affected cytokine concentrations similarly when compared to the higher dose of 10^7 *L. monocytogenes* CFU. Despite the dose, all select cytokines were expressed at lower levels in fetal brain of experimental groups when compared to fetal brains from controls.

In this study, gestation also appeared to affect the levels of select cytokines. IL-5, IL-2, IFN-γ or TNF-α was not detectable in fetal livers or brains from dams exposed to 10^7 *L. monocytogenes* CFU on gd 22. However as seen in dams exposed to *L. monocytogenes* on gd 35 resulted in cytokine expression in both fetal liver and brain. A previous study conducted in our lab (Irving et al., 2008a) showed that IL-5, IL-2, IFN-γ and TNF-α were detected in placentas of dams exposed to *L. monocytogenes* on gd 35. Our inability to detect IL-5, IL-2, IFN-γ and TNF-α in any of the fetal livers and brains of the experimental or control animals suggests that they may not be expressed at such an early gestation or that there is a temporal window in which they are expressed.

Early diagnosis and antimicrobial treatment of listeriosis during pregnancy can result in the birth of a healthy infant (Kalstone, 1991). However, the lack of biomarkers or diagnostic tests results in fetal deaths for a treatable disease. The data from these studies will aid in the development of regulatory practices to alleviate pregnancy-related problems that result from ingesting foods contaminated with *L. monocytogenes* as well as in detecting infection. These data will also contribute to designing more effective strategies for preventing stillbirths and adverse fetal outcomes in women exposed to *L. monocytogenes* during pregnancy.