Listeria monocytogenes strains encoding premature stop codons in \textit{inlA} invade mice and guinea pig fetuses in orally dosed dams

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The reason why this subtype persists is not known, but we have excluded a number of causes, including a higher prevalence in the outdoor environment (Hansen et al., 2006), a better ability to grow or adhere to surfaces (Jensen et al., 2007), higher tolerance to disinfection agents (Kastbjerg & Gram, 2009) and enhanced tolerance to desiccation (Vogel et al., 2010). As this group of strains is both persistent and dominant in several fish-processing plants, it is a repeated source of food contamination and hence the virulence properties of these strains represent a key parameter in assessing risk to the consumer.

The persistent strains (N53-1 and La111) appear to have low virulence in a range of virulence models (Holch et al., 2010; Jensen et al., 2007, 2008a, b). They have low invasive ability in intestinal cells (Caco-2), which may be attributed to the presence of a premature stop codon (PMSC) in inlA (Holch et al., 2010). Internalin A (InlA) is used by L. monocytogenes to invade intestinal epithelial cells expressing the cellular receptor E-cadherin, and thus facilitates the crossing of the human body barriers during the establishment of systemic infection (Lecuit et al., 1997). InlA is a perfect match for the human E-cadherin receptor and also matches guinea pig E-cadherin (Lecuit et al., 2001). In contrast, the mouse E-cadherin is slightly different (Lecuit et al., 1999) and mice may not be an appropriate model for this oral infection. To overcome this, L. monocytogenes strains with an InlA that matches mouse E-cadherin have been constructed (Monk et al., 2010). Internalin B (InlB) is another listerial surface protein and the gene sequence of inlB in our persistent subtypes is 99% identical to inlB from the genome-sequenced L. monocytogenes EGD-e, which results in full invasion of persistent subtypes into fibroblastic L929 cells (Holch et al., 2010).

The food-processing persistent strains caused slower killing of eukaryotic Caenorhabditis elegans or Drosophila melanogaster than did clinical L. monocytogenes strains (Jensen et al., 2008a). These models indicate that the food-processing persistent L. monocytogenes strains are less virulent than clinical strains. We were therefore surprised to discover that persistent L. monocytogenes strains invaded placentas and fetuses in pregnant guinea pigs after oral dosing (Jensen et al., 2008b). We hypothesized that the persistent strains could be more invasive in placental cells than a clinical strain; however, the ability of the persistent strains to invade placental JAR cells (a human chorionicinoma cell line) was low, as was their ability to spread from cell to cell (Holch et al., 2010). Hence, the effective spread into guinea pig placenta and fetuses cannot be explained by known invasion mechanisms.

Few, if any, animal models are a perfect match for human infection, in part because of slight differences in the eukaryotic cell receptors used by L. monocytogenes (Khelef et al., 2006; Lecuit et al., 1999). The surface protein E-cadherin does not bind listerial InlA in mice (Lecuit et al., 1999), and listerial InlB does not activate the Met receptor in guinea pigs (Khelef et al., 2006). Several studies have described the placental invasion and colonization process of L. monocytogenes (Bakardjiev et al., 2004, 2005; Holch et al., 2010; Le Monnier et al., 2006, 2007; Lecuit et al., 2004; Robbins et al., 2010), but the results do not clearly demonstrate which listerial virulence proteins are important in the process, although several studies have pointed to InlA as a key surface protein (Disson et al., 2008; Lecuit et al., 1999; Robbins et al., 2010).

We hypothesized that the high infection rate of guinea pig placentas and fetuses by food-processing persistent strains could occur by circumventing InlA-mediated E-cadherin binding. The purpose of this study was to investigate this hypothesis by comparing the infection potential of selected L. monocytogenes strains in pregnant guinea pigs and pregnant mice, where the interaction between PMSC InlA and mouse E-cadherin is completely inconceivable.

**METHODS**

**Bacterial strains and preparation of inocula.** Four strains of L. monocytogenes were used in this study. Two strains (N53-1 and La111) belong to a molecular subtype that has been found to be the dominant, persistent subtype in several Danish fish-processing factories (Wulff et al., 2006). La111 was isolated in 1996 from cold-smoked salmon (Vogel et al., 2001) and has been tested previously in pregnant guinea pigs (Jensen et al., 2008b). N53-1 was isolated in 2002 from a surface in a fish smoke-house (Wulff et al., 2006). The two strains are indistinguishable by randomly amplified polymorphic DNA and amplified fragment-length polymorphism subtyping (Wulff et al., 2006) and show 99% identity when comparing 3300 predicted proteins from the genome sequences of the two strains (Holch et al., 2013). Furthermore, both strains have a PMSC in inlA (Holch et al., 2010). A human marmoset strain, 4810-98 (Smith et al., 2009), was used as positive infection control in pregnant guinea pigs (having matching E-cadherin) and a murinized strain (EGD-e InlA+; Monk et al., 2010) was used as positive control in infection of pregnant mice. By codon optimization, an EGD-e strain has been modified in inlA to show higher affinity for murine E-cadherin (Monk et al., 2010). The inocula were prepared as described previously (Jensen et al., 2008b). In brief, L. monocytogenes strains were subcultured three times at 24 h intervals in 10 ml tryptic soy broth (Oxoid) at 37 °C. Cultures were harvested (9000 g at 4 °C for 10 min), washed once and resuspended in phosphate-buffered saline (Oxoid). The cell suspensions were diluted to the desired cell density and 38% whipping cream was added to ensure consumption of all bacteria. At 4 ml sterile whipping cream to ensure consumption of all bacteria. At each day (GD) 30, and housed and dosed (at GD36) as described by Jensen et al. (2008b). Briefly, nine animals per bacterial strain were dosed at GD36 as described by Jensen et al. (2008b). Briefly, nine animals per bacterial strain were dosed at GD36 with the L. monocytogenes inoculum containing approximately 10^8 c.f.u. (4 ml whipping cream)~1~, followed by administering 2-4 ml sterile whipping cream to ensure consumption of all bacteria. At each day of killing (GD42, GD45 and GD56), three guinea pigs per bacterial strain were anaesthetized using Hypnorm/Dormicum and killed by decapitation. The liver, gall bladder, spleen, whole blood and placenta were collected from each pregnant animal on the day of death. The brain and liver were collected from each fetus. Nine animals were used for each bacterial strain, with 36 fetuses for N53-1-2, 37 for La111- and 50 for 4810-98-dosed animals. In addition, fresh placental samples (approximately 1 g) were collected for all living animals three times per week.

**Animals and tissue sample collection.** Timed-pregnant Dunkin Hartley guinea pigs were obtained from Charles River on gestation day (GD) 30, and housed and dosed (at GD36) as described by Jensen et al. (2008b). Briefly, nine animals per bacterial strain were dosed at GD36 with the L. monocytogenes inoculum containing approximately 10^8 c.f.u. (4 ml whipping cream)~1~, followed by administering 2-4 ml sterile whipping cream to ensure consumption of all bacteria. At each day of killing (GD42, GD45 and GD56), three guinea pigs per bacterial strain were anaesthetized using Hypnorm/Dormicum and killed by decapitation. The liver, gall bladder, spleen, whole blood and placenta were collected from each pregnant animal on the day of death. The brain and liver were collected from each fetus. Nine animals were used for each bacterial strain, with 36 fetuses for N53-1-2, 37 for La111- and 50 for 4810-98-dosed animals. In addition, fresh placental samples (approximately 1 g) were collected for all living animals three times per week.
Timed-pregnant BALB/c mice were obtained from Taconic on GD14, housed individually in cages and maintained on a 12 h light/dark cycle at 21 °C and 55 ± 15% humidity. After 1 day of acclimatization, mice were dosed intragastrically (using a stainless steel oral oesophageal tube attached to a syringe) with 200 μl of the whipping cream/Listeria innoculata (at GD15). Three animals were killed by cervical dislocation on the following 3 days (GD16, GD17 and GD18) and tissue samples were collected. The liver, gall bladder, spleen, eye blood (small volume of blood collected with a small glass capillary tube from the eye of the mouse), femur and placenta were collected from pregnant animals. Each fetus was defined as one sample, due to the small sample size. Nine animals were used for each strain, with 123 fetuses for N53-1, 77 for 4810-98- and 133 for EGD-e InIA"-dosed animals. In addition, fresh faecal samples (approximately 1 g) were collected each day for all living animals.

Listeria enrichment broth (UVM; Difco) was added to each tissue and faecal sample at a 1:10 ratio (tissue:broth) and the samples were homogenized in a stomacher for 1 min. The fetuses from mice were homogenized using an Ultra-Turrax (IKA), which is a working station for grinding and homogenizing various sample types. The femur bone was not homogenized before incubation in UVM broth.

The normal gestational period for guinea pigs is 65 days. In the present study, guinea pigs were killed on GD42, GD45 and GD56, representing the last trimester of pregnancy. The normal gestational period for mice is 21 days. In the present study, mice were killed at GD16, GD17 and GD18, also representing the last trimester of pregnancy. The day of dosing of the two types of animals (guinea pigs GD36, mice GD15) was approximately at the end of the second trimester of pregnancy.

All animal experiments were approved and conducted according to Danish legislation.

**L. monocytogenes detection and quantification in faecal and tissue samples.** Faecal and tissue samples were analysed qualitatively for *L. monocytogenes* (Jensen et al., 2008b) by enriching the homogenized tissue sample in UVM broth for 24 h at 30 °C. The blood sample was analysed as whole blood without pre-treatment before mixing with the UVM broth. After incubation, 100 μl UVM broth was transferred to 9.9 ml Fraser broth (Oxoid) and the samples were incubated at 37 °C for 24 h. Suspensions were streaked on Oxford plates (Oxoid) from both UVM and Fraser-enriched samples. Oxford plates were incubated at 37 °C for 24 h. Positive samples from Oxford plates were restreaked on brain–heart infusion (BHI) agar and incubated at 37 °C for 24 h. For confirmation as *L. monocytogenes*, colonies on BHI plates were streaked on Rapid* L. mono* plates (Bio-Rad) and incubated at 37 °C for 24 h. Tissue samples were also tested quantitatively by serial dilution and plating of the homogenized samples on BHI plates. Colonies were verified as *L. monocytogenes* by streaking on Oxford and Rapid* L. mono* plates.

**Statistical analyses.** When testing tissue samples on the maternal level, statistical significance was tested with the confidence interval of proportions. When testing placental and fetal samples from guinea pigs, statistical significance was tested by comparing the efficiency of infection, defined as the number of infected tissue samples relative to the total number of tissue samples within single animals and thus providing true independent samples, with the non-parametric Mann–Whitney *U* test. Because of the low number of positive placental and fetal samples in infected pregnant mice, it was not possible to test statistical significance using the non-parametric Mann–Whitney *U* test, where placental and fetal samples were independent of the pregnant animal. GraphPad Prism statistical software and Excel were used for all analyses. Values of *P*<0.05 were considered significant.

**RESULTS**

**L. monocytogenes in pregnant guinea pigs**

Faecal samples were collected from dosed pregnant guinea pigs on selected days during the pregnancy (Fig. 1a). The prevalence of positive faecal samples was between 0 and 100%. A significant difference between the percentage of positive faecal samples of the three tested strains was found on GD38 (*P*=0.0018) and GD41 (*P*=0.0016). N53-1 was shed until GD48, whereas 4810-98 was shed during the whole experimental period. Most of the faecal and tissue samples contained *L. monocytogenes* below the detection limit (10 c.f.u. g⁻¹) and the results are therefore reported as percentage of positive samples out of the total investigated within each sample category.

All three strains crossed the guinea pig intestinal barrier and reached the maternal liver and spleen after oral infection (Fig. 1b). Only La111 and 4810-98 were detected in the gall bladder. N53-1 and La111 were detected in maternal tissue at a prevalence of 18% (five positive maternal tissue samples out of 28 samples) and 32% (9/28), respectively, whereas 4810-98 was found in 56% (18/32) of tissue samples.

Strain 4810-98 was detected in half of the blood samples, where neither N53-1 nor La111 could be detected. La111 and N53-1 were each detected in 5 and 6% (2/37 and 2/36, respectively) of placental samples, and in 34% (17/50) of placenta dosed with the maternofetal strain 4810-98 (*P*=0.0002) (Fig. 1c). Strain 4810-98 also invaded the placenta to a higher level of 1.5×10⁴ c.f.u. g⁻¹ as compared with N53-1 and La111, which in all samples were below the detection limit of 10 c.f.u. g⁻¹. One animal dosed with 4910-98 had a stillbirth and the placental and fetal tissue samples were heavily infected with up to 2.9×10⁵ c.f.u. (g placenta)⁻¹, 1.9×10⁵ c.f.u. (g fetal liver)⁻¹ and 2.1×10⁴ c.f.u. (g fetal brain)⁻¹. The total number of infected fetuses (faeces with *Listeria*-positive liver or brain samples) was 14% (5/36) for strain N53-1, whereas 4810-98 only was detected in 6% (3/50) of fetuses. La111 was not identified in any of the fetal samples, despite invading the placental tissue to the same level as N53-1. In total, the number of positive placental and fetal samples were significantly higher for 4810-98 than for the two food-processing persistent strains (*P*=0.0248).

When summarizing all the data, however, there was no significant difference between the strains with a PMSC in *inlA* (N53-1 and La111) and the strain expressing full-length *inlA* (4810-98) in their ability to invade the tissues of pregnant guinea pigs (sum of maternal and fetal organs).

**L. monocytogenes in pregnant mice**

Faecal samples were collected from pregnant mice every day during the experimental period, which lasted for 3 days (Fig. 2a), and between one-third and all of the samples contained *L. monocytogenes*. The food-processing...
**Fig. 1.** Invasion of *L. monocytogenes* in orally dosed pregnant guinea pigs. The results show the percentage of pregnant guinea pig samples positive for *L. monocytogenes* in faecal samples (*n*=9 per bacterial strain) (a), maternal tissue samples (*n*=9 per bacterial strain) (b) and placenta and fetal samples (both *n*=36 for N53-1, *n*=37 for La111 and *n*=50 for 4810-98) (c). Filled bars, strain N53-1 (food processing); dark shaded bars, strain La111 (food processing); light shaded bars, strain 4810-98 (maternofetal). Sampling was carried out during pregnancy (GD38–56) after dosing of the animals on GD36. The bars show the sum (as percentage) of the number of positive samples detected for all 3 days of killing. Columns with different lower-case letters indicate statistically significant differences (*P*<0.05) between samples per sampling day (faecal samples) or per tissue type. Only three animals per strain were sampled on GD41 and GD48. Because of the low number of animals, no significant differences were seen between strains.

**Fig. 2.** Invasion of *L. monocytogenes* in orally dosed pregnant mice. The results show the percentage of pregnant mouse samples positive for *L. monocytogenes* in faecal samples (*n*=9 per bacterial strain) (a), maternal tissue samples (*n*=9 per bacterial strain) (b) and placenta and fetus samples (both *n*=58 for N53-1, processing persistent; *n*=77 for 4810-98, maternofetal; and *n*=61 for EGD-InlA<sup>m+</sup>, murinized) and fetus samples (c). Filled bars, strain N53-1; shaded bars, strain 4810-98; open bars, strain EGD-InlA<sup>m+</sup>. Sampling was carried out at three following GDs during the pregnancy. The bars show the sum (as percentage) of the number of positive samples detected for all 3 days of killing. Columns with different lower-case letters indicate statistically significant differences (*P*<0.05) between samples per sampling day (faecal samples) or per tissue type. Because of the low number of positive placenta and fetus samples from pregnant mice, it was not possible to test statistical significance using the non-parametric Mann–Whitney *U* test, where placental and fetal samples were independent of the pregnant animal.
persistent strain N53-1 was detected in faecal samples from most animals (78%, 7/9) at GD16; however, this decreased to 33% (1/3) on GD18. The human maternofetal strain 4810-98 showed the same pattern as N53-1. The murinized strain EGD-e InlA<sup>m</sup> was shed from all animals (nine animals at GD16 and three at GD18) on all days except GD17, on which only 67% (4/6) of the animals excreted <i>L. monocytogenes</i>. Each animal had at least 1 day with a positive faecal sample for each <i>L. monocytogenes</i> strain.

All three strains were detected in maternal gall bladder and spleen in 11% (1/9) of the animals (Fig. 2b). Of the maternal livers, one-third (3/9) were infected with EGD-e InlA<sup>m</sup>, 22% (2/9) were infected with 4810-98 and only 11% (1/9) were infected with N53-1. EGD-e InlA<sup>m</sup> was the only strain present in eye blood samples (11%, 1/9) and N53-1 was the only strain present in femur bone (11%, 1/9). However, none of the strains differed significantly from the other strains (P=0.3540 and 0.3540, respectively). Each fetus was considered as one sample and the appurtenant placenta as another sample (Fig. 2c). Only 1% of the placentas (1/77) and none of the fetuses (0/77) were infected with 4810-98, whereas EGD-e InlA<sup>m</sup> was found in 3% (2/61) of the placental samples and in 2% (1/61) of the fetuses. Notably, N53-1, which was detected at low prevalence in faecal material and maternal tissue samples, was found at the highest frequency of the three strains in placentas (6/58, 10%) and fetuses (2/58, 3%).

The number of placental and fetal positive samples showed a higher tendency for strain N53-1 than for 4810 and EGD-e InlA<sup>m</sup>. The number of positive maternal samples varied among animals with no systematic pattern (i.e. one type of positive sample did not appear to predict others being positive). Moreover, we did not find a systematic pattern between the number or types of maternal samples that were positive and the occurrence of infected placentas or fetuses. In addition, positive placental and fetal samples were not necessarily seen in a mother with positive tissue samples.

**DISCUSSION**

The ability of <i>L. monocytogenes</i> to invade the intestinal cell line Caco-2 is traditionally used to assess the virulence potential of different strains. This has often been combined with gene sequencing of inlA and inlB, as the corresponding proteins are mediators of listerial cell invasion (Dramsi et al., 1991). We have previously shown that a strain belonging to a food-processing persistent molecular subtype, which is repeatedly isolated in the fish-processing industry in Denmark, is able to cross the placental barrier in pregnant guinea pigs to the same level as a monkey clinical strain, even though the persistent strain has a PMSC in inlA (Holch et al., 2010; Jensen et al., 2008b). In the present study, this was confirmed using another strain of the same molecular subtype and we moreover demonstrate that this subtype can also effectively cross the placental barrier in pregnant mice. We decided to challenge pregnant guinea pigs with <i>L. monocytogenes</i> suspended in a food matrix to mimic human exposure to food. The concentration of bacteria in the suspension was chosen to simulate the concentration humans may be exposed to, rather than using the very high concentrations (approximately 10<sup>8</sup>) of other studies (Nightingale et al., 2008; Roldgaard et al., 2009; Van Stelten et al., 2011).

When <i>L. monocytogenes</i> interacts with human cells, the listerial surface protein InlA interacts with the human cell receptor protein E-cadherin (Mengaud et al., 1996). E-cadherin varies slightly between mammals (Lecuit et al., 1999) and guinea pig E-cadherin is identical, on the protein level, to human E-cadherin, while E-cadherin from mice has a single mutation and is consequently unable to interact with InlA. The interaction between <i>L. monocytogenes</i> and E-cadherin is essential for translocation of the bacterium across the intestinal barrier (Lecuit et al., 2001). We observed that a food-processing persistent strain of the same <i>L. monocytogenes</i> subtype as investigated previously (Jensen et al., 2008b) was able to invade placental and fetal samples, although to a lower level than a maternofetal strain (4810-98), which can cause the death of a human fetus a few weeks before the due date (Smith et al., 2009). We did not, however, see fetal infection by the strain La111, which has previously been reported to be highly invasive in guinea pig fetuses (Jensen et al., 2008b), but this could be explained by differences in inoculation levels, suppliers or housing facilities. More recent studies have revealed that both housing and breed have an important influence on the gut microbiota of experimental animals (Hildebrand et al., 2013; Hufeldt et al., 2010). As it is also recognized that the microbiota is of importance for the susceptibility of the host to infectious bacteria (Licht et al., 2012; Petersen et al., 2010), it is likely that different housing may cause different susceptibility to <i>Listeria</i> infection.

Despite an ongoing discussion about the importance of the interaction between InlA and E-cadherin in placental/fetal invasion (Bakardjiev et al., 2004; Disson et al., 2008; Robbins et al., 2010), the pronounced ability of strains with truncated InlA to invade placenta and fetus, seen both in the present study and in an earlier study by Jensen et al. (2008b), was somewhat surprising to us. Studies in pregnant animals with strains expressing truncated InlA have only been performed in the present study and in one previous study (Jensen et al., 2008b). Other studies have shown that the virulence of such strains in non-pregnant guinea pigs after oral/intrastragastric infection is lower than in strains expressing full-length InlA (Nightingale et al., 2008; Roldgaard et al., 2009; Van Stelten et al., 2011). In the present study, we show that strains with a PMSC in inlA can invade guinea pigs (maternal and fetal organs) to the same degree as a strain expressing full-length inlA.

We hypothesized that the food-processing persistent strains could use mechanisms other than InlA–E-cadherin when invading the placenta and fetus in a pregnant host, and could therefore infect pregnant mice without an
interaction between InLA and E-cadherin. In faecal shedding and maternal tissue, EGD-e InLA”” showed a tendency to be found in more samples than the strain belonging to the persistent subtype; and the persistent strain was also isolated from placentas and fetuses. This suggests that *L. monocytogenes* may use mechanisms other than InLA-E-cadherin when invading placental and fetal tissues, although we do not know the exact molecular mechanism. In this study, EGD-e InLA”” invaded maternal and fetal tissue in mice to a lower level than seen in a previous study by Monk *et al.* (2010); however, this was likely due to a lower inoculation level of the bacteria.

Robbins *et al.* (2010) have hypothesized that *L. monocytogenes* is transported to the placenta via the maternal bloodstream, where it is carried by phagocytes. The bacteria can invade the syncytiotrophoblastic cell layer, which is the outer cell layer of the placenta, either by cell-to-cell spread or by lysing the phagocyte followed by invasion via InLA-E-cadherin engagements. Several studies have investigated the individual virulence factors that are required by *L. monocytogenes* to cross the fetoplacental barrier (Bakardjiev *et al.*, 2004, 2005; Holch *et al.*, 2010; Le Monnier *et al.*, 2006, 2007; LeCuit *et al.*, 2004; Robbins *et al.*, 2010). The surface protein InLA is essential for invasion into explants from human placentas (LeCuit *et al.*, 2004; Robbins *et al.*, 2010) and human trophoblastic cells (Bakardjiev *et al.*, 2004; Holch *et al.*, 2010; LeCuit *et al.*, 2004); however, in an intravenously dosed pregnant guinea pig model, crossing of the placental barrier was independent of InLA (Bakardjiev *et al.*, 2004). Epidemiological studies have indicated that expression of full-length InLA is of significant importance for maternofoetal infections in humans since no/few strains with truncated InLA have been isolated from fetal infections (Disson *et al.*, 2008; Jacquet *et al.*, 2004). Collectively, these findings could indicate that crossing of the placental barrier differs between humans and guinea pigs, which would make our findings less alarming from a risk perspective.

We conclude that the fish-processing persistent strains are just as invasive as *L. monocytogenes* strains expressing full-length InLA in guinea pig placental and fetal infection, despite the apparent mismatch between InLA and E-cadherin. Hence, the animal placental and fetal infection by these strains appears independent of InLA and, unfortunately, very effective. Our data reveal that virulence assessment, as determined by cell invasion assays or sequencing of virulence genes, may not completely reflect the fetal infection risk of a given strain (Holch *et al.*, 2010; Jensen *et al.*, 2008a). Listerial infection is the result of a complex interplay between immune factors from the host and virulence factors from the invasive bacteria, and no model has (yet) been able to mimic this *in vitro*.

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