Executive Summary

Project Title: Refinement of *Listeria monocytogenes* (*L. monocytogenes*) Low Dose Data from Pregnant Guinea Pigs for Human Risk Assessment

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Submittal Date of Final Report to AMI: October 15, 2010

L. monocytogenes is a foodborne bacterium that has been found on a variety of foods including deli meats, and its occurrence has resulted in many recalls. To investigate its pathogenicity, we used pregnant guinea pigs as an animal model for human listeriosis (Williams et al, 2007) demonstrating that maternal exposure to high concentrations of *L. monocytogenes* resulted in the pathogen crossing the intestinal barrier, colonizing maternal and fetal tissues and eventually resulting in stillbirths, the same as seen in humans. To be useful in assessing human risk from exposure to *L. monocytogenes*, data were needed for exposure to low concentrations. The overall objective of this project was to conduct studies investigating exposures to low doses of *L. monocytogenes*, to identify biomarkers of *L. monocytogenes* infection and to use that information to refine the dose response curve, and ultimately the human health risk assessment for exposure to *L. monocytogenes*. Our specific objectives were: (1) to use *L. monocytogenes* labeled with green fluorescent protein (gfp) to examine the dose-related invasion of maternal liver, maternal spleen, placenta, fetal liver and fetal brain in guinea pigs, (2) to identify sublethal biomarkers for *L. monocytogenes* infection at low doses, and (3) focusing on the low dose area of the dose response curve, to develop a dose response curve for the sublethal endpoints and ultimately compare these dose response curves to those from primates and the FDA/USDA/CDC human risk assessment.

Using our pregnant guinea pig model, we showed that following ingestion of gfp-labeled *L. monocytogenes*, we isolated the pathogen from maternal liver and spleen, placenta, fetal liver and brain. Using fluorescent microscopy, we also demonstrated that the gfp maintained its fluorescence throughout our experimental procedure including and in culture without antibiotic pressure. Biomarkers of *L. monocytogenes* infection were identified including hepatic lesions, placental apoptosis, and changes in cytokine levels.

L. monocytogenes cells invaded maternal tissue in pregnant guinea pigs treated with 10^2 *L. monocytogenes* CFU; yet there was no fetal invasion. Exposure to *L. monocytogenes* at an early gestation did not affect invasion of maternal or fetal tissues. Using data from two previously established animal models, the guinea pig and nonhuman primate, we calculated LD_{50} values of 10^7 CFU which is similar to the estimate from the FAO/WHO (1.9×10^6 CFU) but unlike the 10^{13} CFU estimation by the FDA/USDA/CDC.

Deliverables:

The current study further characterized the guinea pig as a surrogate for human listeriosis. Deliverables from our specific aims are: 1) gfp-*L. monocytogenes* was visible using fluorescent microscopy but was not significantly better than traditional culture methods at low doses, 2) sublethal biomarkers of listeriosis were hepatic lesions, placental apoptosis, and changes in maternal cytokine levels, and 3) at 10^2 CFU *L. monocytogenes* invaded maternal liver and spleen but not fetal tissues. These studies resulted in four publications (plus one in preparation), and three awards for best presentations at national meetings.

Technical Abstract

The overall goal of our research was to use our established pregnant guinea pig model to develop data in the low dose region of the dose response curve and use that data to better predict human risk associated with consumption of *L. monocytogenes*. Individual objectives are presented below along with a brief discussion of the materials and methods, results and conclusions. For more details please refer to the attached manuscripts.

Objective 1. To use *L. monocytogenes* green fluorescent protein (gfp) to examine the dose-related invasion of maternal liver, maternal spleen, placenta, fetal liver and fetal brain in guinea pigs.

To determine the invasion of *L. monocytogenes* at low doses, we first wanted to determine the most sensitive method for confirming the presence of *L. monocytogenes* in maternal and fetal tissues. This study assessed maternal and fetal tissue invasion on post-inoculation days 2, 6, 9 and 21 using fluorescence microscopy and a traditional culturing method following an oral inoculation in pregnant guinea pigs. The dams were treated on gestation day 35 with doses ranging from 10^4-10^8 *L. monocytogenes* CFU. *L. monocytogenes* could be detected by both culturing and microscopy as early as 2 days post-inoculation in maternal and fetal tissues. Maternal spleen invasion by *L. monocytogenes* was similar to that seen in placentas, fetal livers and brains suggesting that it may be a predictor of fetal tissue invasion. Fecal shedding of *L. monocytogenes* was seen in all dams orally challenged with 10^4 CFU by post-inoculation day 7. While those given the higher doses of 10^6 or 10^8 CFU shed *L. monocytogenes* by post-inoculation day 5. When comparing detection methods (culturing vs. microscopy), both methods were about equivalent in confirming the presence of *L. monocytogenes*. However, there was some variation when comparing the two detection methods at different concentrations of *L. monocytogenes*. At the highest dose of 10^8 CFU, microscopy was more sensitive than culturing in detecting *L. monocytogenes* in fetal liver and brain. However at the lowest dose, 10^4 CFU, culturing was more sensitive in confirming the presence of *L. monocytogenes* in maternal spleens. Because we were interested in detecting *L. monocytogenes* at the low end of the dose response curve and because culturing appeared to be more sensitive than microscopy, the remaining experiments were completed using culturing methods. For additional information concerning Objective 1 please see the manuscript entitled "Time Course of Fetal Tissue Invasion by *Listeria monocytogenes* Following An Oral Inoculation in Pregnant Guinea Pigs," (Williams et al, in press).

Objective 2. Identify sublethal biomarkers for *L. monocytogenes* infection at low doses at or below fetalcidal levels (such as apoptotic or necrotic events, activation of cellular enzymes, or changes in cytokine levels).

To identify sublethal biomarkers for *L. monocytogenes* infection, we investigated the occurrence of apoptosis and necrosis in maternal liver, maternal serum cytokine concentrations, and mRNA expression of tumor necrosis factor α (TNFα), interferon γ (INF γ), interleukin-2 (IL-2), interleukin-5 (IL-5) and interleukin-10 (IL-10) in the near-term placenta. To determine the dose dependent trends of immunological and

pathological effects in pregnant guinea pigs after infection with *L. monocytogenes*, timed pregnant guinea pigs were treated on gestation day (gd) 35 with doses of 10^4 to 10^8 CFU and sacrificed on gd 56. Hepatic lesions were found in dams treated with $\geq 10^5$ CFU. Apoptosis was detected in significantly more placentas from dams treated with $\geq 10^6$ CFU compared to controls. Maternal serum TNF-α concentrations were significantly decreased in all dose groups compared to controls.

Placentas were analyzed to determine if treatment and duration of infection with *L. monocytogenes* affected mRNA expression of cytokines within the placenta. We analyzed three inflammatory cytokines, IFN-γ, TNF-α and IL-2 along with two anti-inflammatory cytokines, IL-5 and IL-10. Pregnant guinea pigs were treated on gestation day (gd) 35 with 10^8 *L. monocytogenes* CFU and sacrificed on gd 37, 41, 44, or 55. At gd 41, IFN-γ and IL-2 mRNA expression was significantly decreased in placentas from treated dams (0.0012-fold and 0.131-fold, respectively). At gd 55, TNF- α mRNA expression was significantly decreased (0.19-fold), while IFN- γ mRNA expression was significantly increased (32-fold), and apoptosis was detected in 100% of placentas from treated dams.

In summary, increases in premature delivery, maternal hepatic effects and placental apoptosis along with a decrease in TNF-α concentrations were associated with *L. monocytogenes* infection in pregnant guinea pigs. Inflammatory cytokine mRNA expression was altered and apoptosis was increased in the placenta after treatment with *L. monocytogenes*. Potential sublethal markers identified following ingestion of *L. monocytogenes* were TNF-α in maternal serum, placental IL-2, TNF-α and IFN-γ mRNA expression. For additional details concerning objective 2 please refer to the attached manuscripts, "*Listeria monocytogenes* infection in pregnant guinea pigs is associated with maternal liver necrosis, a decrease in maternal serum TNF-α concentrations, and an increase in placental apoptosis" (Irvin et al, 2008a) and "Immunological and pathological changes in the placenta during infection with *Listeria monocytogenes* in pregnant guinea pigs" (Irvin et al, 2008b).

Objective 3. To focus on the low dose area of the dose response curve, and use information obtained from the previous experiments to develop a dose response curve for these sublethal endpoints in guinea pigs and ultimately compare these dose response curves to our dose response data obtained from primates and to the dose response curves published in the FDA/USDA/CDC human risk assessment.

Data gaps in the low dose region of the dose response curve for *L. monocytogenes* need to be addressed to properly evaluate human risk associated with consumption of contaminated foods. We designed and conducted experiments that focused on exposure of the pregnant guinea pig to doses ranging from 10^2-10^8 *L. monocytogenes* CFU. The objectives of this study were to determine 1) whether low dose or early gestation impact the risk of tissue invasion with *L. monocytogenes,* fecal shedding and birth outcome, and 2) whether maternal treatment with *L. monocytogenes* induces changes in select pro-inflammatory and anti-inflammatory cytokine mRNA expression in fetal liver and brain.

Following ingestion of 10^2 CFU, *L. monocytogenes* was isolated from 29% and 14% of the dams' livers and spleens, respectively. Fecal shedding in dams exposed to 10^2 CFU was significantly higher than control animals. However, there were no fetal deaths or fetal tissue invasion following maternal exposure which suggests that a dose

greater than 10^2 *L. monocytogenes* CFU is needed for the pathogen to transmigrate across the placenta and subsequently infect the fetus.

Exposure during an earlier time of gestation (gd 22) does not increase the invasion of *L. monocytogenes* in maternal or fetal tissues following an oral challenge with the relatively high dose of 10^7 CFU. *L. monocytogenes* was isolated from 75% of both maternal livers and spleens, as well as all placentas, fetal livers and brains. Fecal shedding of *L. monocytogenes* was not detected in any control animals. Of dams with nonviable fetuses, *L. monocytogenes* was isolated from 100% and 33% of their fecal samples.

Of the dams that received 10^7 *L. monocytogenes* CFU on gd 35, the cytokines, IL-5, IL-2, IFN-γ and TNF-α were detected in fetal livers with IL-5 and TNF-α being significantly increased while IL-2 was significantly decreased when compared to controls. Of the fetal brains, IL-5, IL-2, IFN-γ and TNF-α were detected at lower concentrations with IL-5 and TNF-α being significantly decreased. We have now drafted the manuscript for this study, and the manuscript is in internal review. For additional details concerning this study please refer to the manuscript entitled, "Characterization of susceptibilities to *Listeria monocytogenes* exposure in pregnant and non-pregnant guinea pigs" (Williams et al, in preparation).

In our experiments, some animals assumed to be pregnant were not, but these animals were treated the same as pregnant animals. To determine whether the non-pregnant animals were susceptible to *L. monocytogenes* tissue invasion, we analyzed this data. *L. monocytogenes* invaded adult female (non-pregnant) guinea pig tissues following an oral challenge. *L. monocytogenes* was also isolated from their fecal samples. *L. monocytogenes* tissue invasion and fecal shedding were dose dependent. However, the non-pregnant guinea pigs showed no obvious signs of adverse effects from *L. monocytogenes* exposure.

Our final objective was to conduct a risk assessment using standard methodology, and compare dose response information from previous nonhuman primate and guinea pig studies conducted by our lab with dose response curves published in the FDA/USDA/CDC (2003) and FAO/WHO (2004) *L. monocytogenes* risk assessments for humans. Using a dose response curve based on mouse data, the FDA/USDA/CDC estimated a human LD_{50} of 10^{13} *L. monocytogenes* CFU. Recent animal studies using nonhuman primates and guinea pigs have both estimated LD_{50}s of approximately 10^7 *L. monocytogenes* CFU which is comparable to that of 1.9×10^6 CFU based on data from pregnant women consuming contaminated soft cheese (FAO/WHO, 2004). For additional details concerning the risk assessment please refer to the attached manuscript, "Risk assessment for *Listeria monocytogenes*-induced stillbirths based on dose response in pregnant guinea pigs and nonhuman primates" (Williams et al, 2009).

Recommendations for Future Research

Based on our studies *L. monocytogenes* can invade fetal tissues as early as 2 days post-inoculation. To elucidate the mechanism(s) used by *L. monocytogenes* to invade fetal tissues, additional time course studies are needed to identify if the pathogen enters the fetus earlier than 2 days post-inoculation. Recently it has been suggested that the gerbil is a better animal model than guinea pigs for listeriosis due to molecular similarities in the Met receptor. Studies are needed to compare susceptibility of

listeriosis in both gerbil and guinea pig models. Preliminary data suggest probiotics may prevent *L. monocytogenes* invasion of fetal tissues. The mechanisms of how probiotics may protect against listeriosis need to be elucidated using appropriate animal models.

Relevant International Presentations:

Smith, M.A., K. Agyekum and D. Williams. Probiotics reduce *Listeria monocytogenes*-induced tissue invasion and stillbirths in pregnant guinea pigs. Oral presentation at the International Symposium on Problems with Listeriosis (ISOPOL), Porto, Portugal, 2010.

Award: Best Poster Presentation (First Place)
Williams, D., J. Castleman, C. Lee, B. Mote and M.A. Smith. 2009. Risk assessment for *Listeria monocytogenes*-induced stillbirths based on dose response in pregnant guinea pigs and nonhuman primates. Poster presentation at the Teratology Society Annual Meeting, Rio Grande, Puerto Rico, 2009.

Award: Best Platform Presentation by a graduate student
Williams, D., E.A. Irvin, R.A. Chmielewski, J.F. Frank, J.F. and M.A. Smith. Maternal And Fetal Tissue Infectivity Following An Oral Challenge With *Listeria monocytogenes* And An Anti-internalin Peptide In Pregnant Guinea Pigs. Oral platform presentation at the Teratology Society Annual Meeting, Monterey, California, 2008.

Award: Best Platform Presentation by a graduate student
Irvin, E.A., D. Williams, A. Jensen, A.N. Richardson, and M.A. Smith. 2007. Changes in placental Th1 cytokines and apoptosis after infection of guinea pigs with Listeria monocytogenes. To be presented at the Teratology Society Annual Meeting, Pittsburgh, Pennsylvania, 2007.

M.A. Smith, D. Williams, E.A. Irvin, R. Chmielewski and J.F. Frank. "Comparison of listeriosis in pregnant guinea pigs and pregnant nonhuman primates," Oral platform presentation at the International Symposium on Problems of Listeriosis (ISOPOL), March 21-23, 2007, Savannah, GA.

Publications Associated with AMIF Project:

Williams, D., S. Dunn, A.N. Richardson, J.F. Frank and M.A. Smith. 2010. Time course of fetal tissue invasion by *Listeria monocytogenes* following an oral inoculation in pregnant guinea pigs. *Journal of Food Protection* (in press).

Williams, D., J. Castleman, C. Lee, B. Mote and M.A. Smith. 2009. Risk assessment for *Listeria monocytogenes*-induced stillbirths based on dose response in pregnant guinea pigs and nonhuman primates. *Risk Analysis* 11:1495-1505.

Irvin, E.A., D. Williams, K.A. Voss and M.A. Smith. 2008a. *Listeria monocytogenes* infection in pregnant guinea pigs is associated with maternal liver necrosis, a decrease in maternal serum TNF-a concentrations, and an increase in placental apoptosis.

Reproductive Toxicology 26:123-129.

Irvin, E.A., D. Williams, S.E. Hamler and M.A. Smith. 2008b. Immunological and pathological changes in the placenta during infection with *Listeria monocytogenes* in pregnant guinea pigs. *Reproductive Toxicology* 26:151-155.

In preparation:

Williams, D., A.N. Richardson, K. Agyekum, J.F. Frank and M.A. Smith. Characterization of susceptibilities to *Listeria monocytogenes* exposure in pregnant and non-pregnant guinea pigs. To be submitted to *Infection and Immunity*.