THE MONGOLIAN GERBIL AS A MODEL FOR HUMAN LISTERIOSIS: AN ANALYSIS AND COMPARISON OF DOSE-RESPONSE DATA

by

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(Under the Direction of Mary Alice Smith)

ABSTRACT

Listeria monocytogenes is a foodborne pathogen than can cause spontaneous abortion and serious illness in neonates. Dose-response data on invasion and adverse pregnancy outcomes in Mongolian gerbils after oral challenge with *L. monocytogenes* were collected and compared to other animal models of listeriosis. Invasion of maternal organs occurred in dams exposed to $\geq 10^7$ CFU *L. monocytogenes*, while fetal invasion occurred in dams exposed to $\geq 10^5$ CFU. Fetal death occurred only in the highest dose group (10^9 CFU). Using fetal data, an ID_{50} of 2.60×10^6 CFU was calculated, while the LD_{50} was estimated to lie between 5×10^6 and 5×10^8 CFU. These results indicate that the gerbil is not more sensitive to *L. monocytogenes* invasion and adverse outcomes than some of the other animal models of listeriosis, namely the guinea pig and nonhuman primate.

INDEX WORDS: *Listeria monocytogenes*, pregnancy, Mongolian gerbil, dose-response, animal models

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DEDICATION

To my parents, for encouraging my love of science, and to Taylor, Emma, and Luke, scientists in training.

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CHAPTER 1

INTRODUCTION

Listeria monocytogenes is a ubiquitous environmental pathogen responsible for approximately 1600 annual cases of listeriosis in the United States (CDC, 2013; Scallan et al., 2011). Listeriosis is contracted almost exclusively through the oral route, and refrigerated ready-to-eat foods are most often implicated in outbreaks (**Table 1.1**; Lamont et al., 2011). Most immunocompetent people will experience only mild gastrointestinal (e.g., diarrhea) or flu-like symptoms (e.g., muscle aches, fever) if they contract listeriosis (CDC, 2013), but more serious illness can occur in elderly, immunocompromised/immunosuppressed, and pregnant patients. Infection of the central nervous system (e.g., meningitis, encephalitis) and septicemia are two of the more severe outcomes of listerial infection in non-pregnant individuals (Goulet and Marchetti, 1996), while pregnant women exposed to the bacteria have a 20% chance of spontaneous abortion or miscarriage. Babies who survive to birth have a 68% chance of being born with the infection, with 25% of these neonates dying shortly after birth and another 13% experiencing some form of long-term complication such as septicemia, meningitis, or encephalitis (Mylonakis et al., 2002a). Overall, *L. monocytogenes* infection has a 15.9% case fatality rate, making it the third-leading cause of death from a foodborne pathogen in the United States (Scallan et al., 2011). The outcomes of several outbreaks of listeriosis from the past three decades are summarized in **Table 1.1**.

Table 1.1. Summary of several listeriosis outbreaks from 1980 to 2012

Year	Outbreak location	Source	No. of cases	Perinatal cases (%)	Mortality rate (%)	Reference
1980-1981	Maritime provinces (Canada)	Coleslaw	41	83	34	Schlech *et al.*, 1983
1983	New England (US)	Pasteurized milk	49	14	29	Flemming *et al.*, 1985
1985	California (US)	Mexican-style cheese	142	65	34	Linnan *et al.*, 1988
1992	France	Pork rilletes	38	82	32	Goulet *et al.*, 1998
1994	Illinois (US)[a]	Chocolate milk	45	--	--	Dalton *et al.*, 1997
1997	Italy[a]	Corn and tuna salad	1566	--	--	Aureli *et al.*, 2000
1998-1999	United States	Hot dogs, cold cuts	108	12	17	Mead *et al.*, 2006
2002	United States	Turkey deli meat	54	22	20	Gottlieb *et al.*, 2006
2005	Switzerland	Tomme cheese	10	20	50	Bille *et al.*, 2006
2008	Quebec (Canada)	Pasteurized cheese	38	42	5	Gaulin *et al.*, 2012
2008	Canada	Turkey deli meat	57	--	39	Weatherill, 2009
2011	United States	Cantaloupe	147	5	23	CDC, 2013
2012	United States	Ricotta cheese	22	5	14	CDC, 2013

[a] Outbreaks of febrile gastroenteritis with no deaths

Choosing an appropriate animal model is very important to toxicological studies, and key considerations for the study of listeriosis include practicality and similarity to human responses. For example, nonhuman primates are perhaps the best animal model in terms of responding to infection in the same manner as humans, but they are difficult to work with for practical, economical, and ethical reasons (Hoelzer et al., 2012). Rodent models do not suffer from these drawbacks to the same extent, but two of the more common ones, mice and guinea pigs, have

their own shortcomings in the form of differences in the receptor-virulence factor interactions that are considered necessary for successful invasion of *L. monocytogenes* in the human body. Specifically, the pathway that is most important for crossing of the intestinal barrier is not active in mice (Lecuit et al., 1999), while guinea pigs have a receptor that is incompatible with the protein responsible for entry into several other cell types, including hepatocytes (Khelef et al., 2006). Therefore, while most risk assessment literature relies on studies in mice and guinea pigs, there has been some speculation on the suitability of these animals. However, the Mongolian gerbil was recently proposed as a potential new small animal model, as it possesses both pathways considered to be important to human invasion (Disson et al., 2008). The objective of the work presented in this thesis was to orally expose pregnant gerbils to *L. monocytogenes*, investigate invasion and adverse pregnancy outcomes, and compare the resultant dose-response data to other animal models.

The literature review (Chapter 2) provides a detailed look at the implications of listerial infection, the interactions between various virulence factors and their targets within the human body, the current debate on how *L. monocytogenes* crosses the placenta, and the mechanisms underlying adverse fetal outcomes. Additionally, current animal models of listeriosis are compared and their weaknesses described.

The methodology for the main study reported in this thesis necessitated the use of timed-pregnant gerbils. However, these animals could not be readily purchased, and the literature was lacking in methods for timed-breeding, which lead us to develop our own method. Chapter 3 is therefore comprised of a manuscript describing in detail the timed-breeding technique developed and employed in our study.

Chapter 4 investigates the presence of *L. monocytogenes* in the feces, several adult organs, and pregnancy-associated tissues (i.e., fetus, placenta, resorption) of Mongolian gerbils after a single oral exposure to *L. monocytogenes*. Dose-response relationships among and between pregnant and non-pregnant gerbils are explored, and the ID_{50} for fetal invasion and LD_{50} for fetal death are estimated. These results are compared to the previously used nonhuman primate and guinea pig models, with emphasis on comparison to the pregnant guinea pig.

The final chapter of this thesis (Chapter 5) summarizes the findings and conclusions of the gerbil study. Its implications for future risk assessments are noted, and future directions of this research are described.

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CHAPTER 2

LITERATURE REVIEW

Introduction

Listeria monocytogenes, the causative agent of listeriosis, is a common environmental pathogen of special concern to immunocompromised, immunosuppressed, elderly, and pregnant individuals (CDC, 2013). *L. monocytogenes* was first described in rabbits in 1926 (Murray et al., 1926), and was identified as the cause of septicemia in a neonate and meningitis in an adult during the 1930s (Seeliger, 1988). However, despite further evidence of its potential adverse effects published in the 1950s, *L. monocytogenes* was an infrequently-identified cause of disease in humans and domestic animals such as sheep for several decades after its discovery (Seeliger, 1988). This classification changed in 1981 following an outbreak of listeriosis associated with coleslaw in Canada that involved 41 people, 83% of whom were pregnant (Schlech et al., 1983). An increase in recognized outbreaks involving food throughout the 1980s resulted in *L. monocytogenes* being taken seriously as a foodborne pathogen (Schlech et al., 1983). Most human exposures occur through the consumption of contaminated foods, notably because *L. monocytogenes* can grow at refrigeration temperatures (Walker et al., 1990). As a result, refrigerated ready-to-eat foods, deli meats, and soft cheeses are often implicated in outbreaks, as these foods typically do not undergo additional kill steps or processing (e.g., heating) before consumption (Lamont et al., 2011).

Though still only rarely of concern to the immunocompetent, *L. monocytogenes* is currently the third-leading cause of death from a foodborne agent in the United States, claiming

the lives of 15.9% of the approximately 1600 annual cases (Scallan et al., 2011). Additionally, the risk of stillbirth or premature delivery of a fetus, or serious illness in a neonate (e.g. septicemia, meningitis), greatly increases if its mother has been exposed (Mylonakis et al., 2002b), though she herself may experience only mild flu-like symptoms (Benshushan et al., 2002). Mechanisms by which *L. monocytogenes* adheres to and invades cells, escapes degradation by host defenses, replicates within cells, and moves between cells have been extensively studied (reviewed in Camejo et al., 2011), but the methods employed in crossing the placental barrier and inducing toxicity in the fetus are less well understood. This review will summarize the work that seeks to explain how *L. monocytogenes* crosses from mother to fetus, and why this pathogen can prove so deadly to the latter even in the absence of extreme symptoms in the former. Additionally, it will review the use of animal models in the study of listeriosis *in vivo* and the importance of establishing the most appropriate one.

Placental Development, Anatomy, and Physiology

A brief overview of placental development and physiology in humans is necessary to understand *L. monocytogenes* invasion. The onset of ovulation induces changes in the uterine endometrium that allow for implantation of the blastocyst should an egg be successfully fertilized (Stewart et al., 1992; Yoshinaga, 1988). When a blastocyst first attaches to the uterine lining, further changes are induced during the process of decidualisation to create an optimal environment for embryonic development (Aplin, 1991). The outermost layer of the blastocyst is comprised of cells called trophoblasts, which penetrate the epithelial cells of the decidua until they reach the underlying connective tissue of the stroma (Schlafke and Enders, 1975). These trophoblasts in turn undergo changes during the process of implantation, differentiating into an

outer multinucleated, aggregate cell layer termed the syncytiotrophoblast and an underlying layer of cytotrophoblasts that continue to proliferate and fuse to form the syncytiotrophoblast (Mayhew, 2001). Collectively, these two layers comprise a portion of the chorion, which, along with the somatic mesoderm, will eventually form the characteristic villi of the mature placenta (**Fig. 2.1A**). The trophoblasts degrade and absorb extracellular matrix as they continue to invade the decidua, eventually reaching maternal capillaries (Boyd and Hamilton, 1970). By day 9 post-fertilization, the blastocyst, surrounded by its cytotrophoblasts and syncytiotrophoblast shell, is completely embedded within the decidua (Aplin, 1991). Membrane-bound spaces now begin to appear within the syncytiotrophoblast. These so-called lacunae, initially surrounded by syncytiotrophoblast, fill with maternal blood as the decidual venous sinuses are invaded and form the initial interface for nutrient transfer from mother to fetus (Boyd and Hamilton, 1970; Enders, 1989). The lacunae grow in size and fuse with other lacunae to eventually form the intervillous space (**Fig. 2.1A**; Aplin, 1991).

Around day 13, the cytotrophoblasts begin to penetrate into the syncytiotrophoblast to form primary villi. By day 21, the inner non-trophoblastic layer of the chorion comprised of the embryonic mesoderm grows into the primary villi to form secondary villi. Once vasculogenesis (i.e., *de novo* production of fetal arteries and veins) begins within the secondary villi a few days later, they are termed tertiary villi; all subsequent villi are subclasses of tertiary villi (Kingdom et al., 2000). The tertiary villi protrude into the intervillous space and continue to branch, with each branch served by a tributary of umbilical artery and vein looping into a capillary plexus. Nutrients and oxygen from the maternal blood diffuse through the layers of syncytiotrophoblast, cytotrophoblasts, and fetal mesenchyme to reach the fetal veins, while wastes carried into the villi via fetal arteries are able to diffuse out into the intervillous space. This villous tree system

allows for the nourishment of the fetus while keeping maternal and fetal blood supplies separate (Crawford, 1956).

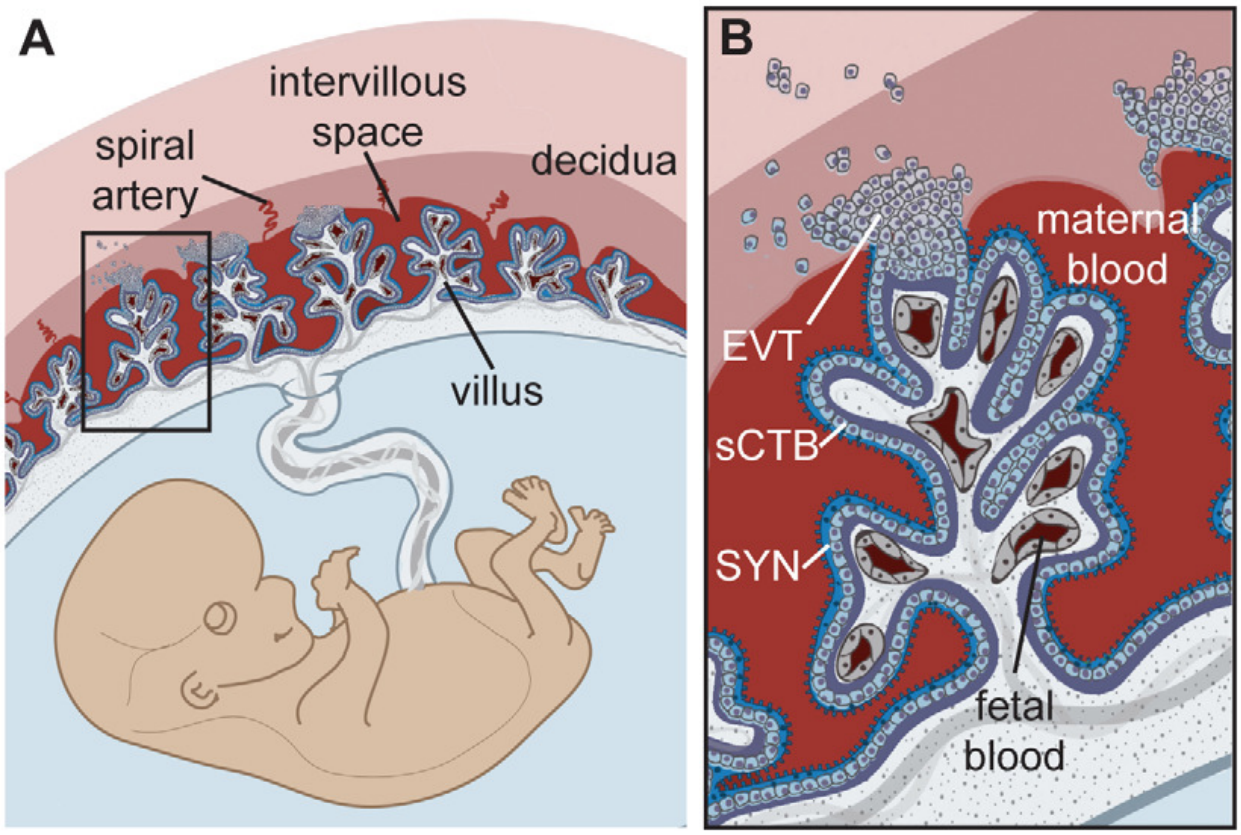

Figure 2.1. Human placental structure. (**A**) Orientation of the fetus and placental villi. Note the villous trees, some of which are "floating" in the intervillous space while others display extravillous trophoblasts (EVT) connecting to the decidua. (**B**) Enlarged box from **A** showing an anchoring villus. Anchoring villi display EVT, while floating villi do not. The villi are covered in a basement membrane (lavender), subsyncytial cytotrophoblasts (sCTB), and the syncytiotrophoblast (SYN) to protect the fetal stroma and circulation (Zeldovich et al., 2011).

The villi described above are often termed floating villi because they are not attached to the decidua, allowing them to "float" freely within the intervillous space (**Fig. 2.1A**). Alternatively, cytotrophoblasts may totally penetrate the syncytiotrophoblast during primary

villus formation and colonize the decidua to form anchoring villi (**Fig. 2.1B**). Villus formation proceeds as usual, but the cytotrophoblasts within the decidua provide a direct link to the interior of the villus in that they are not completely encased in syncytiotrophoblast as are floating villi (Aplin, 1991; Cross et al., 1994). These extravillous trophoblasts (EVT) may play a major role in *L. monocytogenes* placental invasion, and are discussed later.

Contrary to popular belief, the maternal immune system is not so much suppressed during pregnancy as it is modulated, thus learning to tolerate the allogenic fetus (Mor and Cardenas, 2010). Several studies have shown that the aid of cells comprising the mother's innate immunity is necessary for successful invasion of the trophoblast and establishment of the placenta. Likewise, the mother's immune system acts to support and protect the fetus, and the trophoblast itself can recognize pathogens and induce a maternal immune response through the release of cytokines, or protect itself from some pathogens by secreting anti-microbial peptides (reviewed in Mor and Cardenas, 2010). This is in addition to the physical barrier presented by the unique structure of the syncytiotrophoblast. Many pathogens exploit adherens junctions between such cells as intestinal or alveolar epithelium, but the aggregate structure of the syncytiotrophoblast appears to allow for no such access (Robbins and Bakardjiev, 2012; Zeldovich and Bakardjiev, 2012). The syncytiotrophoblast has thus shown resistance to several viral, protozoan, and bacterial pathogens (Robbins and Bakardjiev, 2012), though its interaction with *L. monocytogenes* is currently controversial.

Crossing Barriers: The Virulence Factors

While existing as a saprophyte within the environment, introduction of *L. monocytogenes* into the human body induces the upregulation of virulence factors that transform it into a

potentially deadly pathogen (Toledo-Arana et al., 2009). Despite immune responses and the challenges presented by the syncytiotrophoblast, *L. monocytogenes* is clearly capable of crossing the placental barrier, even if the mechanism by which it accomplishes this is not yet understood. Most research in the area has focused on E-cadherin and the Met receptor, the targets of the *L. monocytogenes* virulence factors InlA and InlB, respectively (Mengaud et al., 1996; Shen et al., 2000). The *inlA* gene was identified by Gaillard *et al.*, who showed that cloning this gene into the normally noninvasive *Listeria innocua* allowed this species to enter host epithelial cells (1991). This group also noted the existence of the *inlB* gene, and several years later demonstrated that InlB was necessary for hepatocyte invasion, though not for intestinal epithelium invasion (Dramsi et al., 1995). These two members of the internalin protein family were the first to be identified as means by which *L. monocytogenes* may enter cells.

The normal function of the transmembrane glycoprotein E-cadherin within the body is to act as an anchor within adherens junctions, and like all anchors, E-cadherin experiences forces and tensions that would cause cells to separate unless it can adapt to withstand these challenges (Smutny and Yap, 2010). It has been demonstrated that E-cadherin acts as a mechanosensor at cell-cell junctions. That is, it transmits forces between its extracellular portion and the F-actin of the cytoskeleton to which is it attached, and responds to increased tensions by causing the cell to stiffen. This cellular stiffening is accomplished through the recruitment of vanculin, a protein located at the ends of actin fibers that can connect to the E-cadherin (le Duc et al., 2010). The InlA of *L. monocytogenes* is able to bind to human E-cadherin and hijack its cytoskeleton reorganization properties by recruiting the α- and β-catenins, p120 catenin, ARHGAP10, and myosin VIIa. Actin polymerization and membrane extensions are induced (Lecuit et al., 2000), while a signaling cascade is triggered to cause caveolin-mediated clustering of E-cadherin. The

clusters are phosphorylated by Src kinase and then ubiquitinated by Hakai, which attracts clathrin to the entry site. The net effect of these processes is the internalization of the pathogen via endocytosis (Bonazzi et al., 2009; Bonazzi et al., 2008).

The interaction between the *L. monocytogenes* virulence factor InlB and host receptors is likewise considered very important to successful invasion. Though InlB has been shown to also interact with gC1qR and glycosaminoglycans (GAGs) (Braun et al., 2000), the receptor tyrosine kinase Met is accepted as the preferential signaling receptor for InlB. The normal function of Met is to act as a receptor for hepatocyte growth factor/scatter factor (HGF/SF), a ligand that encourages either mitosis in liver cells or migration of epithelial cells. The exact mechanism through which HGF/SF binds and is admitted into cells remains elusive, but it is believed that a dimerization is required for full activation, meaning that two HGF/SF ligands must bind to two Met complexes in order for HGF/SF to be admitted. This same arrangement may be necessary for *L. monocytogenes* entry via InlB interaction with Met (Basilico et al., 2008; Niemann, 2011), though other research offers evidence that InlB instead acts as a monomer in which the N-cap of the LRR region binds to Met and the GW regions bind to GAGs. As these regions comprise the two ends of InlB, the net effect is for the virulence protein to detach from the bacteria. The LRR region elicits bacterial entry, while the GW region acts almost as a hormone to induce signaling pathways that will activate the receptor (Banerjee et al., 2004). Once Met is activated, it undergoes autophosphorylation, which attracts various adaptor proteins. These proteins activate P13-kinase to start a cascade that culminates in actin rearrangement and internalization of the pathogen via clathrin-coated endocytosis (Pizarro-Cerdá et al., 2010; Veiga and Cossart, 2005).

Though the InlA/E-cadherin and InlB/Met interactions receive the most attention in the literature, ActA is another important virulence factor involved in placental invasion. ActA has

been shown to contribute, at least in part, to the successful invasion of epithelial cells. Cloning this gene into *L. innocua* rendered the normally noninvasive species capable of invading epithelial cells to a certain extent. However, this internalization could not be accomplished in fibroblasts or hepatocytes, suggesting that ActA uses a pathway unique to epithelium (Suárez et al., 2001). It is thought that ActA helps *L. monocytogenes* adhere to and enter cells through the recognition of its heparan sulfate binding site by host cell heparan sulfate proteoglycans (Alvarez-Dominguez et al., 1997). However, it is the motility and cell-to-cell spreading that ActA confers upon *L. monocytogenes* that is generally of most interest when considering this virulence factor. Actin polymerization is an important product of many *L. monocytogenes* virulence factor mechanisms, but only ActA allows these filaments to be gathered at one pole, forming a tail that allows propulsion through host cytosol and into neighboring cells (Kocks et al., 1995). ActA can directly activate the host Arp2/3 complex that nucleates actin, thus beginning the process of actin polymerization. It does this by mimicking the interactions of the complex's normal activator, the WASp family proteins. The N-terminal of ActA is structurally and chemically very similar to WASp proteins, allowing it to be mistaken for the proper activator (Boujemaa-Paterski et al., 2001). The central domain of ActA recruits Ena/VASP proteins that serve to (1) increase bacterial speed by decreasing the amount of branching the actin filaments undergo and (2) confer persistent directionality, though exactly how this is done is unclear. Both of these are necessary for the successful invasion of neighboring cells (Auerbuch et al., 2003; Samarin et al., 2003). The propulsion of *L. monocytogenes* into the cell membrane creates a protrusion into the neighboring cell, which will pinch off into a vacuole with a double membrane (Tilney and Portnoy, 1989). Furthermore, ActA has been recently shown to aid *L. monocytogenes* in the formation of biofilms and aggregates within the cecum and colon of mice,

contributing to the pathogen's persistence in the host and transmission back into the environment (Travier et al., 2013).

These three virulence factors all require the formation of a vacuole for entry into cells, which necessitates the ability to escape from said vacuole. To this end, *L. monocytogenes* possesses the pore-forming toxin listeriolysin O (LLO). Though the exact mechanism through which LLO works remains unknown, the prevailing theories involve the binding of pore-forming complexes to phagosome membranes, resulting in membrane dissolution by the membrane-rupturing phospholipases C (Schnupf and Portnoy, 2007). LLO activity is greatest at the acidic pH of phagosomes, but is mostly inactive at the neutral pH of the cytoplasm, presumably to prevent excessive host cell damage (Beauregard et al., 1997). Some evidence also suggests that LLO is involved in causing transient network fragmentation in host cell mitochondria during the early stages of invasion, to the effect of temporarily disrupting host cell functions (Stavru et al., 2011). Additionally, LLO has been shown to degrade Ubc9, an enzyme essential to the SUMO pathway, both *in vitro* and *in vivo*; this interferes with the post-translational processing of several host cell proteins and allows for persistence of the infection (Ribet et al., 2010).

Special Delivery: How *L. monocytogenes* makes its Way to the Placental Interface

While cell-to-cell spread is important in the pathogenesis of *L. monocytogenes*, it is insufficient to fully explain how the bacteria find their way to such diverse areas of the body in relatively short periods of time. The answer can be found in a hematogenous route of dissemination comprised of macrophage-associated bacteria and occasionally free bacteria in the bloodstream. The hematogenous route was demonstrated in a study utilizing nonpregnant guinea pigs where *L. monocytogenes* could be found in the liver just 4 hours after oral inoculation,

presumably having been delivered there directly from the intestine by the hepatic portal vein (Melton-Witt et al., 2012).

Occasionally, *L. monocytogenes* will forego its intracellular existence for the bloodstream. Bacteremia is observed in about half (48%) of nonperinatal listeriosis cases worldwide, and a study focusing on cases in Israel found 55% of pregnant women who had stillbirths were bacteremic (Siegman-Igra et al., 2002). In these cases where the pathogen has become extracellular, antibodies against *L. monocytogenes* may be produced (Berche et al., 1990; Bhunia, 1997), but humoral immunity is generally, by itself, ineffective against any bacteria contained within cells. The host's defenses at this point are therefore dependent on cell-mediated immunity, including macrophages (Mackaness, 1969). *L. monocytogenes* does not have to work to invade macrophages, relying simply on the cells' innate function of phagocytosis for internalization (Drevets, 1999). While many bacteria will perish in phagosomes, LLO allows some to escape into the macrophage's cytoplasm (Schnupf and Portnoy, 2007), where it can then spread to any cell the macrophage comes into contact with via the actin polymerization and propulsion conferred by ActA (Tilney and Portnoy, 1989). *L. monocytogenes* may therefore rapidly disperse to distant areas of the body via the highway of the bloodstream, either as free bacteria in cases of bacteremia and/or contained within the cytosol of phagocytes (Drevets, 1999). In this manner, *L. monocytogenes* may be delivered to the intervillous space bathing the placenta in maternal blood (Bakardjiev et al., 2005; Lecuit et al., 2004).

It is worth mentioning that colony-stimulating factor 1 (CSF-1), a glycoprotein essential to the proliferation and differentiation of mononuclear phagocytes (Stanley et al., 1983), may play an important role in how macrophages interact with *L. monocytogenes*. Mouse macrophages have been shown to dramatically increase their phagocytic behavior in response to CSF-1

stimulation without a concurrent increase in bactericidal activity (Cheers et al., 1989). CSF-1 is produced in large amounts at the maternal-fetal interface, implying an important role for CSF-1 in placental development (Pollard et al., 1987). Taken together, an influx of macrophages to the placenta that are prone to phagocytizing bacteria without destroying them may allow for more effective delivery of viable *L. monocytogenes* to the placenta.

Crossing the Placental Barrier: The Trophoblast Challenge

When *L. monocytogenes* reaches the placenta, it faces the challenge of crossing yet another protective barrier. The InlA/E-cadherin interaction has long been deemed to be extremely important when it comes to *L. monocytogenes* effectively and efficiently crossing the intestinal barrier, where it interacts with the luminally-accessible E-cadherins around goblet cells and within villus epithelial folds, as well as with the E-cadherin made temporarily accessible during enterocyte extrusion (Nikitas et al., 2011). This is evidenced by the difficulty of the mouse, which has an E-cadherin incompatible with InlA, to acquire the infection through the oral route (Lecuit, 2007), though interestingly this difficulty can be partially overcome if the mice are anesthetized prior to intragastric inoculation (Czuprynski et al., 2003). However, there is conflicting evidence on this virulence factor's importance in crossing the placental barrier. It should not be surprising that the vast majority of human clinical isolates of *L. monocytogenes* express a full-length InlA when compared to food isolates (96% vs. 65%), given that InlA is so important in allowing the pathogen access to the body (Jacquet et al., 2004). However, while some isolates from cases of listeriosis in patients with meningitis or bacteremia failed to display the full-length InlA, every isolate from cases in pregnant women displayed the full virulence

factor (Jacquet et al., 2004). These observations lead some researchers to investigate how InlA may be involved in crossing the placenta.

Experiments examining human placentas found that E-cadherin is strongly expressed on the surface of cytotrophoblasts, readily allowing invasion and cell-to-cell spread within this layer of the placenta (Lecuit et al., 2004; Robbins et al., 2010). However, its expression on the more relevant syncytiotrophoblast is open to debate. One group found E-cadherin to also be expressed on the apical surface of the syncytiotrophoblast, albeit not as strongly as on the cytotrophoblasts (Lecuit et al., 2004), while another could find no such evidence of E-cadherin expression on the syncytiotrophoblast (Robbins et al., 2010).

Lecuit *et al.* performed several experiments using full-term placental villous explants, cells derived from these full-term placentas, and differentiated BeWo cells (a cell line derived from a malignant choriocarcinoma) in which they stained E-cadherin and observed *L. monocytogenes* crossing the syncytiotrophoblast from the maternal blood space using these stained E-cadherins (2004). However, E-cadherins at this location appear to have no real physiological function. That is, the apical surface is not anchored to another cell, so there is no need of E-cadherin at this location. This group therefore hypothesize that these E-cadherins are remnants from the differentiation of cytotrophoblasts into the syncytiotrophoblast, and that any *L. monocytogenes* that has made its way into the maternal blood space may interact with the E-cadherins of the syncytiotrophoblast to gain admittance to the underlying cytotrophoblasts (Lecuit et al., 2004).

Conversely, Robbins *et al.* could find no evidence of E-cadherin on the syncytiotrophoblast using first trimester placental explants (2010). This group's experiments showed very little invasion of the syncytiotrophoblast, with *L. monocytogenes* instead focusing

its efforts on the more receptive extravillous trophoblasts (EVT). Unlike the floating villi that are totally encased in syncytiotrophoblast and bathed in maternal blood, villi with EVT are directly connected to the maternal decidua, and have been shown to strongly express E-cadherin (Robbins et al., 2010). In placental explants, 75 – 100% of anchoring villi were infected with *L. monocytogenes* after 72 hours, spreading to the subsyncytial cytotrophoblasts, floating villi, and fetal stroma during this time; the syncytiotrophoblast, however, remained largely uninfected (Robbins et al., 2010). This group therefore holds that while InlA is indeed important to placental invasion, invasion occurs through its interaction with E-cadherin of the EVT while the syncytiotrophoblast resists infection. *L. monocytogenes* is delivered to the intervillous space, and from there invades the maternal decidua, engaging in cell-to-cell spread until it reaches the EVT (Robbins et al., 2010). However, this group later found that the EVT are not totally permissive. Approximately 80% of invading bacteria were unable to escape from EVT vacuoles due to impaired function of LLO in this cell type, and were degraded within 24 hours (Zeldovich et al., 2011). The conclusion drawn from these findings suggest that EVT are preferentially colonized but also serve as a bottleneck (Zeldovich et al., 2011).

Other research implicates InlB located on the syncytiotrophoblast working in concert with InlA as being necessary to *L. monocytogenes* invasion of the placenta, at least in species that are susceptible to both of these pathways (Disson et al., 2008). This was demonstrated both in gerbils and in mice expressing human E-cadherin. *L. monocytogenes* mutants lacking InlA, InlB, or both were severely compromised in their ability to cross the placenta and thereby infect the fetus (Disson et al., 2008).

The focus on entry via InlA, InlB, or the conjugated action of the two has perhaps overshadowed two important aspects regarding fetoplacental invasion: (1) *L. monocytogenes*

mutants lacking one or both of these virulence factors could still be found in the fetus, though in far fewer numbers than their wild type counterparts (Disson et al., 2008; Robbins et al., 2010), and (2) species that are non-permissive to one of the pathways (i.e., mice to InlA and guinea pigs to InlB) are nonetheless susceptible to *L. monocytogenes*-induced stillbirths. In fact, though mice are susceptible to the InlB pathway and guinea pigs to the InlA, neither of these pathways are utilized in fetoplacental invasion in their respective species (Bakardjiev et al., 2004; Le Monnier et al., 2007). Even accounting for invasion enabled by ActA does not offer a full explanation. In mice, ActA is heavily involved in placental and fetal invasion; however, ActA mutants injected into mice were still able to somewhat invade the placenta and fetus despite the absence of both InlA and InlB pathways (Le Monnier et al., 2007). Placental invasion in guinea pigs was unaffected by the deletion of ActA, though successful fetal invasion was inhibited in mutants (Bakardjiev et al., 2005). These observations would indicate that at least one as-yet-unidentified invasion pathway is at work. Thus, a better understanding of vertical transmission apart from InlA, InlB, and ActA is needed if fetoplacental invasion is to be fully understood.

The Gift that Keeps on Giving: What Happens after Successful Placental Invasion

Regardless of how it accomplishes the crossing, there is evidence that the placenta acts as a sort of reservoir for *L. monocytogenes* once it is successfully invaded, a place conducive to the pathogen's growth that also serves to re-seed maternal organs (Bakardjiev et al., 2006). Intravenous inoculation of guinea pigs revealed that maternal organs were originally seeded with 10^3 – 10^4 bacteria for every 1 that managed to seed the placenta, but that this ratio rapidly equalized over the next 24 hours due to replication within the placenta. Furthermore, dams with infected placentas were unable to clear *L. monocytogenes* from their organs and showed

bacteremia by 72 hours post-injection (Bakardjiev et al., 2006). In contrast, non-pregnant guinea pigs were able to clear *L. monocytogenes* from their spleens and had no detection of bacteremia, and dams without infected placentas behaved in much the same fashion (Bakardjiev et al., 2006). Further mathematical modeling demonstrated that this response would be expected if the bacteria were being trafficked from the placenta back to the maternal organs. This re-seeding phenomenon has therefore been proposed as a contributing factor to the increased susceptibility of pregnant women to listeriosis (Bakardjiev et al., 2006).

L. monocytogenes exerts fetal toxicity in several ways. Infection of the placenta can have serious implications, and could be what triggers a preterm delivery, miscarriage, or stillbirth. Inflammatory and anti-inflammatory cytokines are tightly regulated during pregnancy, but placental infection disrupts their regulation (Keelan et al., 2003). Specifically, one experiment showed that expression of the inflammatory cytokine IFN-γ in guinea pigs increased as *L. monocytogenes* infection of the placenta progressed, which coincided with significantly increased apoptosis in the placenta (Irvin et al., 2008). Similarly, an increase in IFN-γ is often seen in humans prior to preterm labor (Makhseed et al., 2003), and this same increase was associated with brucellosis-induced abortion in mice (Kim et al., 2005). Given that a heavily-infected placenta serves as a means of maintaining maternal infection, the early expulsion of the fetus from the mother's body may be a form of defense mechanism (Bakardjiev et al., 2006). Even if the pregnancy is carried to term, a placenta that is significantly damaged through apoptosis will not function as well, potentially leading to insufficient nutrient and waste exchange between the mother and fetus.

Amniotic epithelial cells are also known to be infected by *L. monocytogenes* (Lecuit et al., 2004), and this infection can cause necrosis of these cells (Ammendolia et al., 2009).

Amniotic epithelial cells line the innermost layer of the fetal membranes, are bathed in the cushioning amniotic fluid, and are responsible for secreting immunosuppressive factors (Li et al., 2005). They undergo autophagic and apoptotic cell death to rupture the amnion in full-term placentas prior to delivery (Shen et al., 2008), so it may be possible that the unintentional necrotic death of these same cells earlier in the pregnancy could lead to amniotic rupture and preterm delivery.

Finally, *L. monocytogenes* may invade fetal tissues to directly cause problems. The fetus and neonate have a naïve adaptive immune system and an incompletely developed innate immune system. This makes them particularly susceptible to infection, especially if the pathogen is an intracellular one (Marodi, 2006). Thus, *L. monocytogenes* may invade fetal tissues and spread throughout the body without inciting an immune response (Marodi, 2006). Though it does not necessarily result in fetal death, this unchecked invasion translates into the meningitis, septicemia, and encephalitis seen in severe cases of neonatal listeriosis (Mylonakis et al., 2002b).

Stand-Ins Needed: Animal Models of Listeriosis

Much of what is known about the mechanisms of placental interaction and fetal toxicity associated with *in utero* exposure to *L. monocytogenes* comes from the study of the infection within various animal models (reviewed in Hoelzer et al., 2012), and a few of these animals (i.e., mice, guinea pigs, gerbils) have already been mentioned. When choosing a model, it is of utmost importance to choose an animal with responses to the pathogen that are as similar to humans as possible. In the case of *L. monocytogenes*, this requirement can be assessed by determining LD_{50} values, defined as the doses at which either 50% of adult animals die or 50% of dams have stillbirths. These values are then compared with the appropriate human numbers. Ideally, the

doses should be approximately the same; large differences are indicative of varying sensitivities to the pathogen. This review is concerned with the 50% perinatal morbidity value. In humans, this value is estimated at 1.9×10^6 CFU (FAO-WHO, 2004), and was determined using data from a 1985 listeriosis outbreak involving Mexican-style cheese that resulted in 93 pregnancy-associated cases and 30 fetal or neonatal deaths (Linnan et al., 1988). The 50% perinatal morbidity values for several animal models are reported in **Table 2.1**.

Table 2.1. Perinatal morbidity values for four animal models of listeriosis

Model	LM[a] Strain	Exposure Time	Exposure Route	Endpoint	LD_{50} (CFU)	Reference
Nonhuman primate (pregnant)	12443 (serotype 1/2a)	GD[b] ~111 of 164	Nasogastric	Fetal death	8.45×10^7	Smith *et al.*, 2008
Mouse (CD-1, pregnant)	F6214-1 (serotype 4nonb)	GD 7.5 of 19 - 21	Intragastric	Maternal death	1.5×10^8	Hamrick *et al.*, 2003
Rat (neonate)	serotype 4b	PND[c] 3	Subcutaneous	Neonatal death	6×10^5	Bortolussi *et al.*, 1984
Guinea pig (pregnant)	12443 (serotype 1/2a)	GD 35 of 59 - 72	Ingestion	Fetal death	1.999×10^7	Williams *et al.*, 2007

[a] LM = *Listeria monocytogenes*
[b] GD = gestation day
[c] PND = postnatal day

Each of the animal models in **Table 2.1** suffers from one or more drawbacks that call into question their suitability as models for human listeriosis. Nonhuman primates are perhaps the most appropriate model due to their genetic similarity to humans, but ethical and economical reasons often make it difficult to obtain these animals in large enough numbers to perform

adequate studies (Hoelzer et al., 2012). These same reasons make it difficult to sacrifice the mothers and/or babies, leading to the loss of important information on exactly what is happening within the primate's body during infection. Studies using nonhuman primates can therefore often only rely on fecal shedding of *L. monocytogenes* throughout the pregnancy and the occurrence of stillbirths to draw conclusions (Smith et al., 2003; Smith et al., 2008).

The difficulties associated with nonhuman primates are not as problematic for rodents, making them more popular as potential models. However, the drawbacks for both mice and guinea pigs have already been alluded to: lack of one of the targets for *L. monocytogenes* internalins. Mouse E-cadherin, though sharing 85% sequence identity with human E-cadherin, cannot be bound by InlA (Lecuit et al., 1999). This has been attributed to a single amino acid substitution at residue 16 of the first extracellular portion of E-cadherin: whereas humans harbor a proline in this position, mice (and rats) have a glutamic acid (Lecuit et al., 1999). The inability of InlA to bind to murine E-cadherin therefore severely compromises *L. monocytogenes* in its invasion of the intestinal epithelium, as evidenced by the higher doses required to establish infection in mice via the oral route compared to intravenous or subcutaneous injection routes (Audurier et al., 1980; Hoelzer et al., 2012). Any crossing of the mouse intestinal barrier therefore occurs only through the InlA-independent route provided by the M-cells of Peyer's patches (Lecuit et al., 2007; Marco et al., 1997). Most studies involving mice therefore administer the dose via an injection route. However, injection removes an extremely important barrier (i.e., the intestine), introduces free bacteria directly into the bloodstream where they may incite humoral immune responses that are not often active during listeriosis, and is decidedly different from the way humans are exposed to the pathogen. Furthermore, while a transgenic mouse model expressing humanized E-cadherin in the intestine has been developed, these mice

express their normal E-cadherin at all other places in the body (Lecuit et al., 2001); thus, while *L. monocytogenes* may enter the body using the route most relevant to humans, any other InlA-dependent interactions are still irrelevant in these mice. The fact that mice are susceptible to listeriosis only when exposed to doses several orders of magnitude greater than that required for humans is a recognized deficiency in using these animals for risk assessment purposes (FAO-WHO, 2004). Taken together, these facts show mice to be less than ideal for modeling human listeriosis, especially during pregnancy.

Guinea pigs and rabbits, on the other hand, possess a different Met receptor from humans, one that is not conducive to InlB binding (Khelef et al., 2006). While InlB knock-out mutants of *L. monocytogenes* are compromised in their ability to invade murine tissues compared to wild-type strains, no difference in invasive capabilities are detected between knock-out and wild-type *L. monocytogenes* in either guinea pigs or rabbits; transfection with human *Met* and *gc1qr* genes, however, permits InlB-dependent entry in guinea pigs (Khelef et al., 2006). *In vitro* invasion studies utilizing *L. innocua* expressing InlB to permit interaction with Met have shown guinea pig and rabbit cells to allow 3-fold less entry than human HeLa cells (Khelef et al., 2006). This decreased permissiveness perhaps contributes to the order of magnitude difference between the doses at which 50% of either guinea pigs or humans will have stillbirths, with guinea pigs requiring the higher dose (**Table 2.1**; Williams et al., 2007). Other studies have reported the higher doses required to establish fetoplacental infection within guinea pigs (Bakardjiev et al., 2006), but guinea pig dams otherwise respond to infection similarly to humans. That is, dams can have *L. monocytogenes*-induced stillbirths without showing clinical manifestations of the disease themselves (Bakardjiev et al., 2006; Williams et al., 2007). Though not quite as susceptible as humans, guinea pigs have therefore been used as a model for human listeriosis.

Recent years have seen the Mongolian gerbil rise in popularity as a potential model, mainly due to the similarities between human and gerbil E-cadherin and Met that allow *L. monocytogenes* to use both InlA- and InlB-mediated pathways (Disson et al., 2008). However, LD_{50}s in these animals have yet to be determined.

Conclusions and Future Directions

The intracellular pathogen *Listeria monocytogenes* presents a serious risk to pregnant women and their unborn children. Through a combination of actin-exploiting and phagosome-escaping virulence factors, *L. monocytogenes* gains access to cells and crosses the placental barrier. Once entrenched within the placenta, the pathogen rapidly replicates. Apoptosis, necrosis, and inflammation within the placenta may encourage the mother's body to prematurely expel the fetus, while direct invasion and challenge to the underdeveloped fetal immune system can result in severe complications to the fetus itself.

While great strides have recently been made in determining how *L. monocytogenes* crosses the placental barrier, more research is needed to determine which pathways are utilized and how/where the invasion occurs, as the current state of the research provides incomplete and occasionally conflicting explanations. Future research should look beyond the InlA, InlB, and ActA pathways to see if there are any others involved, as a complete understanding of the various invasion pathways is needed to devise effective therapies. This will be especially important in eradicating *L. monocytogenes* from its placental reservoir and stopping the processes that ultimately result in miscarriage, stillbirth, preterm delivery, and post-birth complications. Additionally, establishing an animal model that responds to *L. monocytogenes*

invasion in the same manner as humans, especially pregnant women, while being economical and practical to use, is of utmost importance.

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CHAPTER 3

OBTAINING TIMED-PREGNANT MONGOLIAN GERBILS FOR USE IN DEVELOPMENTAL STUDIES [1]

[1] Roulo, R.M., J.D. Fishburn, L. Alworth, A.M. Hoberman, and M.A Smith. Accepted by *Lab Animal*. Reprinted here with permission of the publisher.

ABSTRACT

The developing fetus is differentially susceptible to chemicals and pathogens depending on the stage of development. It is therefore essential for developmental studies to expose embryos/fetuses at the same stage during gestation. This is accomplished through the use of timed-pregnant animals, but though many such animals are readily available from animal suppliers, timed-pregnant Mongolian gerbils cannot currently be purchased. Because no method for breeding virgin female gerbils in a timed fashion has been described in the literature, we developed a novel method. Early breeding attempts consisted of pairing of one female with one male for one day, but only 14% of animals became pregnant using this method. Further efforts were made to increase mating success. Gerbils were housed individually in separate rooms according to sex to prevent the influence of male pheromones on the estrus cycle. Male gerbils remained on the same bedding for a minimum of 10 days to allow for accumulation of their pheromones within the cage, while females were given a minimum of 7 days to acclimate to their new surroundings. Cage tops modified with a metal partition dividing the cage into two equal areas were used in preparation for breeding. Three days prior to breeding, a female was placed into the male cage with the partition lowered between the pair, allowing the gerbils to occupy the same cage but with complete physical separation. After three days, the partition was removed and the gerbils were allowed to interact. To determine sexual receptivity, the female was monitored for lordosis one hour after lights-out. Ten of 15 females (67%) performed lordosis, and 9 of these (90%) became pregnant. When lordosis was not observed, none of the females became pregnant. These results represent a significant increase in mating success over the previous method ($p < 0.05$) and indicate lordosis as a reliable predictor of sexual receptivity and subsequent successful mating in the Mongolian gerbil. This method has allowed us to obtain

timed-pregnant gerbils in sufficient quantity and at the appropriate developmental stage to conduct our research.

INTRODUCTION

Adverse outcomes resulting from fetal exposure to certain pathogens, chemicals, or pharmacological agents often vary depending on the stage of development at which the exposure occurs. Because of these differing windows of susceptibility, developmental exposure studies that administer potentially harmful substances or organisms to a pregnant animal require accurate knowledge of the developmental stage of the embryo/fetus, as determining susceptible periods and synchronizing exposures can only be accomplished if gestational age or stage is known. This is necessary for both the reliability and reproducibility of a study, and is especially important in rodents with short gestation periods, where a single day can see relatively large differences in development. However, developmental stage cannot often be determined *in utero* without using stressful techniques such as ultrasound, which requires anesthesia. Gestational day (GD) of fetuses is therefore used as a surrogate. For these reasons, timed-pregnant animals, which are actively monitored for mating and then removed from their partners, are used in developmental exposure studies, as the timed-pregnant method allows researchers to know the time of conception within a few hours.

Our laboratory performs dose-response research with chemicals and pathogens investigating adverse effects to an exposed fetus. We have consistently used timed-pregnant mice[1], guinea pigs[2], and primates[3] as animal models in our studies. However, recent information required that we conduct experiments with the foodborne pathogen *Listeria monocytogenes* using timed-pregnant Mongolian gerbils[4].

Obtaining timed-pregnant animals often relies on either exploitation of the female's post-partum estrus period or on the observation of vaginal plugs. In rodent species where the female has a fertile post-partum estrus period, timed-pregnant animals can be obtained by mating sows or dams immediately following the births of their litters[5]. However, this technique is necessarily reliant upon when litters are born. In large breeding colonies, probability and density dictate that several animals will give birth on the same day and can thus be bred again all at the same time, but smaller breeding colonies will have too few synchronous births to obtain sufficient numbers of timed-pregnant animals for most experimental designs. While Mongolian gerbils do undergo post-partum estrus[6], there are currently no suppliers of gerbils in the United States with a breeding colony large enough to supply us with timed-pregnant gerbils in sufficient numbers using the post-partum estrus method.

Initial timed-breeding attempts for our experiments therefore concentrated on looking for vaginal plugs. Charles River Laboratories, International (Wilmington, MA) shipped us all females plugged within a 24-hour period, but only 3 of 22 animals were actually pregnant (14%). Thinking that the lack of pregnancies might result from the stress of shipping and its interference with the gerbils' 7-day implantation period[7], we decided to begin breeding in-house with some modifications to the technique employed by Charles River, as establishing our own large, ongoing breeding colony to utilize the post-partum estrus method was not feasible.

Here we have described a method for reliable timed-breeding of virgin Mongolian gerbils that exploits the induced estrus of the female gerbil in response to male pheromones[8]. Furthermore, the female's performance of the lordosis reflex was used to confirm her sexual receptivity. The objective of our study was to develop a method to reliably increase the number

of timed pregnancies from a relatively small number of breeding pairs for use in developmental dose-response studies.

TIMED-BREEDING TECHNIQUE

All animal work was done in full compliance with federal regulations, including the Animal Welfare Act, and all procedures were approved by the Institutional Care and Use Committee (IACUC) at the University of Georgia. The University of Georgia's Animal Care Program is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Animals

Fifteen virgin female Mongolian gerbils (Charles River), aged 80 – 95 days at the time of breeding, were used in this study. Their weights at the start of the breeding process averaged 71.6 g (SD: 4.7 g; range: 61.4 – 79.2 g). Five inexperienced male gerbils over 90 days of age at the time of breeding were obtained from Charles River, and were proven fertile through breedings in our laboratory prior to this study. No more than five females were bred at any one time, resulting in three separate breeding groups. Each male was paired with a single female during each breeding cycle.

Housing conditions prior to breeding

All gerbils were housed individually, for identification purposes, in solid-bottom polycarbonate rat cages (45 cm long x 23 cm wide x 21 cm tall) upon arrival. Males and females were kept in separate rooms to prevent male pheromones from impacting the female estrus cycle.

Each room was set to a 14:10 h light:dark cycle, as a longer light cycle is more conducive to breeding[6, 9, 10]. Gerbils were provided PicoLab Rodent Diet 20 5053* (PMI Nutrition, St. Louis, MO) and water *ad libitum* via an overhead wire-top feed hopper with water bottle, and were given two to three Nestlets (Ancare, Bellmore, NY) with which to build nests. Males remained on the same Bed-o'Cobs Combination chopped corncob bedding (The Andersons, Maumee, OH) for a minimum of 10 days prior to breeding to allow for accumulation of pheromones. Females were allowed a minimum of 7 days to acclimate to their new setting apart from the males.

Breeding

Gerbils are spontaneous ovulators with a 4 – 6 day estrus cycle, during which they are sexually receptive for 12 – 15 hours[6, 11]. Females enter estrus within 7 days of being paired with a male, and almost half will enter estrus after three days[8]. Given these observations, each female involved in this study was placed into one male's cage (one female with one male) for three days before breeding, although the pair was physically separated during this time by a solid metal partition welded to the middle of the cage top. This partition could be lowered to divide the cage transversally, and later raised to allow for gerbil interaction. The metal bar used for raising or lowering the partition could be hooked into either a "locked down" or "up" position. Gerbils on both sides of the partition could easily reach food, and a water bottle was provided to both sides of the cage so that both animals could drink freely at all times. All gerbils were paired according to weight: the heaviest female was paired with the heaviest male, the second heaviest female with the second heaviest male, etc.

This method of sharing a cage with a partition allowed each gerbil to become accustomed to the olfactory and auditory stimuli from another gerbil in preparation for physical contact,

while initially avoiding interaction. The 10-day accumulation of male pheromones within the cage was intended to induce estrus in the females, while the three-day separation allowed the gerbils to acclimate to one another until the female reached the stage of her estrus cycle where she was most likely to be sexually receptive[8].

After three days of sharing a divided cage, the partitions were lifted in the late afternoon to allow the gerbils to interact. All pairs were observed for a minimum of 20 minutes to ensure that no fighting occurred. Any aggressive behavior witnessed during this time would have required immediate and permanent separation of the pair, though no aggression occurred between any gerbils during this study. All gerbil pairs were left uninterrupted until one hour after lights-out, at which time mating attempts will be witnessed if the female is sexually receptive. Because gerbils cannot see red wavelengths of light[12], all nighttime observations were made under a red light to minimize disruption of the gerbils' light:dark cycle. As there were never more than 5 breeding pairs to observe, gerbils were monitored in real-time by a single researcher for a minimum of 30 minutes for the female's performance of the lordosis reflex. This position, in which the female arches her back downwards to lift her hindquarters for several seconds upon being mounted, was interpreted as confirmation that she was sexually receptive to the male. In receptive females, mating and lordosis were observed several times over the observation period, sometimes as often as 3 – 4 times per minute. These pairs remained together until the next morning, allowing a total interaction time of ~ 17 hours. The male was then removed and placed into a new cage next to his paired female. These pairs were not reunited during this study.

Females who did not go into lordosis but instead rebuffed the males' advances were deemed not receptive after 30 minutes of observation. This period of time was sufficient to observe multiple attempts from the male and multiple refusals from the female. For five of the

females who did not perform lordosis during this time, the male was allowed to remain with the female overnight and was removed the next morning. Mating was not attempted on subsequent nights. These females served as negative controls to ensure that successful mating and pregnancy would not occur given the absence of an observed lordosis reflex. For the remaining cases where lordosis was not observed an hour after lights-out, the partition was again placed between the pair, and the process was repeated again over the next few nights until the female performed lordosis.

Because gerbils are prone to losing their pregnancies, potentially because of their relatively long, 7-day implantation period[7], several steps were taken to ensure the survival of the pregnancies. Females were not handled during the first week of gestation and were thereafter handled as little as possible to reduce stress. Additionally, the male pheromones were maintained within the female cages by (1) leaving each female in her breeding cage until cage change; (2) mixing some of the used bedding from the breeding cage (~ ¼ cup) with the new bedding when a cage was cleaned; and (3) leaving each male next to his paired female for two weeks, after which males were necessarily removed prior to experimental treatment of the females.

OUTCOME

It has been noted that gerbils, particularly those paired before 7 weeks of age, tend to form monogamous pairs, and that it can therefore be difficult to introduce a surviving partner to a new mate after the death or removal of the previous mate[10]. However, this behavior is not always observed[6, 13], and no problems in pairing proven males with new female partners were experienced in our study. No overt aggression between pairs was ever witnessed, and all males displayed courtship behavior (e.g., stomping, mutual grooming) even toward females who were

not sexually receptive. This observation confirms that monogamous pairs are not necessarily formed and that it is possible to use male gerbils as studs.

Lordosis was observed on the first breeding attempt (Night 1) in 6 of the 15 females (40%). Three females (20%) performed lordosis on the third breeding attempt (Night 3), and 1 female (7%) on the fourth breeding attempt (Night 4; **Table 3.1**). For these 10 gerbils, 9 (90%) became pregnant (**Table 3.2**). The dams gained an average of 10.1 g (SD: 5.1 g; range: 3.9 – 18.6 g) throughout the first two weeks of their pregnancies, although it was not possible to determine the health of these pregnancies beyond this point (i.e., how many implantations resulted in miscarriages or stillbirths), as the dams were exposed to *L. monocytogenes* or vehicle control on GD 15 as part of our primary experiment. Of the five females who did not perform lordosis and were given only one night to breed, none (0%) became pregnant (**Table 3.1**). This, along with appropriate staging of fetuses as observed upon sacrifice of the dams, gave confidence that the dams that did not perform lordosis on the first night but were given more chances to breed on subsequent nights did not become pregnant before the lordosis was observed. These results indicate performance of lordosis as an accurate predictor of sexual receptivity, and that these animals will likely become pregnant. Conversely, absence of lordosis indicates the female is unreceptive and will likely not mate or become pregnant.

Statistical analysis of the numbers of pregnant versus nonpregnant gerbils obtained using this method compared to the initial breeding attempts utilizing vaginal plugs as indicators reveals a significant increase in breeding success ($p < 0.05$) using a Chi-square test for independence (**Table 3.2**).

OTHER CONSIDERATIONS

Prior to employing the timed-breeding method described above, our inability to confirm pregnancy status before treating animals with *L. monocytogenes* on GD 15 resulted in the unnecessary exposure of many nonpregnant animals. By observation alone, we could not determine whether or not a gerbil was pregnant until GD 17 or 18, at which point the animals had already been treated, and weight gain during the two weeks post-mating was not always clearly indicative of pregnancy. For example, one dam carrying three fetuses gained 3.9 g during the first two weeks of her pregnancy, whereas a nonpregnant counterpart gained 5.1 g during the same time period. Likewise, attempts at abdominal palpation before GD 15 were not successful.

Some researchers have had success in monitoring progesterone levels throughout pregnancy using various immunoassays[14, 15]. We therefore attempted to predict pregnancy by evaluating progesterone concentrations in each gerbil, as progesterone increases from a pre-pregnancy baseline of 75 ng/mL to 185 ng/mL by its peak at GD 12[14]. We collected both urine and serum samples from presumptive pregnant gerbils on GD 10, when progesterone is approximately 175 ng/mL[14], for analysis of elevated progesterone concentrations using a 96-well Progesterone EIA Kit (Cayman Chemical Company, Ann Arbor, MI). However, this method was inconclusive as a pregnancy test for our gerbils, and collecting samples from dams resulted in extra handing and potential stress. Monitoring for the lordosis reflex was much more reliable as an indicator of sexual receptivity and subsequent pregnancy, with the added benefit of putting less stress on the animals.

CONCLUSIONS

Timed-pregnant animals are necessary for developmental studies where exposures must be given at specific developmental stages during gestation. We could not find any published literature describing a method for timed-breeding of virgin gerbils, so we developed a method in which a male and female gerbil pair occupy separate living quarters of the same cage for three days to induce estrus and are then monitored for performance of the lordosis reflex as an indicator of sexual receptivity and subsequent mating. Observation of lordosis was highly correlated (90%) with pregnancy and was assumed to represent mating. This method was effective in producing pregnancies with confidence in the date of conception and stage of development. Furthermore, lordosis was shown to be a simple and reliable indicator of sexual receptivity.

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Table 3.1. Summary of breeding results according to performance of lordosis[a]

When was lordosis performed?	No. females (n)	Paired >1 night?	No. pregnant (%)	Appropriate staging of fetuses upon sacrifice?
Never	5	No	0 (0%)	N/A
Night 1[b]	6	No	6 (100%)	Yes
Night 2	0	N/A	N/A	N/A
Night 3	3	Yes	2 (66.7%)	Yes
Night 4	1	Yes	1 (100%)	Yes

[a] All females spent three days on male bedding prior to breeding, though they remained physically separated from the males.
[b] Night 1 refers to lordosis performed on the first breeding attempt; Night 2 to the second breeding attempt; etc.

Table 3.2. Breeding success according to mating indicator

Mating indicator	**No. females (n)**	**No. pregnant (%)**
Plugs	22	3 (14%)
Lordosis	10	9 (90%)

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CHAPTER 4

DOSE-RESPONSE OF *LISTERIA MONOCYTOGENES* INVASION, FETAL MORBIDITY, AND FETAL MORTALITY AFTER ORAL CHALLENGE IN PREGNANT AND NON-PREGNANT MONGOLIAN GERBILS (*MERIONES UNGUICULATUS*) [2]

[2] Roulo, R.M., J.D. Fishburn, M. Amosu, and M.A. Smith. To be submitted to the *Journal of Food Protection*.

ABSTRACT

Listeria monocytogenes is a foodborne pathogen that can cause adverse pregnancy outcomes ranging from preterm delivery and spontaneous abortion of a fetus, to septicemia and encephalitis in a neonate exposed *in utero*. Nonhuman primates, mice, and guinea pigs have traditionally been used in the study of *L. monocytogenes*, but the Mongolian gerbil was recently proposed as being the most appropriate small animal model. The objective of this study was to orally expose pregnant gerbils to *L. monocytogenes*, investigate invasion and adverse pregnancy outcomes, and compare the resultant dose-response data to other animal models. Additionally, differences in the responses of pregnant vs. non-pregnant gerbils were investigated. Gerbils were orally exposed to control, 10^3, 10^5, 10^7, or 10^9 CFU *L. monocytogenes* in whipping cream. *L. monocytogenes* was recovered from 6.7, 66.7, and 100% of fecal samples; 2.1, 42.5, and 74.4% of adult organs; and 25, 50, and 100% of litters from gerbils exposed to 10^5, 10^7, or 10^9 CFU, respectively. While no differences between pregnant and non-pregnant animals were seen in fecal shedding, dams exposed to 10^9 CFU had more invaded organs and higher concentrations of *L. monocytogenes* in almost all organs than non-pregnant animals. Using fetal invasion data, an ID_{50} of 2.60×10^6 CFU was calculated using a logistic fit model. Adverse pregnancy outcomes occurred only in the dams treated with 10^9 CFU, where 75% of litters were affected, and while an exact LD_{50} could not be determined, these results indicate that it falls within the range of 5×10^6 and 5×10^8 CFU. This range includes the guinea pig and nonhuman primate LD_{50}s of 2×10^7 and 8×10^7 CFU, respectively, but the observation that *L. monocytogenes*-induced stillbirths could be seen in guinea pigs exposed to as low as 10^6 CFU and primates exposed to 10^3 CFU would indicate that gerbils are not any more sensitive to *L. monocytogenes* invasion than are guinea pigs or primates.

INTRODUCTION

The foodborne pathogen *Listeria monocytogenes* is responsible for approximately 1600 cases of listeriosis every year in the United States. While this number is small in comparison to foodborne illnesses caused by other agents, the 15.9% case fatality rate of listeriosis makes *L. monocytogenes* the third-leading cause of death from a foodborne pathogen *(19)*. Humans are usually exposed through the consumption of refrigerated ready-to-eat foods, deli meats, and soft cheeses *(11)*. At-risk individuals include the elderly, persons who are immunocompromised or immunosuppressed, and the fetuses of pregnant women, with one in six listeriosis cases (17%) occurring during pregnancy *(5)*. The risk of miscarriage, stillbirth, or premature delivery to a fetus, or serious illness in a neonate (e.g. septicemia, meningitis), greatly increases if its mother has been exposed to *L. monocytogenes (15)*, though she herself may experience only mild flu-like symptoms *(2)*. One review of historical data reported that 36 of 178 maternal listeriosis cases (20.2%) resulted in spontaneous abortion or stillbirth. Of the remaining 142 cases, 97 neonates (68.3%) were born with the infection. Data available on 94 of these neonates reported that 23 of the affected neonates (24.5%) died, and another 12 (12.8%) experienced some form of serious long-term complication *(15)*. Listeriosis is therefore of great concern to pregnant women and their unborn children.

L. monocytogenes possesses almost 50 virulence factors that help it adhere to, invade, replicate within, and spread among cells *(4)*. Of the invasion virulence factors, InlA and InlB are most often the focus of research. InlA binds to human E-cadherin, hijacking this mechanosensor's cytoskeleton reorganization capabilities, and inducing membrane extensions that allow *L. monocytogenes* to enter the cell via endocytosis *(14)*. InlB binds preferentially to the human Met receptor, initiating a cascade that culminates in actin rearrangement and

internalization of the pathogen via clathrin-coated endocytosis *(17, 22)*. The InlA/E-cadherin interaction is important to the efficient crossing of the intestinal barrier *(12)*, while the InlB/Met interaction is thought to be important for the entry of *L. monocytogenes* into other mammalian cell types *(3, 20)*, and could play a role in enhancing intestinal invasion *(16)*. Some animal models of listeriosis differ from humans in either the E-cadherin or Met targets, and determining the appropriateness of these animals for modeling human listeriosis is important for the study of the disease.

Similarity to human listeriosis and ease of study are chief considerations for the study of *L. monocytogenes* invasion *in vivo*. Ideally, the model should be (1) similar to humans in every aspect important for *L. monocytogenes* invasion and spread within the body, and (2) easily obtainable and capable of being timed-bred in numbers large enough for studies involving pregnancy. Various animals have been used as models, but some of the most common are lacking in one or both of these areas. Listeriosis in nonhuman primates is perhaps most similar to listeriosis in humans *(8)*. However, it can be difficult to acquire large numbers of primates for a single study, the studies are expensive, and the primates are not usually sacrificed, resulting in a loss of valuable information that addresses what is happening within tissues. Mice and rats possess an E-cadherin with a single amino acid difference from humans *(13)*, and because *L. monocytogenes* is incapable of binding to this altered E-cadherin, mice and rats are highly resistant to oral exposure *(13)*. While these rodents can contract listeriosis if injected with the pathogen, injection removes the first barrier *L. monocytogenes* must cross (i.e., the intestine), introduces the bacteria directly into the bloodstream, and is otherwise less-than-ideal in that this exposure route is not applicable to humans. Guinea pigs and rabbits posses a polymorphism in

their Met receptor that affects invasion of cells such as hepatocytes, potentially making guinea pigs and rabbits less sensitive than humans to listeriosis *(10)*.

The Mongolian gerbil (*Meriones unguiculatus*) has recently been proposed as the small animal model of choice because both InlA and InlB entry pathways are operative in gerbils *(6)*, making them theoretically similar to humans in both exposure route and invasion susceptibility. Additionally, gerbils are easily purchased, can be timed-bred *(18)*, and can be sacrificed for analysis of maternal and fetal tissues. However, no dose-response curve has been calculated for gerbils, and extrapolation of gerbil data to humans may be inappropriate if the LD_{50} values are significantly different. The objective of this research is therefore to provide dose-response data for fetal morbidity and mortality after oral exposure to *L. monocytogenes* in Mongolian gerbils. Furthermore, we compare *L. monocytogenes* invasion in pregnant animals to their non-pregnant counterparts.

MATERIALS AND METHODS

Animals. Thirty-eight female and 8 male Mongolian gerbils (*Meriones unguiculatus*) were obtained from Charles River Laboratories, International (Wilmington, MA). All gerbils were housed individually upon arrival at the animal facility, with males and females kept in separate rooms; both rooms were set to a 14:10 h light:dark cycle conducive to breeding. Gerbils were provided PicoLab Rodent Diet 20 5053* (PMI Nutrition, St. Louis, MO) and water *ad libitum*.

Animals were bred in-house according to a method developed for breeding timed-pregnant gerbils and described in Roulo et al *(18)*. Briefly, male gerbils aged 90+ days and female gerbils aged 80 – 95 days and averaging 71.0 g (± 8.4 g) acclimated in separate rooms for

a minimum of 7 days before overnight breeding. Males were removed the following morning, but all gerbils remained together in the same room, though physically separated, for the two weeks prior to treatment. On gestation day (GD) 15, females were moved from the breeding room to a treatment room, where they remained until sacrifice. A microisolator was placed on each cage to prevent cross-contamination between gerbils.

All females were weighed every three to four days beginning on GD 7 as a means of monitoring pregnancy. After treatment, females were also monitored three times daily for adverse effects such as lethargy, pre-term labor, or death.

Bacterial preparation and treatment. All media and the whipping cream vehicle were sterilized by autoclaving at 121°C for 20 min. Cells were prepared as previously described by Williams et al. *(23)*, with some minor modifications. *Listeria monocytogenes* strain 12443 (serotype 1/2a), an isolate known to cause stillbirths in primates *(21)* and guinea pigs *(9)*, was grown at 37°C for 24 h in 10 mL Bacto tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) and activated by two subcultures at 24 h intervals. Cells were harvested by centrifugation (3600 × g at 15°C for 10 min) and washed three times in 10 mL BBL phosphate buffered saline (PBS; BD). The final pellet was resuspended in 1 mL PBS, then diluted with an additional 9 mL of either PBS or ultrapasteurized heavy whipping cream (Publix, Lakeland, FL). This mixture was serially diluted into either PBS or whipping cream to give concentrations ranging from 10^9 to 10^3 CFU/mL *L. monocytogenes*. The control dose was prepared by diluting 1 mL PBS into 9 mL whipping cream. Exposure doses were confirmed by duplicate plating onto Difco tryptic soy agar (TSA; BD) and enumeration after incubation at 37°C for 48 h.

All female gerbils were assumed to be pregnant and were orally exposed to 0.5 mL of the whipping cream containing either control, 10^3, 10^5, 10^7, or 10^9 CFU/mL *L. monocytogenes* via 18G × 1.25" animal feeding needle (Cadence Science, Cranston, RI) on GD 15. Average total CFU administered to gerbils are reported in **Table 4.1**.

Tissue collection and analysis. Fecal samples were collected every day, starting with a pretreatment sample collected immediately prior to exposure and ending the day before sacrifice. With the exception of one high-dose dam who died on GD 21, gerbils were sacrificed by CO_2 overdose on GD 22, two to four days short of full-term gestation (GD 24 – 26), allowing *L. monocytogenes* a total invasion period of just under 7 full days. All fetuses, placentas, and resorptions were collected from dams. Fetuses were directly checked for viability (i.e., movement and breathing when removed from their amniotic sacs), as well as visually inspected for differences in coloration, size, and development when compared to their littermates. Adverse effects were defined as fetal death (stillbirth or resorption) or underdevelopment compared to littermates (small size and earlier stage of development). The liver, spleen, brain, intestine, cecum, colon, uterus, kidneys, adrenal glands, and gallbladder of each adult animal were harvested, regardless of pregnancy status. Blood samples available from exsanguination were also saved for analysis in non-heparinized blood collection tubes (BD). Additionally, mesenteric lymph nodes were collected when they could be found. Only portions of the liver, intestine, and colon were saved for analysis due to the large size of these organs; all other organs were analyzed in their entirety. All samples were weighed upon collection, placed into individual 24 oz. WHIRL-PAK bags with filters (Nasco, Fort Atkinson, WI), and transferred to ice. Processing of samples was completed within 24 hours.

Individual fecal or tissue samples were homogenized (Bio-Gen PRO200, BioExpress, Kaysville, UT) on ice in a volume of Difco UVM modified *Listeria* enrichment broth (UVM; BD) equal to 10 times their weight until no individual fecal pellet or piece of organ was discernible. Each entire fetus, placenta, or resorption was processed individually. Blood samples were allowed to clot at room temperature for at least 1 hour before centrifugation (1000 × g at 4°C for 20 min). After discarding the serum, the remaining blood components were processed as described above. Sample and/or sample dilutions were plated in duplicate onto Difco modified Oxford *Listeria* selective agar (Oxford; BD) to obtain direct counts; these plates were incubated at 37°C for 48 h prior to enumeration. Of the remaining homogenized sample, 1 mL was diluted into 9 mL Difco Fraser *Listeria* selective enrichment broth (FB; BD); in cases where less than 1 mL was available, all remaining sample was placed into FB. FB tubes were incubated at 37°C for 24 h, then plated in duplicate onto Oxford and incubated at 37°C for 48 h to determine presence or absence of *L. monocytogenes*. Oxford plates from FB were considered positive if any *L. monocytogenes* colonies were present.

Direct counts were used to calculate final numbers of *L. monocytogenes* in CFU/g. Any sample that did not have counts but was positive for FB enrichment was set at the detection limit for counts (50 CFU/g). Any sample that did not have counts and was negative for FB enrichment, including control samples that were presumably negative for *L. monocytogenes*, was set at the detection limit for FB (10 CFU/g). As a final *L. monocytogenes* confirmation, a random sampling of colonies from both direct count plates and FB plates was re-plated onto RAPID'*L.Mono* agar (Bio-Rad Laboratories, Hercules, CA) and incubated at 37°C for 24 h.

Statistical analysis. Overall, 265 fecal samples, 372 organ and blood samples, 150 fetuses, 148 placentas, and 23 resorptions across the dose groups were analyzed. After

calculating the amount of *L. monocytogenes* present in each sample, all values were log-transformed for statistical analysis. Fetal data was combined within litters before analysis to control for within-litter similarities; thus the sample size is equal to the number of dams. Both positive/negative data and count data were analyzed using a Kruskal-Wallis test for multiple comparisons combined with a Sidak correction test (Stata, College Station, TX) to investigate potential differences between dose groups. Relationships between pregnant and non-pregnant animals within a single dose group were analyzed using two-sample t-tests, while relationships between fetuses and their placentas were analyzed with paired t-tests. All t-tests were performed with Microsoft Excel (Redmond, WA). The significance level was set at $\alpha = 0.05$. The dose-response curve was created using a user-defined logistic fit model in PSI-Plot (Pearl River, NY), with an equation of $y(x) = 1/(1 + \exp(-A*x-B))$, where parameter A = 0.9752, parameter B = -6.2557, and the goodness-of-fit correlation is 0.986.

RESULTS

The purpose of this study was to collect dose-response data on invasion and adverse fetal outcomes in Mongolian gerbils after a single oral exposure to *L. monocytogenes* to investigate their suitability as a small animal model for human listeriosis. Results from fecal shedding, adult organs, and pregnancy-associated tissues (fetuses, placentas, and resorptions) are reported below. Each of these was analyzed using two different methods: amount of *L. monocytogenes* recovered directly from each sample and presence/absence of *L. monocytogenes* through sample enrichment to check for *L. monocytogenes* presence where colonies could not be enumerated.

Fecal shedding. None of the 38 animals shed *L. monocytogenes* prior to treatment, and control animals remained negative for *L. monocytogenes* throughout the study period. Likewise,

dams exposed to 10^3 CFU did not shed *L. monocytogenes* at any point during fecal collection (**Table 4.2**). Dams exposed to 10^5 CFU shed only on the first day post-treatment (PTD 1), while at least 50% of dams exposed to 10^7 CFU and all dams receiving 10^9 CFU shed every day throughout the collection period (**Table 4.2**). Looking at each day individually, dams showed a trend toward dose-dependent increases in both the numbers of fecal samples positive for *L. monocytogenes* ($p < 0.05$; **Table 4.2**) and the amount of *L. monocytogenes* shed. Dams exposed to 10^9 CFU shed significantly more *L. monocytogenes* than all other groups on every day except for PTD 3, when they were not different from the 10^7 CFU dams (**Fig. 4.1A**). In a day-by-day analysis of positive samples, the only significant differences between days could be seen in the dams exposed to either 10^5 or 10^9 CFU: dams treated with 10^5 CFU *L. monocytogenes* had more positive fecal samples on PTD 1 than on any other day, and dams treated with 10^9 CFU had significantly more positive samples on all PTDs than during pretreatment (data not shown). Furthermore, *L monocytogenes* was shed in higher numbers on PTD 6 in dams exposed to 10^9 CFU than on either PTD 2 or 3 (**Fig. 4.1A**).

In non-pregnant animals, many of the relationships between when *L. monocytogenes* was shed, the number of days shed, and the amount shed were the same as for pregnant animals (**Table 4.2**). One notable difference occurred in non-pregnant animals exposed to 10^5 CFU, where shedding occurred only on the final day post-treatment as opposed to only on PTD 1; the only significant difference between numbers of samples positive for *L. monocytogenes* between pregnant and non-pregnant animals occurred on PTD 1 in this dose group (**Table 4.2**). Non-pregnant animals who received 10^7 CFU shed significantly more *L. monocytogenes* on PTD 1 compared to pretreatment, whereas the dams did not (**Fig. 4.1B**). Within each dose group, the

total number of positive fecal samples (**Table 4.2**) and the amount of *L. monocytogenes* shed on each day post-treatment (**Fig. 4.1**) was not different between pregnant and non-pregnant animals.

Adult organs. As with fecal shedding, *L. monocytogenes* was not isolated from any of the organs of the control dams. Additionally, it was not isolated from any organs of dams exposed to 10^3 or 10^5 CFU (**Table 4.3**). A trend toward a dose-dependent increase in both the number of positive organs and the amount of *L. monocytogenes* isolated from dams was observed, and the overall number of positive samples showed a dose-response (**Table 4.3**). As expected, the dams receiving the highest dose (10^9 CFU) had significant more ($p < 0.05$) intestine and brain tissues positive for *L. monocytogenes* than any other group (**Table 4.3**); *L. monocytogenes* was also recovered in significantly higher numbers in these organs (**Fig. 4.2A** and **4.2D**). Likewise, liver and spleen samples from the 10^9 CFU treated dams were different from the two low-dose groups in both number of sample from with *L. monocytogenes* was isolated (**Table 4.3**) and the total amount recovered (**Fig. 4.2B** and **4.2C**), but these organs were not different from the dams treated with 10^7 CFU. No differences were seen between gallbladders harvested from any group (**Table 4.3**; **Fig. 4.2E**).

L. monocytogenes was also isolated from 50 – 75% of cecum, colon, uterus, kidney, adrenal gland, and blood samples collected from dams treated with 10^7 CFU *L. monocytogenes*, and from all of these same tissues collected from 10^9 CFU treated dams. It should be noted, however, that colon and cecum samples can be cross-contaminated with fecal material, while uterine tissues can be cross-contaminated with amniotic fluid; thus, these positive tissues may be reflective of *L. monocytogenes* presence in other sample types. Although difficult to obtain, mesenteric lymph nodes were collected from two 10^7 and three 10^9 CFU treated dams, but only the samples from the higher dose group were positive for *L. monocytogenes* (data not shown).

Dose-dependent increases in either the number of organ samples positive for *L. monocytogenes* or the amount recovered were not as pronounced in non-pregnant animals as they were in pregnant animals. Again, no *L. monocytogenes* was recovered from non-pregnant controls, but one liver sample in the 10^5 CFU treated group was positive for *L. monocytogenes* (**Table 4.3**). While the number of positive samples for the intestine, liver, and spleen all increased with increasing dose, only the liver showed a significant difference between animals treated with 10^9 CFU and the other groups (**Table 4.3**). As with the dams, gallbladder samples collected from non-pregnant animals did not differ between dose groups (**Table 4.3**; **Fig. 4.2E**). The overall number of positive samples did not show as clear of a dose-response as did the sample from pregnant animals, with the two high dose groups (10^7 and 10^9 CFU) differing from the control and 10^5 CFU treated groups but not from each other (**Table 4.3**).

Within dose groups, no differences could be seen between numbers of positive samples collected from pregnant and non-pregnant animals, with the exceptions of brain samples (100 vs. 20%) and overall samples (95 vs. 58%) in the groups treated with 10^9 CFU *L. monocytogenes*. In both of these cases, the dams had significantly more samples from which *L. monocytogenes* was isolated than the non-pregnant animals (**Table 4.3**). More interesting, however, is the dramatic difference in the amount of *L. monocytogenes* isolated from pregnant and non-pregnant animals. In the intestine, liver, spleen, and brain tissues of animals treated with 10^9 CFU, *L. monocytogenes* was isolated in far greater numbers in pregnant animals than in non-pregnant ones ($p < 0.05$; **Fig. 4.2**).

Pregnancy-associated tissues. Each experimental group was comprised of 4 dams. Most dams gained weight steadily throughout their pregnancies, though the dams exposed to 10^9 CFU *L. monocytogenes* experienced a significant net loss of weight indicative of maternal toxicity

(**Table 4.4**). One dam treated with 10^9 CFU died on GD 21, and her fetuses averaged only 0.48 g, a significant difference from the other two litters of this dose group analyzed only a day later that averaged 1.45 g and 0.99 g ($p < 0.05$). Resorptions were seen in 100% of control litters, 50% of 10^3 CFU litters, and 50% of 10^9 CFU litters. One litter from a dam treated with 10^9 CFU was totally resorbed (**Table 4.4**). Other summary characteristics of the dams and their litters are presented in **Table 4.4**.

As with the fecal and maternal organ samples, *L. monocytogenes* was not isolated from any fetus, placenta, or resorption from dams in the control or lowest dose group. In dams exposed to 10^5, 10^7, and 10^9 CFU *L. monocytogenes*, 25, 50, and 100% of litters, respectively, contained at least one fetus from which *L. monocytogenes* was isolated (**Table 4.5**). In the two higher dose groups, if *L. monocytogenes* was isolated from one fetus, it was isolated from all fetuses within that litter; however, *L. monocytogenes* was isolated from only one of the three fetuses in the positive litter exposed to 10^5 CFU. The placentas showed the same trend as the fetuses: in every fetal-placental pair analyzed (n = 148), every positive fetus also had a positive placenta (**Table 4.5**). However, two placentas of the positive 10^5 litter, including the one associated with the one positive fetus of this litter, could not be analyzed. Resorptions were not seen in either the 10^5 or 10^7 CFU treated groups, but two litters of dams exposed to 10^9 CFU had resorptions, including one that was totally resorbed. All resorptions from this high-dose group tested positive for *L. monocytogenes* (**Table 4.5**).

Although a trend toward increased isolation of *L. monocytogenes* from fetuses, placentas, and resorptions could be seen with increasing dose, only those pregnancy-associated tissues from dams treated with 10^9 CFU were significantly different from those of the other dose groups (**Fig. 4.3**). On an individual basis, placentas of both 10^7 and 10^9 CFU treated groups, if positive for *L.*

monocytogenes, were always more invaded than their corresponding fetuses, though this trend was also not statistically significant when dams were compiled into their dose groups (**Fig. 4.3**). Resorptions were invaded to the same extent as placentas (**Fig. 4.3**).

Dose-response curve. A dose-response curve based on fetal invasion data was created using a logistic fit model with the following equation (**Fig. 4.4**):

$$p = \frac{1}{1+\exp[(-Ax)-B]} \quad \Rightarrow \quad x = \frac{\ln(1/p - 1) + B}{-A}$$

where p represents the invasion rate, x represents the log dose, A = 0.9752, and B = -6.2557. Using this formula, the log 50% infectivity dose for fetuses (log ID_{50}) is estimated to be 6.415. The ID_{50} is therefore 2.599×10^6 CFU *L. monocytogenes* (95% confidence limits of 4.457×10^5 and 1.442×10^7).

DISCUSSION

The primary objective of this research was to investigate maternal and fetal invasion of *L. monocytogenes* within the Mongolian gerbil, construct its dose-response curve for fetal morbidity and/or mortality, and evaluate its appropriateness for modeling human listeriosis. The official FAO-WHO risk assessment for listeriosis during pregnancy estimates an LD_{50} of 1.9×10^6 CFU for human fetuses *(7)*. Previous studies using guinea pigs and nonhuman primates with fetal death as an endpoint have yielded LD_{50}s of 1.999×10^7 CFU and 8.45×10^7 CFU, respectively *(21, 23)*. The 95% confidence intervals of these three dose-response curves overlap, but the inability of *L. monocytogenes* to interact with the guinea pig Met receptor during invasion has lead some to believe that guinea pig susceptibility to the pathogen may not be equivalent to that of humans *(10)*. The gerbil has therefore been proposed as the small animal model of choice

for the study of listeriosis, as its InlA/E-cadherin and InlB/Met receptor interaction pathways, the two invasion pathways considered most important to successful invasion of *L. monocytogenes*, are similar to humans *(6)* in ways that mice and guinea pigs are not *(10, 13)*. This study adds to the knowledge of *L. monocytogenes* invasion within gerbils and allows for a primary comparison with the guinea pig and nonhuman primate models.

The dose-response curve presented in **Figure 4.4** has been drawn using data from the invasion of fetuses, with a calculated ID_{50} of 2.599×10^6 CFU *L. monocytogenes*. However, the presence of *L. monocytogenes* within a given sample does not necessarily mean there will be an adverse effect. Indeed, large concentrations of *L. monocytogenes* (up to 5.0×10^8 CFU/g) could be found in some of the viable fetuses of dams exposed to 10^7 CFU without any concurrent overt signs of illness or adverse developmental effects. In adults, up to 6.3×10^3 CFU *L. monocytogenes* could be isolated from a single brain without any apparent change in the health or behavior of the animal. Looking directly at adverse effects is therefore more appropriate, and while the exact LD_{50} for gerbil fetuses cannot be calculated using the results of this study, an estimated range can be given. No adverse outcomes were seen in any of the litters exposed to 10^3, 10^5, or 10^7 CFU, but 75% of 10^9 CFU litters contained resorptions and/or nonviable fetuses positive for *L. monocytogenes*. The LD_{50} can therefore be tentatively said to lie somewhere between 5.68×10^6 and 5.08×10^8 CFU.

One of the more interesting findings of this study was the almost complete lack of *L. monocytogenes* invasion and adverse pregnancy outcomes within the two lower dose groups (10^3 and 10^5 CFU) when compared to guinea pigs and nonhuman primates. Given that gerbils are theoretically more susceptible than guinea pigs to successful *L. monocytogenes* invasion due to the presence of both InlA- and InlB-mediated invasion pathways as opposed to only the InlA

pathway *(6)*, we expected to see more positive samples in the gerbils exposed to these lower doses. However, only a single fetus in these groups was positive for *L. monocytogenes*, out of over 100 samples of maternal organs and pregnancy-associated tissues analyzed. In contrast, *L. monocytogenes* was recovered from 25% of livers collected from guinea pig dams exposed to 10^4 CFU and 64% of livers collected from 10^5 CFU dams *(23)*. Additionally, only 50% of litters from 10^7 CFU treated gerbils were invaded, none of which showed *L. monocytogenes*-induced adverse outcomes, whereas guinea pigs had fetal invasion in dams exposed to 10^5 CFU and stillbirths in all groups exposed to $\geq 10^6$ CFU. These results can be further contrasted with those of nonhuman primates, a model that is also permissive to both InlA and InlB invasion pathways due to its close phylogenetic relationship to humans *(8)*, where stillbirths occurred in animals exposed to as little as 10^3 CFU *(21)*; if invasion in gerbils is indeed similar to invasion in primates and humans, we would have expected more stillbirths to occur at lower doses that 10^9 CFU in gerbils. Taken together, these results indicate that permissiveness to InlA- and InlB-dependent invasion pathways is insufficient to explain the susceptibility of various animals to *L. monocytogenes*, and that some other mechanism or pathway is involved.

Another striking observation from the data is the extreme variability present in the dams exposed to 10^7 CFU *L. monocytogenes*. The dams of this group ranged from being negative for *L. monocytogenes* in every sample collected to having several tens of thousands CFU *L. monocytogenes* isolated from almost every sample. This variability may reflect variation within individuals in their responses to invasion with *L. monocytogenes* and presents the opportunity for future studies to determine why some individuals are susceptible at lower concentrations than others.

Finally, while no differences could be seen between pregnant and non-pregnant animals in the number of days or amount/day *L. monocytogenes* was shed in feces, *L. monocytogenes* was isolated in significantly higher numbers from pregnant organs than from non-pregnant organs in the highest dose group. Additionally, the dams treated with 10^9 CFU appeared to be less active than the non-pregnant members of this dose group, with one dam dying prematurely. It has been hypothesized that the placenta may act as a reservoir for *L. monocytogenes* growth and spread within the maternal body, and that this may be the reason pregnant women are more susceptible to listeriosis than the general population *(1)*. A closer look at the dams from which *L. monocytogenes* was isolated revealed that on an individual basis, *L. monocytogenes* was isolated in higher numbers from each placenta than from any other tissue sample in all but one of the dams; however, the placenta did not harbor significantly higher concentrations ($p > 0.05$) when examined by group.

In conclusion, the foodborne pathogen *L. monocytogenes* can cause adverse outcomes in the pregnant Mongolian gerbil, though *L. monocytogenes*-induced fetal deaths were seen only in the highest dose group (10^9 CFU). While a dose-response curve for fetal mortality could not be drawn, the LD_{50} falls somewhere between 5.68×10^6 and 5.08×10^8 CFU, and a threshold for lethality is seen. The ID_{50} is calculated to be 2.599×10^6 CFU *L. monocytogenes*. These results indicate that the gerbil is not more sensitive to *L. monocytogenes*, and may be less sensitive, than the guinea pig and nonhuman primate models of listeriosis for both invasion and adverse pregnancy outcomes. More research is therefore needed to elucidate which pathways are involved in fetoplacental invasion of *L. monocytogenes*, as InlA- and InlB-mediated pathways alone are insufficient to explain the differences between susceptibility among the various animal models.

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Table 4.1. Average confirmed doses administered to gerbils during oral challenge with *L. monocytogenes*.

Dose group	No. pregnant	No. non-pregnant	Average confirmed dose (CFU ± SD)
Control Animals	4	3	0
10^3 Animals	4	0	$6.11 \times 10^2 \pm 1.73 \times 10^2$
10^5 Animals	4	6	$5.36 \times 10^4 \pm 1.95 \times 10^4$
10^7 Animals	4	4	$5.58 \times 10^6 \pm 1.58 \times 10^6$
10^9 Animals	4	5	$5.28 \times 10^8 \pm 1.43 \times 10^8$

Table 4.2. Isolation of *L. monocytogenes* in fecal samples of pregnant and non-pregnant gerbils during the 6 post-treatment days (PTD) leading up to sacrifice.

Pregnant animals							
Dose (CFU)	**PTD 1 (%)**[a,b]	**PTD 2 (%)**[a]	**PTD 3 (%)**[a]	**PTD 4 (%)**[a]	**PTD 5 (%)**[a]	**PTD 6 (%)**[a]	**Total (%)**[a]
Control	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/24 (0)^{A}
10^3	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/24 (0)^{A}
10^5	3/4 (75)^{B}	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	3/24 (13)^{A}
10^7	3/4 (75)^{B}	3/4 (75)^{B}	2/4 (50)^{AB}	3/4 (75)^{B}	2/4 (50)^{AB}	2/4 (50)^{AB}	15/24 (63)^{B}
10^9	4/4 (100)^{B}	4/4 (100)^{B}	4/4 (100)^{B}	4/4 (100)^{B}	4/4 (100)^{B}	3/3 (100)^{B}	23/23 (100)^{C}
Non-pregnant animals							
Dose (CFU)	**PTD 1 (%)**[a,b]	**PTD 2 (%)**[a]	**PTD 3 (%)**[a]	**PTD 4 (%)**[a]	**PTD 5 (%)**[a]	**PTD 6 (%)**[a]	**Total (%)**[a]
Control	0/3 (0)^{A}	0/3 (0)^{A}	0/3 (0)^{A}	0/3 (0)^{AB}	0/3 (0)^{AB}	0/3 (0)^{A}	0/18 (0)^{A}
10^5	0/6 (0)^{A}	0/6 (0)^{A}	0/6 (0)^{A}	0/6 (0)^{B}	0/6 (0)^{B}	1/6 (17)^{AB}	1/36 (3)^{A}
10^7	4/4 (100)^{B}	3/4 (75)^{B}	3/4 (75)^{B}	2/4 (50)^{A}	2/4 (50)^{A}	3/4 (75)^{BC}	17/24 (71)^{B}
10^9	5/5 (100)^{B}	5/5 (100)^{B}	5/5 (100)^{B}	5/5 (100)^{C}	5/5 (100)^{C}	5/5 (100)^{C}	30/30 (100)^{C}

[a] Number of positive fecal samples/total fecal samples collected (% positive)
[b] Groups with different letters are significantly different ($p < 0.05$)

Table 4.3. Isolation of *L. monocytogenes* in adult organs of pregnant and non-pregnant gerbils.

Pregnant animals

Dose (CFU)	Intestine (%)[a,b]	Liver (%)[a]	Spleen (%)[a]	Brain (%)[a]	Gallbladder (%)[a]	Total (%)[a]
Control	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/3 (0)^{A}	0/3 (0)^{A}	0/18 (0)^{A}
10^3	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/20 (0)^{A}
10^5	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}	0/3 (0)^{A}	0/19 (0)^{A}
10^7	2/4 (50)^{A}	3/4 (75)^{B}	2/4 (50)^{AB}	1/4 (25)^{A}	1/4 (25)^{A}	9/20 (45)^{B}
10^9	4/4 (100)^{B}	4/4 (100)^{B}	4/4 (100)^{B}	4/4 (100)^{B}	2/3 (66)^{A}	18/19 (95)^{C}

Non-pregnant animals

Dose (CFU)	Intestine (%)[a,b]	Liver (%)[a]	Spleen (%)[a]	Brain (%)[a]	Gallbladder (%)[a]	Total (%)[a]
Control	0/3 (0)^{AB}	0/3 (0)^{A}	0/3 (0)^{A}	0/3 (0)^{AB}	0/2 (0)^{A}	0/14 (0)^{A}
10^5	0/6 (0)^{A}	1/6 (17)^{A}	0/6 (0)^{A}	0/6 (0)^{A}	0/4 (0)^{A}	1/28 (4)^{A}
10^7	2/4 (50)^{AB}	1/4 (25)^{A}	1/4 (25)^{A}	3/4 (75)^{B}	1/4 (25)^{A}	8/20 (40)^{B}
10^9	4/5 (80)^{B}	5/5 (100)^{B}	3/5 (60)^{A}	1/5 (20)^{AB}	1/4 (25)^{A}	14/24 (58)^{B}

[a] Number of positive tissue samples/total tissue samples collected (% positive)
[b] Groups with different letters are significantly different ($p < 0.05$)

Table 4.4. Summary characteristics of gerbil pregnancies.

Maternal dose	Maternal weight gain (g ± SD)	Implantations per litter (n ± SD)	Fetuses per litter (n ± SD)	Litters with ≥1 resorption (%)	Litters totally resorbed (%)
Control	18.5 ± 3.7	9.5 ± 1.3	7.5 ± 1.7	4/4 (100)	0/4 (0)
10^3 CFU	18.2 ± 4.3	10.3 ± 1.5	9.8 ± 1.9	2/4 (50)	0/4 (0)
10^5 CFU	15.8 ± 7.6	5.8 ± 2.8	5.8 ± 2.8	0/4 (0)	0/4 (0)
10^7 CFU	16.1 ± 4.0	8.5 ± 0.6	8.5 ± 0.6	0/4 (0)	0/4 (0)
10^9 CFU[a]	-1.5 ± 10.7	9.3 ± 2.2	6.0 ± 4.1	2/4 (50)	1/4 (25)

[a] This group includes one dam who died prematurely (GD 21).

Table 4.5. Isolation of *L. monocytogenes* from pregnancy-associated tissues.

Maternal dose	Fetus (%)[a,b]	Placenta (%)[a]	Resorption (%)[a]
Control	0/4 (0)^{A}	0/4 (0)^{A}	0/4 (0)^{A}
10^3 CFU	0/4 (0)^{A}	0/4 (0)^{A}	0/2 (0)^{A}
10^5 CFU	1/4 (25)^{AB}	0/4 [c] (0)^{A}	N/A [d]
10^7 CFU	2/4 (50)^{AB}	2/4 (50)^{AB}	N/A
10^9 CFU	4/4 (100)^{B}	4/4 (100)^{B}	2/2 (100)^{B}

[a] Number of litters with ≥1 positive tissue/total litters from which the tissue was collected (% positive)
[b] Groups with different letters are significantly different ($p < 0.05$)
[c] Two placentas, including the placenta paired with the one positive fetus of this group, could not be analyzed
[d] N/A = not applicable; no resorptions were present in these groups

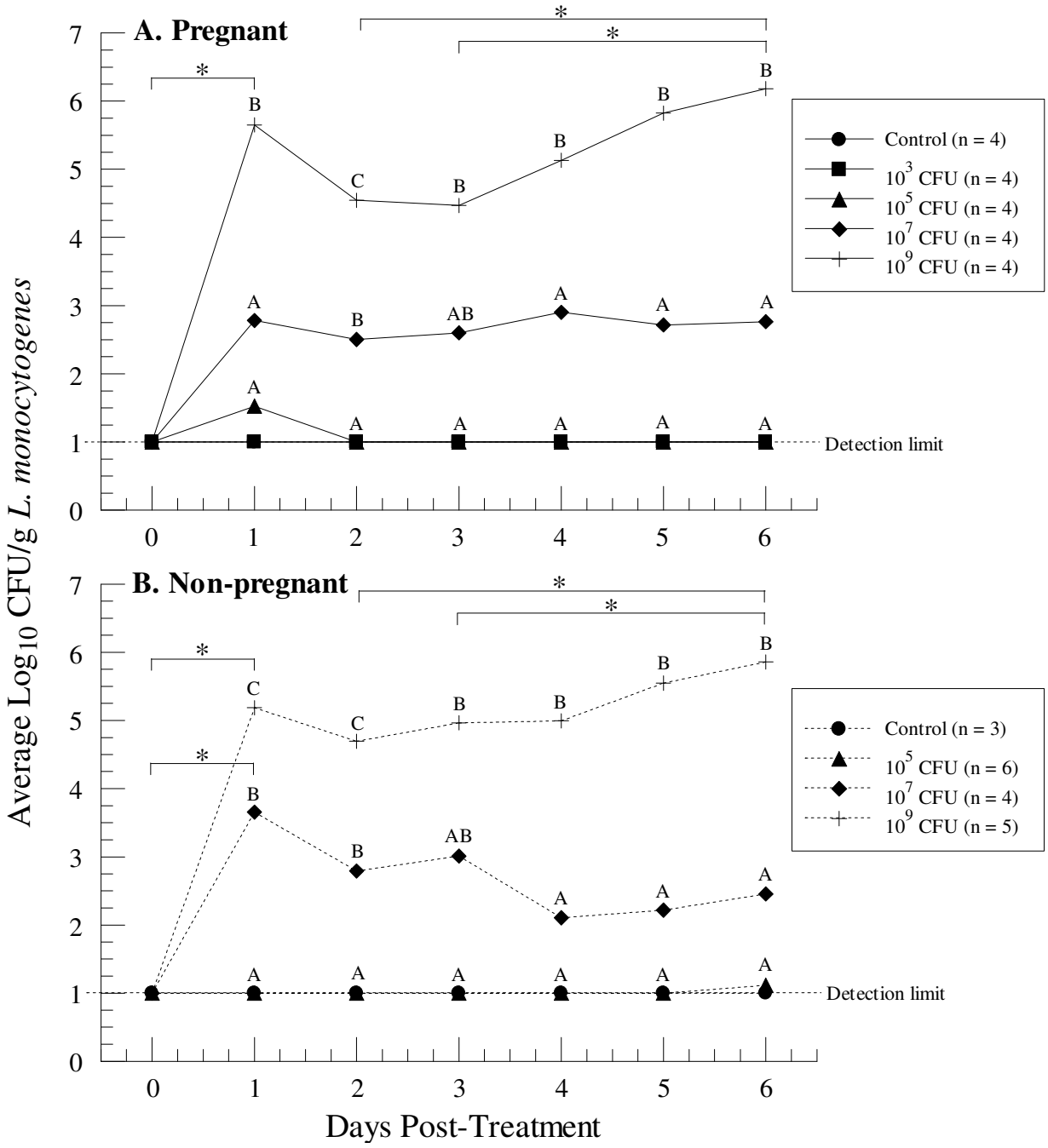

Figure 4.1. Fecal shedding of *L. monocytogenes* from pretreatment (Day 0) to the day prior to sacrifice (Day 6). Fecal material from every dam (**A**) and non-pregnant animal (**B**) was gathered over 7 24-hours periods. Dose groups significantly different from one another ($p < 0.05$) on the same day are denoted by different letters. Control, 10^3, and 10^5 CFU treated groups in **A**, and control and 10^5 CFU treated groups in **B**, have been given a single set of letters, as these groups were not different from each other on any post-treatment day. Days that differ significantly from one another within dose groups are denoted by bars with asterisks. Samples that had no countable colonies and were negative for enrichment were set at $\log_{10}(10 \text{ CFU/g}) = 1.00$, the detection limit for the enrichment method.

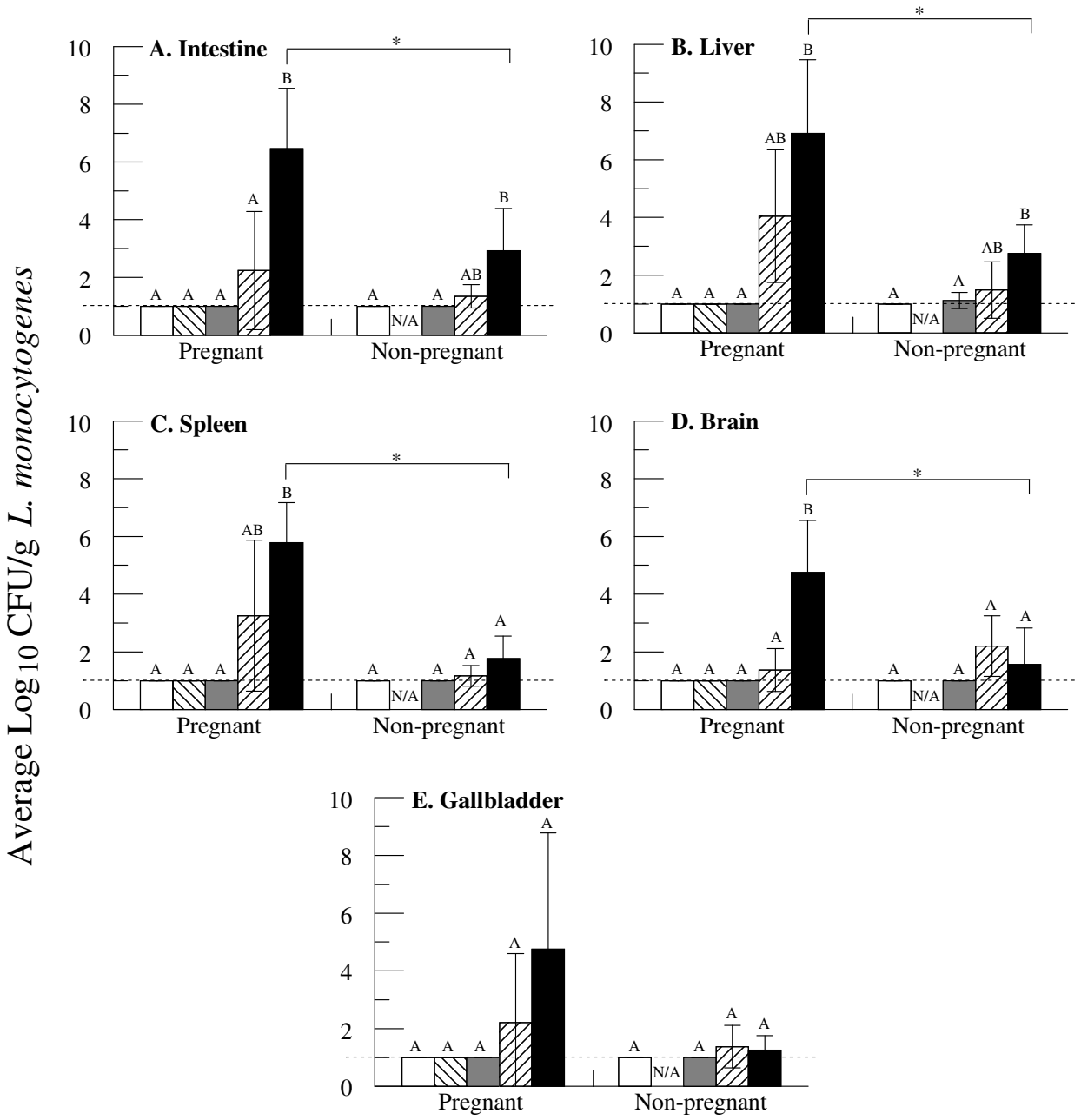

Figure 4.2. Isolation of *L. monocytogenes* from adult organs after a single oral challenge. All organs were harvested and analyzed 7 days post-challenge, with the exception of one 10^9 CFU treated dam whose organs were harvested a day earlier due to her premature death. Dose groups significantly different from one another ($p < 0.05$) are denoted by different letters; pregnancy groups that are significantly different from one other are denoted by a bar with an asterisk. Samples that had no countable colonies and were negative for enrichment were set at $\log_{10}(10$ CFU/g) = 1.00, the detection limit for the enrichment method, denoted by the dotted lines. $n \geq 3$ for each dose group comprised of pregnant animals. $n \geq 3$ for each dose group comprised of non-pregnant animals, except for control gallbladders, where n = 2. There were no non-pregnant animals treated at 10^3 CFU. □ Control, ▨ 10^3 CFU, ■ 10^5 CFU, ▨ 10^7 CFU, ■ 10^9 CFU

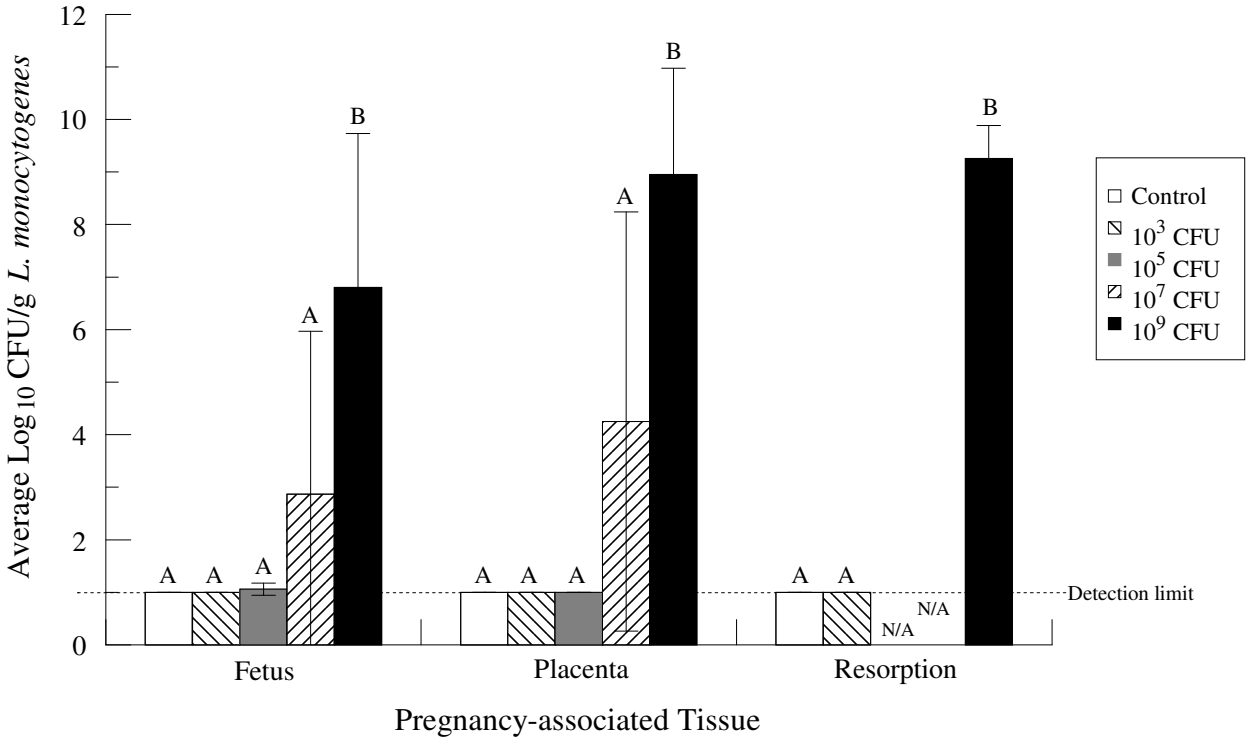

Figure 4.3. Invasion of pregnancy-associated tissues 7 days after maternal challenge with *L. monocytogenes*. Dose groups significantly different from one another ($p < 0.05$) are denoted by different letters. Samples that had no countable colonies and were negative for enrichment were set at $\log_{10}(10 \text{ CFU/g}) = 1.00$, the detection limit for the enrichment method. The sample size is equal to the number of dams. For fetus and placenta data, n = 4 for each dose group. For resorptions data, n = 4, 2, and 2 for control, 10^3, and 10^9 CFU groups, respectively.

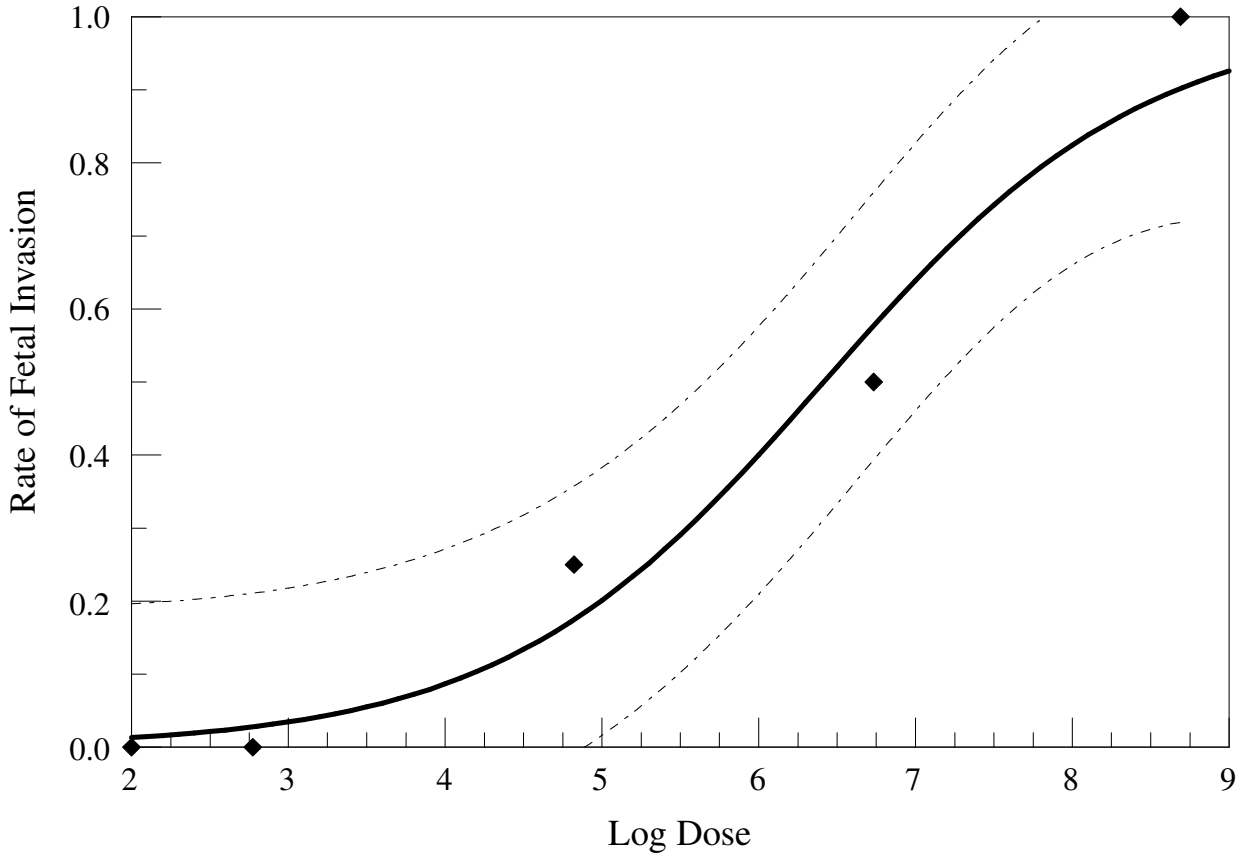

Figure 4.4. Dose-response of *L. monocytogenes* invasion in gerbil fetuses. A logistic model was used to fit the data (solid line) on the basis of dose resulting in fetal invasion. The estimated ID_{50} is 2.599×10^6 CFU *L. monocytogenes*. Solid dots represent the average invasion for each dose group. Dashed lines represent 95% confidence limits.

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CHAPTER 5

CONCLUSION

The Mongolian gerbil (*Meriones unguiculatus*) was recently proposed as the most appropriate small animal model for the study of listeriosis, the disease caused by the foodborne pathogen *Listeria monocytogenes*. The work presented in this thesis was concerned with studying the effects of *L. monocytogenes* invasion, particularly with regards to dose-dependent increases in adverse pregnancy outcomes, and comparing these effects among several animal models used in the study of listeriosis.

In the first project (Chapter 3), a method for time-breeding gerbils was developed to obtain the animals required for the main study. This method relied on inducing estrus in female gerbils by placing them on male bedding for three days prior to mating, then monitoring females for the performance of the lordosis reflex as an indicator of sexual receptivity. Using this technique, 9 of 10 females (90%) in which lordosis was observed became pregnant, while none of the animals in which lordosis was not observed became pregnant. Monitoring for lordosis was therefore employed during all remaining breeding cycles. To our knowledge, this is the first complete description for obtaining timed-bred gerbils, and this technique can be easily implemented by future researchers who require timed-pregnant gerbils.

In the main study (Chapter 4), gerbils were orally inoculated with a single dose *L. monocytogenes* in order to collect dose-response data on maternal and fetal invasion, fetal morbidity and mortality, and differences in the responses of pregnant versus non-pregnant animals. Significant ($p < 0.05$) dose-dependent increases were seen both in the number of fecal

samples positive for *L. monocytogenes* and in the number of adult organs positive for *L. monocytogenes*. A trend toward a dose-response was seen in the amounts of *L. monocytogenes* recovered from each sample, though significant only at the highest dose (10^9 CFU). Interestingly, no differences between pregnant and non-pregnant members of the same dose group were observed in either the number of fecal samples positive for *L. monocytogenes* or the amount of *L. monocytogenes* recovered in feces; however, significant differences both in the number of positive organ samples and in the amount recovered from almost all organs could be seen in the animals exposed to 10^9 CFU. In this highest dose group, *L. monocytogenes* was found in 94.7% of pregnant organs but only 58.3% of non-pregnant organs, and the intestine, liver, spleen, and brain tissues collected from pregnant animals had concentrations of *L. monocytogenes* that were 3 to 4 orders of magnitude higher than the concentrations found in non-pregnant animals. This pronounced difference in the invasion of adult organs was not expected, and would be interesting to study further, as a satisfactory explanation for why the fetus is at greater risk of contracting listeriosis than the general population has yet to be offered.

L. monocytogenes was isolated from fetuses of dams exposed to $\geq 10^5$ CFU *L. monocytogenes*, but fetal death was seen only in the highest dose group (10^9 CFU). Therefore, an ID_{50} of 2.599×10^6 CFU *L. monocytogenes* could be calculated, but the LD_{50} could only be estimated as lying somewhere between 5.68×10^6 and 5.08×10^8 CFU. While this LD_{50} range does include the LD_{50}s for both guinea pigs (1.999×10^7 CFU) and nonhuman primates (8.45×10^7 CFU), fetal deaths occurred in guinea pigs and nonhuman primates exposed to far lower doses of *L. monocytogenes* (10^6 and 10^3 CFU, respectively) than those required to see adverse pregnancy outcomes in gerbils (10^9 CFU). These results indicate that the gerbil is no more

sensitive, and may be less sensitive, than either the guinea pig or nonhuman primate models for invasion and adverse pregnancy outcomes caused by *L. monocytogenes*.

The findings of the dose-response study may prove useful to researchers and risk assessors when choosing an animal model for the study of listeriosis. Future studies should focus on examining the variability seen amongst individuals to help explain why some individuals are more susceptible to *L. monocytogenes* at lower doses than others. Potential threshold effects should also be investigated. Finally, more mechanistic research into the pathways utilized during *L. monocytogenes* invasion should be performed, as the current pathways considered to be most important to successful and efficient invasion are insufficient to explain differences, or lack thereof, between guinea pig, nonhuman primate, and gerbil models of listeriosis.