Research article

Characterisation of *Listeria monocytogenes* isolates from cattle using a bovine caruncular epithelial cell model

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ABSTRACT

Listeria monocytogenes is an important foodborne pathogen in human and veterinary health, causing significant morbidity and mortality including abortion. It has a particular tropism for the gravid uterus, however, the route of infection in reproductive tissues of ruminants (i.e. placentome), is much less clear. In this study, we aimed to investigate a bovine caruncular epithelial cell (BCEC) line as a model for *L. monocytogenes* infection of the bovine reproductive tract. The BCEC infection model was used to assess the ability of 14 different *L. monocytogenes* isolates to infect these cells. Lysozyme sensitivity and bacterial survival in 580 µg lysozyme/ml correlated with attenuated ability to proliferate in BCEC (p = 0.004 and p = 0.02, respectively). Four isolates were significantly attenuated compared to the control strain 10403S. One of these strains (AR008) showed evidence of compromised cell wall leading to increased sensitivity to ß-lactam antibiotics, and another (7644) had compromised cell membrane integrity leading to increased sensitivity to cationic peptides. Whole genome sequencing followed by Multi Locus Sequence Type analysis identified that five invasive isolates had the same sequence type, ST59, despite originating from three different clinical conditions. Virulence gene analysis showed that the attenuated isolate LM4 was lacking two virulence genes (*uhpT*, *virR*) known to be involved in intracellular growth and virulence.

In conclusion, the BCEC model was able to differentiate between the infective potential of different isolates. Moreover, resistance to lysozyme correlated with the ability to invade and replicate within BCEC, suggesting co-selection for surviving challenging environments as the abomasum.

1. Introduction

The zoonotic intracellular pathogen *Listeria monocytogenes* causes a range of clinical presentations including listeriosis, meningitis, septicaemia and abortions, in both cattle and humans. In humans and a number of animals, *L. monocytogenes* is known to be able to invade the placenta during pregnancy, leading to spontaneous abortion or neonatal infection with high mortality rates [1, 2]. Listeriosis is of major veterinary importance in cattle due to its negative impact on animal health resulting in premature death or reproductive failure responsible for economic losses [3].

The route by which *Listeria* spp. infect the ruminant placenta is unclear. Most studies have focused on infection of humans and rodents, and distinct species differences in placental structures as well as interhemal barriers mean that making comparisons between ruminants and infection in other species is erroneous [4]. In the placenta, maternal and fetal tissues interact. In hemochorial placentas that are present in humans or guinea pigs, maternal blood comes into direct contact with fetal trophoblast cells. In contrast, in synepitheliochorial placentas found in cattle and sheep, the maternal and fetal blood are separated by several cell/tissue layers which any pathogen must cross to cause fetal infection [5]. In addition, the ruminant placenta is composed of multiple

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placentomes throughout the uterus, with each placentome formed from fetal cotyledons interdigitating with maternal caruncules. The latter are formed by multiple layers of stromal cells covered in a single layer of caruncular epithelial cells that interact with the fetal trophoblasts [5]. *L. monocytogenes* have been isolated from infected bovine placentomes post abortion and identified as the causative agent of disease [1]. In addition it has been shown that *L. monocytogenes* is able to invade and

replicate in trophoblastic cells derived from a bovine chorioallantoic membrane explant model [6].

Listeria spp. invasion is primarily mediated by the interaction of the surface Internalin (Inl) proteins A and B with host cell receptors E-cadherin and c-Met tyrosine kinase (c-Met), respectively. For InlA-dependent entry into cells, proline at position 16 of E-cadherin is critical; in rats and mice if this proline is replaced by glutamic acid, then InlA-dependent
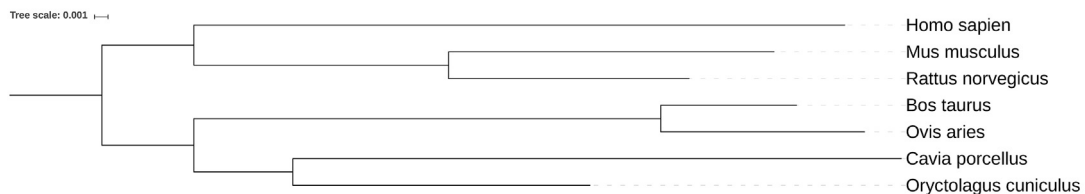

Figure 1. Bovine E-cad and cMet sequence comparisons and expression. (A) Multiple sequence alignment of the Pro16 residue of E-cad correlating with probability of invading the host cell [12], emboldened letters indicate amino acid substitutions previously identified in other studies and red letters indicate amino acid substitution unique to rodents. (B) Multiple sequence protein alignments of the region of c-MET, emboldened letters indicate amino acid substitutions which have previously been identified as a primary interface between InlB and c-MET [19], blue letters indicate relatedness of amino acid substitutions between certain species and red letters indicate amino acid substitutions discovered in this work. (C) Maximum likelihood tree of c-Met. E-cad (D) and c-MET (E) transcript levels in BCEC cells stimulated with 1 μg ml^{-1} LPS or infected with *L. monocytogenes* (MOI = 200) for 4, 8 or 24 h.

entry into cells is prevented [7]. Whereas InlB-dependent cell invasion via c-Met does not occur in rabbits and guinea pigs but is functional in both mice and humans [8]. However, InlB also has a secondary role of activating Phosphoinositide 3-kinase (PI3–K), which is required for InlA-dependent invasion in tissues that do not constitutively express this enzyme activity [9]. InlA and InlB may also act in a concerted manner during invasion of the placenta [10]. Given this complex interplay, it is not surprising that the *inlA* and *inlB* genes are arranged in an operon and can either be expressed as one bi-cistronic mRNA or independently expressed from promoters [11] and there are multiple promoters which are controlled by both the virulence regulator, PrfA [11], and the stress sigma factor, Sigma B [12]. Generally, *inlA* mRNA levels are slightly higher than those for *inlB,* and expression of both genes is higher in the stationary phase of growth or under other environmental stress conditions [13]. However, it is also widely reported that many environmental strains may contain mutations in InlA which result in a less invasive phenotype [14], therefore, sequencing of the gene is required as well as monitoring mRNA levels to fully characterise the virulence potential of strains [15].

Recently, genome wide analysis has led to the identification of InlP as a virulence factor linked to tissue tropism in the gravid uterus [16]. Secreted InlP targets cytosolic afadin and, after host cell invasion mediated by InlA or InlB, facilitates bacterial spread from infected epithelial monolayers into an underlying compartment during placental infection [17].

In this study, we investigated a bovine caruncular epithelial cell (BCEC) line as a model for *L. monocytogenes* infection of the bovine reproductive tract. BCECs, epithelioid shaped cells, express cytokeratin, zonula occludens-1 and vimentin, while lacking desmin and alpha smooth muscle actin; and form tight junctions with trans epithelial resistance increasing with advancing confluence [18, 19]. To predict the likelihood of interaction of InlA and InlB with bovine E-cadherin and c-Met, respectively, the sequence and mRNA expression of the genes encoding these receptors were analysed. We hypothesized that *L. monocytogenes* isolates from bovine abortions might more readily infect bovine caruncules and replicate within these cells. Therefore, the ability of a range of *L. monocytogenes* isolates from different clinical or environmental sources to infect the bovine caruncular epithelial cell lines was investigated. In addition, whole genome sequencing was used to further characterise the genome content of these isolates.

2. Results

2.1. *Sequence comparisons of host receptors E-cadherin and c-Met tyrosine kinase receptors in caruncular cells*

For this study a bovine caruncular epithelial cell (BCEC) line was chosen as model a for *L. monocytogenes* infection of the bovine reproductive tract. Since host specificity towards InlA-dependant entry into cells depends on the presence of proline at position 16 of E-cadherin in the first extracellular domain [7], alignment of the E-cad region of a range of species containing residue 16 was performed (Figure 1A). This showed that bovine E-cadherin has proline at position 16 and suggests that bovine and ovine E-cadherin will interact with InlA in a similar way to human and guinea pig E-cadherin and can act as a receptor for *L. monocytogenes* in ruminant species. Interactions between InlB and c-Met are less well defined; in c-MET the Sema, PSI and Ig1 region have shown to play a role in interaction with InlB [20]. Alignment of amino acids in these c-Met regions derived from bovine, ovine, human, murine, rabbit and guinea pig genome sequences showed that bovine c-Met does not cluster closely to rabbit and guinea pig c-Met (Figure 1C). There were no consistent amino acid substitutions evident in the six amino acids of the Ig1 region that interacts with InlB (Figure 1B), indicating that there is no obvious structural reason why InlB-dependent cell entry would not occur when *L. monocytogenes* interacts with bovine cells.

Expression of *E-cadherin* and *c-Met* mRNA in BCEC cells was then verified and mRNA for both were detected in these cells in the presence and absence of *L. monocytogenes* infection (Fig. 1D & E). No difference in *E-cadherin* and *c-Met* mRNA expression level was observed when these cells were exposed to four different *L. monocytogenes* isolates (Fig. 1D & E) indicating that infection with these bacteria did not down-regulate these receptors. Taken together, these results suggest that *L. monocytogenes* interaction with BCECs was likely to utilise the InlA/B receptor-mediated uptake pathway.

2.2. *L. monocytogenes infection of bovine caruncular epithelial cells*

The *L. monocytogenes* strains chosen for this study included the well characterised reference strain 10403S and isolates from different bovine samples (Table 1). These included 6 strains from bovine abortions, 4 strains from bovine eyes, 1 strain from a case of bovine meningitis and 2 strains isolated from bovine milk. Initial experiments carried out to establish an infection method using the BCEC cells used a range of MOIs. This revealed that MOI of at least 200 was required to achieve consistent bacterial recovery from BCEC cells 2 h post infection (data not shown), which is high but not unexpected as placental tissues are not easily or immediately invaded by *L. monocytogenes* [22]. Thus, all subsequent infections of BCEC cells were carried out using a MOI of 200. After 2 h of infection, very low levels of intracellular bacteria (mean 0.78–1.5 $\log_{10}$ CFU per 2×10^5 BCEC cells per well) were recovered and high levels of variability were observed between replicate infections. Although, the level detected was close to the detection limit (0.7 $\log_{10}$ CFU per 2×10^5 BCEC cells per well), all isolates tested were able to invade BCEC 2h post infections to a similar extent (Fig S1 A). A preliminary time course of 4–24 h incubation post-infection showed that 24h yielded the most consistent and reproducible levels of bacterial recovery (Fig S1 B), therefore this was used for further experiments.

Using this infection model, the ability of the different *L. monocytogenes* isolates to invade BCEC cells was investigated. Of the 14 isolates tested, four were significantly attenuated compared to the control strain (10403S). These were an isolate from a healthy bovine eye (AR008, P < 0.001), an isolate from a milk processing plant (LM4 P < 0.01) and two abortion isolates (7644 P < 0.05, C07754 P < 0.001) (Figure 2) and the percentage of intracellular bacteria recovered compared to the control were 1.9 %, 8 %, 0.5% and 33 %, respectively (Figure 2). No induction of extensive cell death was observed microscopically that would account for these low invasion rates.

Differences in the ability of *Listeria* strains to internalise may be due to the variation in *inlA* or *inlB* expression levels therefore *inlA* and *inlB* mRNA levels were measured in the cultures used to inoculate the BCEC cells. All isolates expressed *inlA* and *inlB* mRNA and this was not found to vary between strains (Figure 3). As expected, *inlA* and *inlB* mRNA levels were positively correlated (r = 0.58, p = 0.03) and as previously reported the levels of *inlB* mRNA were consistently lower than those of *inlA* [23]. Thus, there was no evidence that differences in InlA or InlB levels in these strains would account for the differences in levels of intracellular bacteria recovered, although differences in gene sequences could not be ruled out at this stage.

2.3. *Sensitivity to lysozyme correlates with attenuated ability to proliferate in BCECs*

We have previously reported the lysozyme sensitivity of these strains [21]. This was found to be an important factor in determining the ability of most *L. monocytogenes* strains to infect a bovine conjunctiva explant model [21]. Interestingly, the number of recovered bacteria from BCEC cells after 24 h of infection again correlated strongly with levels of lysozyme resistance (MIC [r = 0.82, p = 0.004]; bacterial survival in 580 µg lysozyme/ml [r = 0.72, p = 0.02]). Growth rate of the strains was determined in laboratory culture to see if this was a factor that might affect the number of intracellular bacteria recovered. However, there was

Table 1. *Listeria monocytogenes* isolates used in this study.

Strain number	Source[a]	PCR Serotype[c]	PCR Lineage[d]	ST/CC[e]		Generation time [min][f]	Source/Reference
10403S	Skin Lesion	1/2a	II	85	CC7	50 ± 7.5	(Bishop and Hinrichs, 1987) [55]
AR008	Healthy eye	1/2a, 3a[b]	II[b]	12	CC7	83.33 ± 13.89[g]	(Warren et al., 2015) [21]
C00938	Kerato-conjunctivitis	1/2a, 3a[b]	II[b]	20	CC20	43.48 ± 0.76	APHA
R06262	Kerato-conjunctivitis	1/2b, 3b[b]	I[b]	59	CC59	50 ± 10	APHA
C02118	Kerato-conjunctivitis	4b[b]	I[b]	6	CC6	45.45 ± 2.07	APHA
LM7644	Abortion	1/2a, 3a[b]	II[b]	122	CC9	62.5 ± 11.72[h]	APHA
C08389	Abortion	1/2a, 3a[b]	II[b]	7	CC7	58.82 ± 17.3	APHA
C08078	Abortion	1/2b, 3b	I	59	CC59	52.63 ± 5.54	APHA
C07872	Abortion	1/2b, 3b	I	59	CC59	55.56 ± 6.17	APHA
C04949	Abortion	1/2b, 3b	I	59	CC59	38.46 ± 8.88	APHA
C07754	Abortion	1/2a, 3a	II	91	CC14	38.46 ± 2.96	APHA
G03652	Meningitis	1/2b, 3b[b]	I[b]	59	CC59	52.63 ± 5.54	APHA
LM4	Milk	1/2b, 3b[b]	I[b]	1009	CC5	66.67 ± 4.44[i]	(Lawrence et al., 1995) [56]
LM6	Milk	4b[b]	I[b]	1	CC1	52.63 ± 5.54	(Lawrence et al., 1995) [56]

APHA: Animal and Plant Health Agency.

[a] All isolates are from bovine sources except for the human isolate 10403S.

[b] Warren et al., 2015 [21].

[c] Serotypes were determined using the PCR-based method of Doumith et al (2004). This method in conjunction with the lineage typing cannot distinguish between serotypes 1/2a and 3a or 1/2b and 3b. However, serotypes 3a and 3b are not commonly isolated.

[d] Lineages were determined using the PCR-based method of Ward et al. (2004).

[e] Institute Pasteur *Listeria* MLST data base.

[f] Growth rates in broth culture presented as mean +/- SD (n = 3).

[g] Growth rate reduced compared to C04949 (p = 0.001).

[h] Growth rate reduced compared to C04949 (p = 0.024).

[i] Growth rate reduced compared to 10403S (p = 0.039).

no correlation with growth rate of these strains (r = 0.49, p > 0.05) or with the ability of the these strains to infect conjunctiva explants (r = 0.55, p > 0.05) (Table 2; for MIC and survival in lysozyme see [21]).

To investigate the basis of differences in sensitivity to lysozyme of these strains, isolates were challenged with β-lactam antibiotics (ampicillin, penicillin G and cefuroxime) to test cell wall integrity and also a cationic peptide (mCRAMP) to test membrane integrity. Only one isolate, AR008 (isolated from a healthy bovine eye), showed significant increased sensitivity to ampicillin (p = 0.04), penicillin G (p = 0.004) and cefuroxime (p = 0.0001) compared to the reference strain 10403S, suggesting that a compromised cell wall may contribute to the lysozyme sensitivity of this isolate (Figure 4 A–C). Of the three lysozyme-sensitive isolates, only isolate 7644 showed sensitivity to mCRAMP, indicating

that compromised cell membrane integrity may contribute to the lysozyme sensitivity of this isolate (Figure 4 D–F).

2.4. Analysis of L. monocytogenes sequence types, core genomes and virulence genes

To further characterise these isolates, WGS was carried out on all the uncharacterised isolates and these sequence data were added to the open access Pasteur MLST database to determine sequence types (ST) (Table 1, Figure 5A). From the ten identified, eight were single STs, while five isolates belonged to ST59. Interestingly, while the ST59 isolates were collected over several years (1999–2012) and from three different clinical presentations (keratoconjunctivitis (n = 1), meningitis (n = 1),
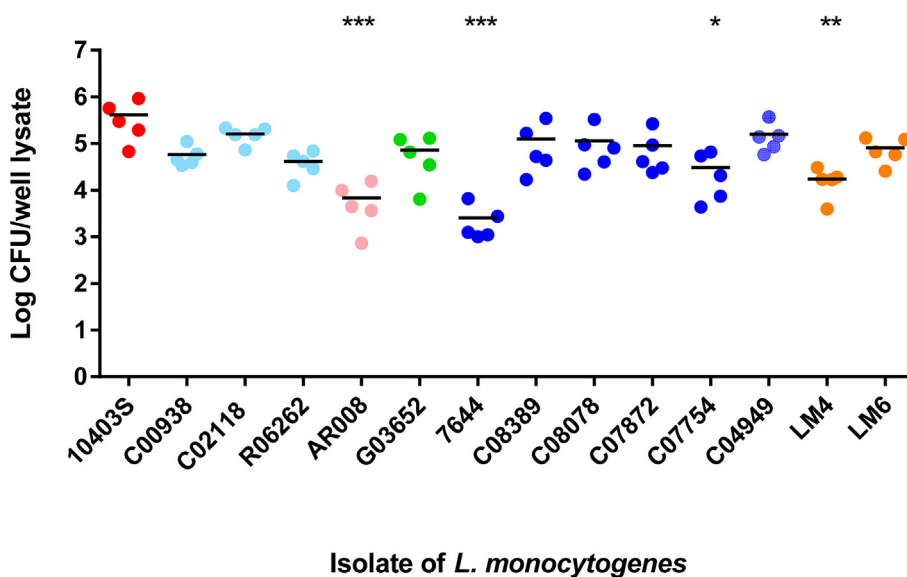
Figure 2. Infection of BCEC cells. BCEC cells were infected with an MOI = 200 with *L. monocytogenes* isolates for 24 h at 37 °C; For each isolate 5 independent experiments were performed, as represented by each symbol. Dark blue indicates isolates from cases of bovine abortion, pale blue indicates isolates from bovine keratoconjunctivitis, orange indicates isolates from an environmental source, green indicates isolates from a case of meningitis, pink indicates an isolate from a healthy eye and red indicates the control strain 10403S originally a human isolate from a skin lesion. All data points and mean are shown. Statistical significance is shown compared to 10403S: *p < 0.05, **p < 0.01, ***p < 0.001, (Kruskal-Wallis test followed by Dunn's multiple comparisons test).

● *inlA*
△ *inlB*
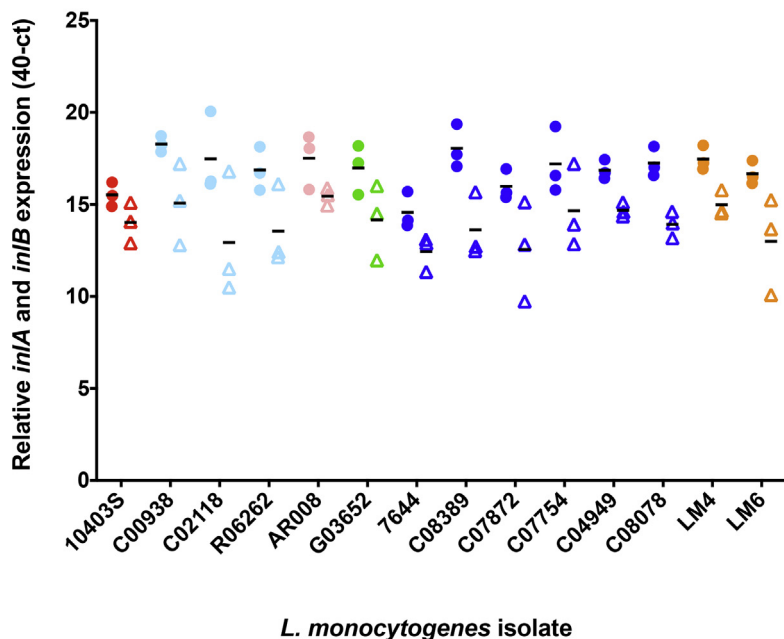
Figure 3. InlA and InlB expression. *inlA* and *inlB* transcript levels in *L. monocytogenes* isolates grown to late log phase in HI medium. For each isolate 3 independent experiments were performed, as represented by each symbol. Dark blue indicates isolates from cases of bovine abortion, pale blue indicates isolates from bovine keratoconjunctivitis, orange indicates isolates from an environmental source, green indicates isolates from a case of meningitis, pink indicates an isolate from a healthy eye and red indicates the control strain 10403S originally a human isolate from a skin lesion (Kruskal-Wallis test followed by Dunn's multiple comparisons test).

abortion (n = 3)), they were all able to infect and replicate inside BCEC cells at levels comparable to 10403S (Figures 2, 5).

Core genome analysis was carried out for the 128 isolates in the MLST database where a genome sequence was available (accessed 24/7/2017, Table S1). This showed that isolates C00938 (ST20), C07754 (ST91), C02118 (ST6), AR008 (ST12) and LM6 (ST1) all cluster with other isolates from these sequence types (Figure 6). Some clusters contained more than one sequence type, for instance LM7644 (ST122) clustered with sequence types ST9, ST622 and ST441. C08389 (ST7) is part of a cluster that also contains ST58 isolates (including 10403S) and ST98. Isolates LM4 (ST1009) and LM7 (ST220) were the only isolates of that sequence type present in the database but clustered closely with ST5 and ST194, respectively (Figure 6).

Further analysis of the WGS data for virulence gene content (presence and absence of genes as well as sequence similarity), showed that they clustered according to their linage and serotype as expected (Figure 7, Table S2). Sequence identity in general was high, between 91.3-100%, except for *inlK* (90.1–100%) and genes encoding the sRNA family *lhrC* (90.1–100 %) (Figure 7, Table S2). Five genes were only present in the six isolates of the 1.2a, 3a serotype: *inlL* (adherence), *ami* (adherence), *vip* (invasion) and two genes of the *lhrC* family (non-coding regulatory sRNA) (Figure 7, Table S2). The absence of those genes did not correlate with attenuation in the context of BCEC infection. In contrast, the attenuated isolate LM4 was the only one in this study that lacked two virulence genes known to be involved in intracellular growth and virulence. These were the *uhpT, the* sugar phosphate antiporter important for intracellular proliferation [24, 25] and *virR*, a transcriptional two component response regulator implicated in cell invasion and virulence

in vitro and *in vivo* [26, 27] (Table S2). In addition, LM4 had three other virulence genes (*srtB* (surface display), *sipZ* (intracellular survival) and *inlC* (internalin)) with the lowest reported level of sequence identity to the reference genome (Table S2).

Analysis of the *inlA* sequences of these isolates for known changes that would be predicted to reduce levels of InlA (i.e. frameshifts causing premature stop codons or mutations in the promoter region [28]) was also performed. In agreement with the results gained from the *inlA* mRNA analysis (Figure 3), no differences were identified in the *inlA* and *actA* promoter regions that might contribute to the lower levels of cell invasion and intracellular replication recorded. Similarly, InlP had 94% identity at the protein level across all isolates and therefore this did not seem to provide an explanation for the variation seen in the ability of these strains to infect the BCEC cells.

3. Discussion

L. monocytogenes has been isolated from placentomes of infected cattle [1]. The maternal caruncle contains a dense network of blood vessels [29] allowing *Listeria* access to the maternal side of the placenta through the blood stream. The caruncle is also in close contact with the fetal chorion, meaning infection of the uterus can lead to endotoxaemia, an increased prostaglandin synthesis and subsequent lysis of the corpus luteum, leading to abortion. Alternatively, placentitis itself can disrupt the metabolic exchange of nutrients to the fetus, triggering the abortion [30]. While BCECs have been used for *L. monocytogenes* infections as a comparison to other tissues previously [31, 32] this study presents BCEC cells as infection model to characterise bovine *L. monocytogenes* isolates

Table 2. Pearson correlations.

|	r	95% conf interval	R^2	p-value
mRNA expression: *inlA* vs *Inl*B	0.58	0.07 to 0.85	0.34	**0.03**
Intracellular bacteria count (log CFU/well with:				
MIC lysozyme	0.82	0.40 to 0.96	0.67	**0.004**
Survival in 580 µg/ml lysozyme (log CFU/ml)	0.72	0.16 to 0.93	0.52	**0.02**
Growth rate in HI broth	0.49	-0.05 to 0.81	0.24	0.073
Intracellular bacteria counts in conjunctiva	0.55	-0.12 to 0.88	0.31	0.097

p-values <0.05 were deemed significant and highlighted in bold.
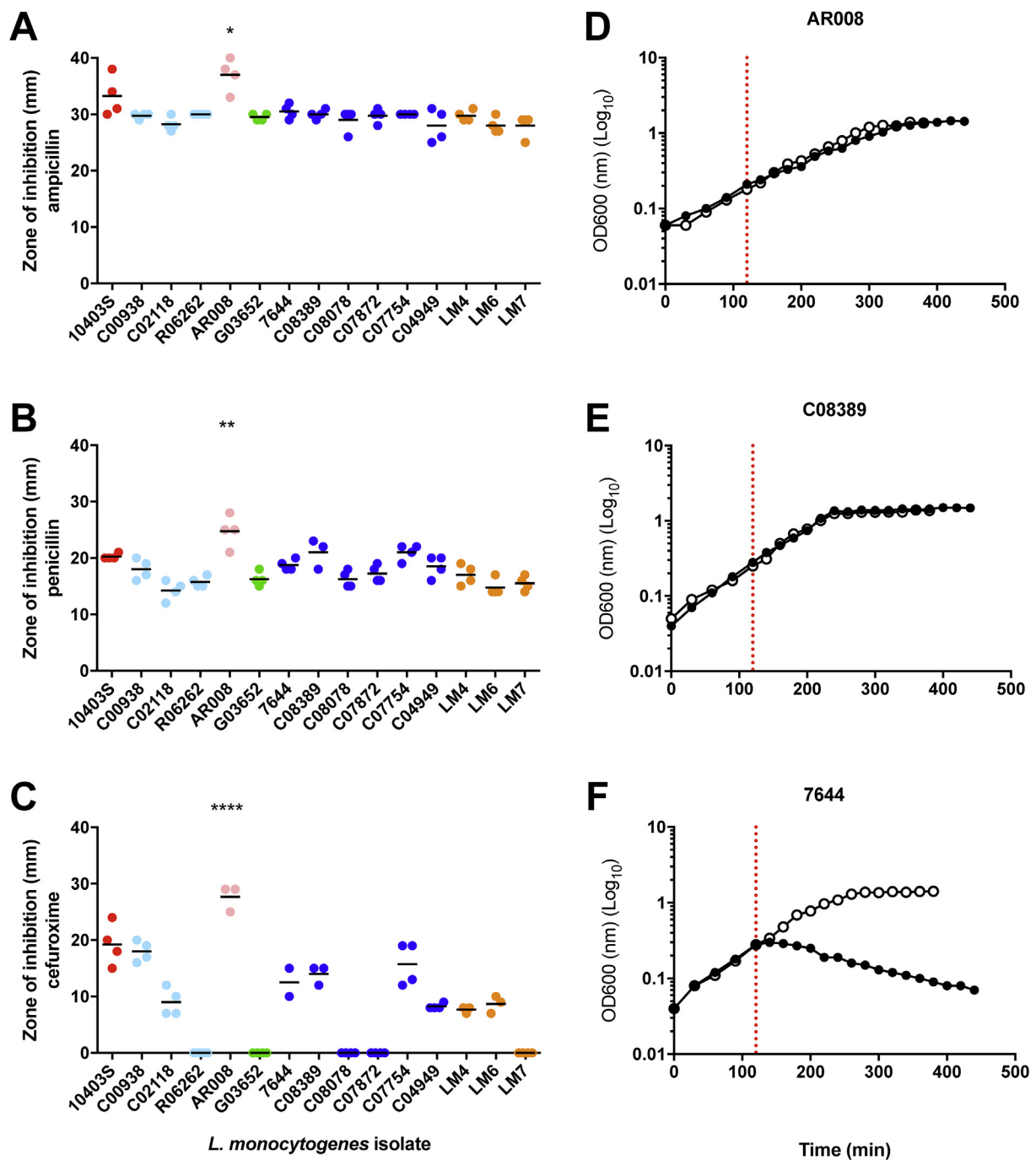
Figure 4. Treatment of *L. monocytogenes* with cell wall acting antibiotics and CRAMP. To investigate cell wall integrity, overnight cultures were plated on heart infusion agar and disks containing 1U penicillin G (A), 25μg ampicillin (B) or 30μg cefuroxime sodium (C). The plates were incubated overnight at 37 °C, and zones of inhibition were measured. Dark blue indicates isolates from cases of bovine abortion, pale blue indicates isolates from bovine keratoconjunctivitis, orange indicates isolates from an environmental source, green indicates isolates from a case of meningitis, pink indicates an isolate from a healthy eye and red indicates the control strain 10403S originally a human isolate from a skin lesion. For each isolate 5 independent experiments were performed. All data points and mean are shown. Statistical significant increase in susceptibility is shown compared to 10403S using a. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (One Way ANOVA followed by Dunnett's multiple comparisons test). To assess cell membrane integrity, duplicate cultures of *L. monocytogenes* isolates AR008 (D), 7644 (E), C08389 (F) were grown to log phase in HI broth at 37 °C and stimulated with a final concertation of 10 mg ml^{-1} CRAMP/DMSO (closed circles) or DMSO alone (open circles). The red dashed line indicates time of stimulation. Absorbance at 600 nm was measured in 20 min intervals. Data are representative of at least duplicate experiments.

from different clinical presentations and sources. Genome sequencing of the isolates used in this study revealed that within our set of bovine clinical isolates, collected across the UK over several years and from different disease presentations, the MLST sequence type ST59 was over represented, with 5 out of the 10 clinical isolates belonging to that sequence type. Core MLST analysis further confirmed that they are

closely related, forming a distinct cluster with other ST59 isolates. This sequence type has been previously reported to be isolated in France predominantly associated with human infections [33]. In this study it was noted that the clones most prevalent in human patients were also those most frequently associated with bovine listeriosis, and the ST59 strains in this study were isolated from cases of Kerato-conjunctivitis,
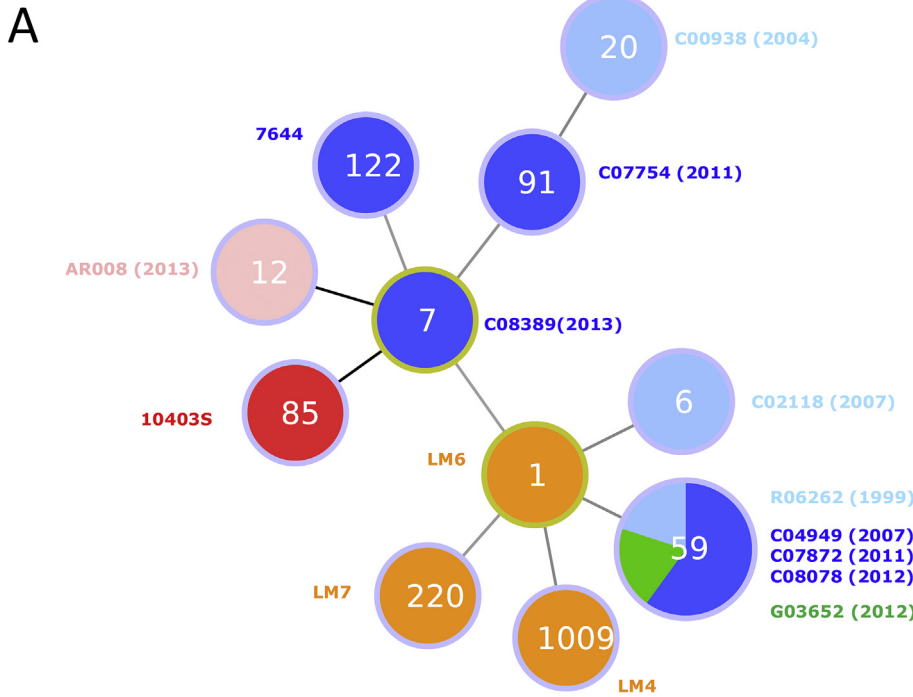
Figure 5. Epidemiological analysis of *L. monocytogenes* isolates based on MLST. (A) goeBURST analysis of the isolates used in this study; dark blue indicates isolates from cases of bovine abortion, pale blue indicates isolates from bovine keratoconjunctivitis, orange indicates isolates from an environmental source, green indicates isolates from a case of meningitis, pink indicates an isolate from a healthy eye and red indicates the control strain 10403S originally a human isolate from a skin lesion. Numbers in the circles denote the sequence type. (B) Maximum likelihood tree of the UK isolates present in the MLST database. Shaded areas correspond to the isolates used in this study, where green identifies linage I and blue identifies linage II.
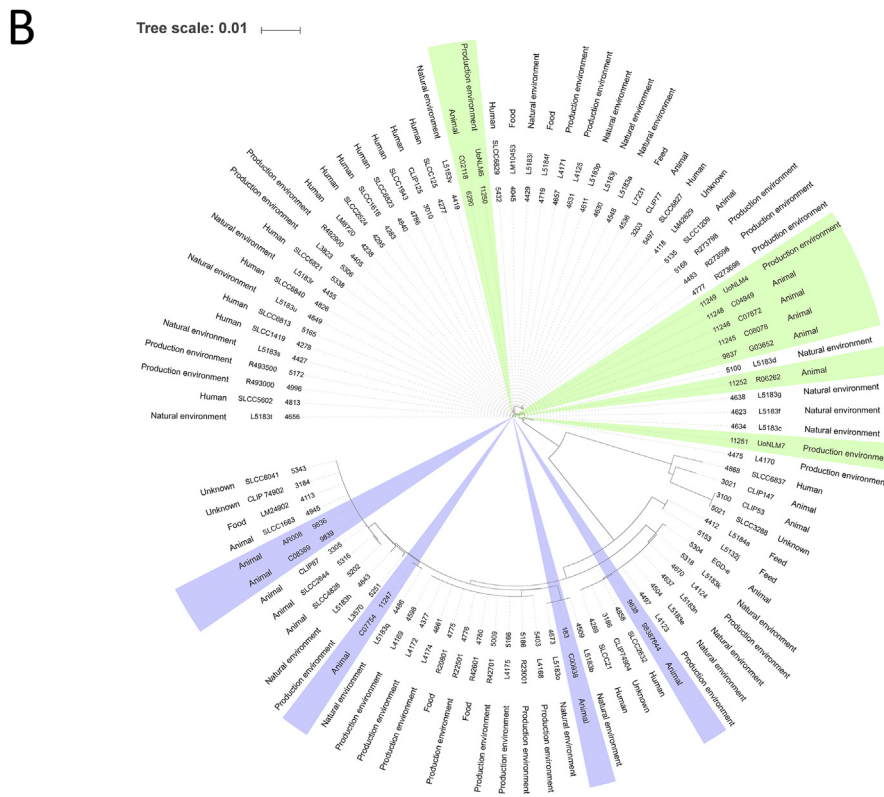
abortion (3 isolates) and meningitis. This suggests that these bovine strains may be transmitted to human through the food chain [33]. Interestingly, one of our cattle isolates has the MLST type ST6 (C02118, keratoconjunctivitis isolate, 2007), which is the sequence type identified in the large outbreaks of human disease in Europe and South Africa during 2017/2018. As sequence type and core genome analyses revealed that isolates from different clinical diseases, as well as from different species (human/cattle) cluster together, this suggests that it is less likely

that the ability of *L. monocytogenes* to infect different host species is due to species-specific virulence factors, but more subtle variation in gene sequence influencing host interactions of this pathogen.

L. monocytogenes infected BCECs at low efficiency and required a high MOI. In other species, such as pregnant guinea pigs, colonisation of the placenta was initially slow with 10^3-10^4 fewer bacteria seen in the placenta than in the liver and spleen immediately after intravenous inoculation [22]. This suggests that placental tissues are not easily or
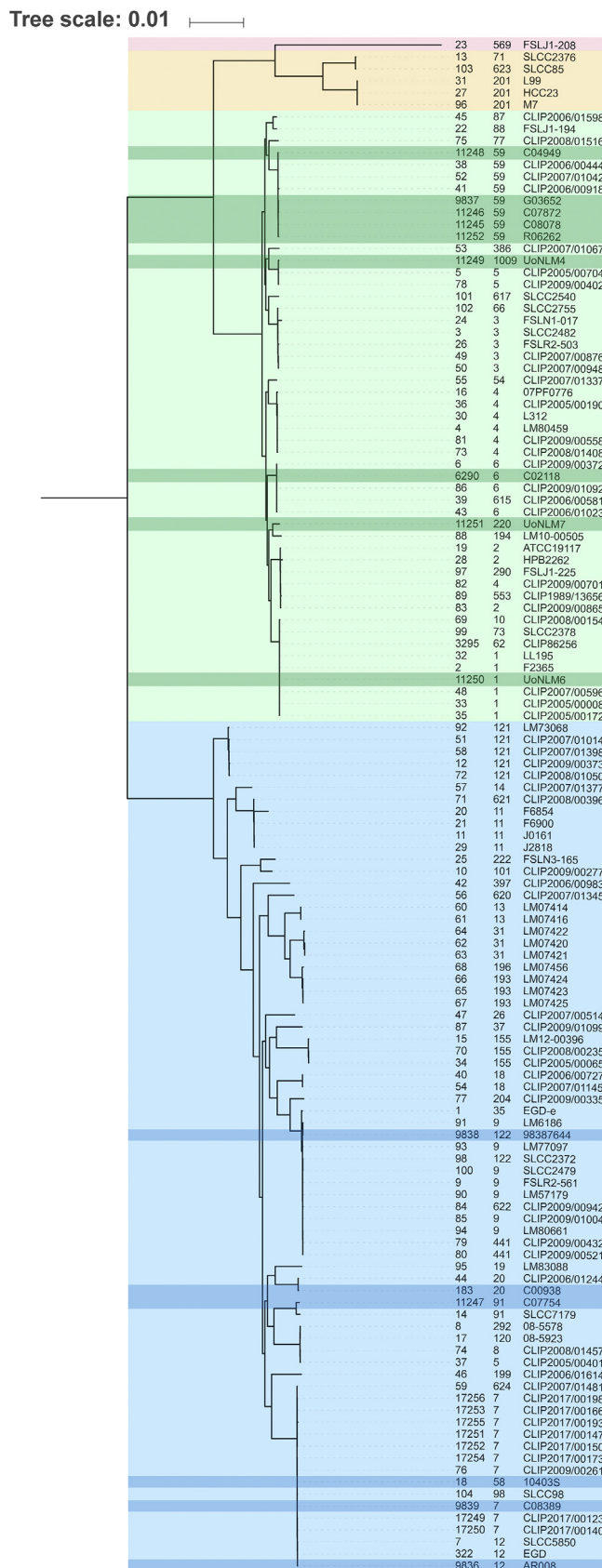
Figure 6. ***L. monocytogenes*** **core genome comparison.** Maximum likelihood tree has been generated using the core genome of isolates in the MLST database. Shaded areas correspond to linages, where green indicates linage I, blue indicates linage II, orange indicates linage III and red indicates linage IV. Darker shading highlights the isolates used in this study.

immediately invaded by *L. monocytogenes*. This is consistent with our findings that low numbers of bacteria were recovered from caruncular cells 2 h post infection with a wide range of variation. However, the invasion of a single bacterium into the placenta of guinea pigs can be sufficient to cause an abortion. Once colonised, there is poor bacterial clearance from the placenta and replication allows *Listeria* spp. to recolonise maternal tissues [22]. This is consistent with our findings that at 24 h post infection, higher numbers of bacteria were recovered from BCECs with less variation between infection experiments. This also suggests that any isolates able to invade BCECs may be able to cause an abortion *in utero* if they are able to grow within these tissues. Entry points towards systemic infections in cattle may include small breaches of oral mucosa from rough feeding material which may lead to repeat exposure of *L. monocytogenes* through contaminated silage [34].

The use of the BCEC infection model allowed us to identify strains with different potential to infect this cell type. Surprisingly, given that this isolate originated from a bovine abortion case, isolate 7644 was highly attenuated in the BCEC infection model. However, this strain was found to belong to ST9 and these isolates have been reported to be hypovirulent and are most commonly isolated from food sources rather than clinical infections [15]. Since identification of *Listeria* from aborted foetuses is problematic, the possibility exists that this isolate was a post-abortive environmental contaminant rather than the causative agent of infection. Alternatively, the animal may have been challenged with a high infectious dose. This assumption was previously proposed for a field isolate from a bovine abortion, which had a truncated PrfA, and was strongly attenuated in infection experiments with a wide range of cell types [32]. However, loss of infectivity may also be due to mutations accumulated during long term culture of the bacteria in a laboratory environment. In our previous study, both LM4 and 7644 were attenuated in a Caco2 infection model, whereas AR008 was able to infect Caco2 cells at similar levels to the control strain 10403S [21]. The fact that AR008 was attenuated in the BCEC model when other strains were able to infect successfully suggests that there are specific factors in these bovine placental cells involved in the interactions with *L. monocytogenes*.

Previously, we have shown that resistance to lysozyme was a positive predictive factor for infection of bovine conjunctiva explant model [21] but genome analysis performed in this study did not reveal any differences between the isolates used in the genes suspected to be involved in lysozyme resistance (*pdgA, oatA, degU*) [21,35, 36]. Interestingly, there was again a strong correlation between *L. monocytogenes* replication in BCECs 24 h post-infection and their level of lysozyme resistance. In cattle, lysozyme activity in most tissues is relatively low compared to other species, except for the abomasum [37] and tear fluid [38] which both have high levels of lysozyme activity. This may explain the co-selection for high levels of lysozyme resistance found in isolates from conjunctivitis as well as from other sites (reproductive tissues/fetus, brain and milk) that require the bacteria to survive passage through the abomasum. In addition, degradation of *L. monocytogenes* cell wall by lysozyme leads to the release of peptidoglycan and its breakdown products that are ligands for the pattern recognition receptors of the innate immune system, such as Nod1, Nod2 and Toll-like receptor (TLR) 2 [39, 40, 41, 42]. This is illustrated by *L. monocytogenes* lacking *pgdA*, which was not only highly attenuated in its virulence *in vivo* and *in vitro* but also elicited a strong TLR2 and Nod1 dependent interferon-ß response [43]. This suggests that lysozyme resistance may contribute to *L. monocytogenes* virulence in two different manners, by increasing bacterial survival as well as modulating the host response [44].

WGS identified the absence of virulence genes *virR* and *uhpT* in LM4 which potentially explains the attenuation of this isolate in the BCEC infection model. VirR is part of a two-component regulator (VirR–VirS) which is required for the virulence of *Listeria in vivo* [27]. It was also found that *virR* mutants are affected in their entry into Caco2 cells [26] and we have previously reported that LM4 also has a reduced capacity to invade this cell type [21]. The sugar phosphate antiporter UhpT promotes the uptake of phosphorylated hexoses during cytosolic growth
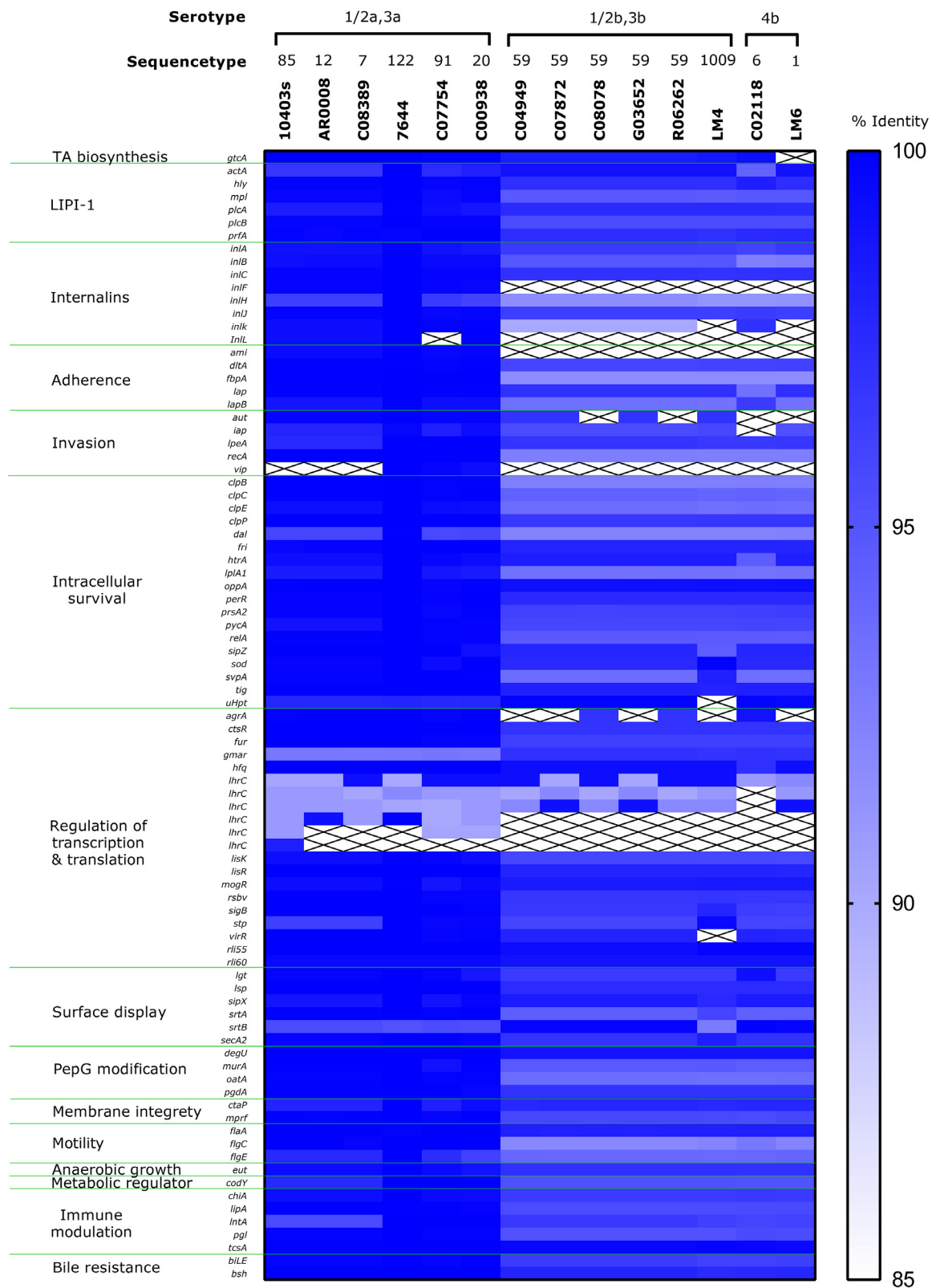
Figure 7. *Virulence gene analysis.* Heat map illustrating percentage identity of 87 *L monocytogenes* virulence genes in comparison to isolate EDG-e determined through virulence finder [64], with crosses denoting the absence of genes. The gene matrix represents from top to bottom, genes involved in teichoic acid biosynthesis *(gtcA)*, located in pathogenicity island LIPI-1 *(actA, hly, mpl, plcAB, prfA)*, genes coding for internalins *(inlABCFHLKL)* and other genes involved in adherence *(ami, dltA, fbpA, lap, lapB)*, invasion *(aut, iap, lpeA, recA, vip)*, Intracellular survival *(clpBCEP, dal, fri, htrA, lplA1, oppA, perR, prsA2, pvcA, relA, sipZ, sod, svpA, tig, uHpt)*, regulation of transcription and translation *(ctsR, fur, gmar, hfg, lhrc, lisKR, mogR, rsbv, sigB, stp, virk, rli55, rli60)*, surface display *(lgt, lsp, sipX, srtAB, secA2)*, peptidoglycan modification *(degU, murA, oatA, pgdA)*, membrane integrity *(ctap, mrpf)*, motility *(flaA, flgCE)*, anaerobic growth *(eut)*, regulation of metabolism *(codY)*, immuno-modulation *(chiA, lipA, lnyA, pgl)* and bile resistance *(bile, bsh)*.

[25] and deletion of this gene also leads to impaired intracellular proliferation in Caco2 cells [24]. Therefore, as LM4 lacks these two genes, it would be predicted that it would be less able to grow in BCEC cells. Interestingly, in our previous study, LM4 was not significantly attenuated in its ability to invade and proliferate in bovine conjunctiva tissues [21] but perhaps in that infection model the high level of resistance to lysozyme may compensate for any reduced intracellular growth. VirRS is also known to control the expression of a set of 17 genes, several of which affect bacterial cell wall and membrane integrity and, virR mutants are reported to be more sensitive to some beta-lactam antibiotics, including penicillin and cefuroxime [45]. However, LM4 did not show increased levels of sensitivity to these two antibiotics, or to challenge with cationic peptides, suggesting in the absence of VirR the genes in this operon are regulated in a different manner.

4. Conclusion

L. monocytogenes is a highly versatile and adaptive bacterium, with the ability to not only infect a wide range of tissues within a host, but also to infect a wide range of physiologically distinct animal hosts. The placentome cell model provides a novel tool to characterise the infection processes carried out by *Listeria* spp. in a different host, where different host factors may influence the infection process. Correlating WGS analysis with infection models provides more insights into the underlying basis of differences in strain of *Listeria*.

5. Materials and methods

5.1. Bacterial culture

Listeria monocytogenes strains used in the infection studies are listed in Table 1. Bacteria were cultured overnight (approximately 17h) at 37 °C in 5ml Heart infusion (HI) broth or on HI agar plates (Oxoid, UK). Growth was monitored using optical density (OD_{600nm}) and cultures were diluted depending on the multiplicity of infection (MOI) required for infection experiments. The precise CFU/ml of the inoculum was then determined by serial dilution and plating on HI agar. To determine the growth rates and generation times of the isolates, overnight cultures were diluted in HI broth to OD600 = 0.01. Growth was monitored using optical density (OD_{600nm}) and serial dilution plated on HI agar.

5.2. Multiplex PCR assay for Listeria monocytogenes serotyping

Multiplex PCR was performed in order to separate the four major serovars (1/2a, 1/2b, 1/2c, and 4b) and three main lineages (I, II, III) of *L. monocytogenes* [46, 47]. To prepare template DNA, three to six colonies resuspended in 1 ml of sterile water were incubated at 90 °C for 10min and then chilled on ice for 10min; 1µl of this was used as template DNA for each PCR reaction.

5.3. Cell culture and infections

Bovine caruncular epithelial cell line BCEC-1 (BCEC), provided by Prof. C. Pfarrer [18], were grown in DMEM/Ham's nutrient mixture F12 1:1 (Sigma-Aldrich, UK) with 10% (v/v) fetal calf serum (Sigma), 2mM L-Glutamine and 100 U/ml penicillin/streptomycin (Gibco) at 37 °C with 5% CO_2 [19]. BCECs were seeded into 24-well plates (Thermo Scientific, UK) in 500µl of complete medium and grown to confluence. One hour before infection, the complete medium was replaced with antibiotic-free medium and the plate incubated at 37 °C. Cells were infected with an MOI of 200 (n ≥ 5, for details see result section), and incubated for 1h. Medium was then removed from the wells and replaced with medium containing 100 µg/ml gentamycin (Sigma) to kill extracellular bacteria. After a further 1h incubation, the medium was replaced with medium containing 5 µg/ml gentamycin and incubated for 2–24hr post-infection. To enumerate intracellular bacteria, cells were washed three times with pre-warmed (37 °C) PBS and lysed by addition of 100µl of ice-cold 0.5% (v/v) Triton-X-100 (Fisher Scientific, UK) per well. This was incubated on ice for 20min and the resultant lysate serially diluted in PBS before 10µl samples were plated using the Miles Misra technique onto HI agar and incubated at 37 °C overnight. Then, the CFU/ml of lysates was calculated.

5.4. Antibiotic resistance screening

Samples (100µl) of each of the 14 isolates of *L. monocytogenes* were spread onto individual HI agar plates. Disks of penicillin G (1U), cefuroxime/sodium (30µg), oxacillin (1µg), ampicillin (25µg) and ciprofloxacin (1µg) (Oxoid Ltd, Basingstoke, UK) were immediately placed on top of the spread culture. The plates were then incubated at 37 °C overnight and the zones of inhibition were measured (mm).

5.5. Antimicrobial peptide challenge assay

The antimicrobial peptide challenge was performed as outlined by Burke *et al.* (2014) [35] using mouse cathelicidin-related antimicrobial peptide (H-GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ-OH; Isca Biochemicals, Exeter, UK) [48] at a final concentration of 10 µg/ml (stock concentration: 1 mg/ml in dimethyl sulfoxide (DMSO)).

5.6. Isolation of RNA, cDNA synthesis and quantitative (q) PCR

Late log phase culture containing approximately 10^9 CFU/ml was centrifuged at 13000x*g* for 2 min at room temperature. The pelleted cells were suspended in 1ml RNAlater (Sigma Aldrich) and incubated for 1h at room temperature. The suspension was centrifuged at 13000x*g* for 5min and the supernatant removed. The pelleted cells were suspended in 375µl of freshly prepared cell wall disruption buffer (30 U/ml mutanolysin, 10 mg/ml lysozyme in 10ml of 10mM Tris, 1mM EDTA buffer, pH 8), incubated at 37 °C for 30 min and then centrifuged at 13000x*g* for 5 min at room temperature. RNA was extracted using NuceleoSpin®RNA isolation kit (Macherey-Nagel, UK) following manufacturer's instructions.

For BCEC RNA extractions, the supernatant was removed and cells were lysed with 350µl of RNA lysis buffer (Nucleospin®RNA isolation kits, Machery-Nagel, UK) followed by RNA isolation according to manufacturer's instructions. Eluted RNA was quantified using Qubit (Qiagen) and stored at -80 °C. RNA was diluted in water and cDNA was synthesized using MMLV reverse transcriptase (Promega, Madison, USA) according to manufacturer's instructions. The final volume of each reaction was diluted in RNAse/DNAse free water (Fischer Scientific, UK).

All quantitative real time PCR (qPCR) experiments were designed and performed to comply with the quality controls detailed in the MIQE guidelines (Bustin et al., 2010). Quantitative PCR was performed using a LightCycler® 480 (Roche, Hertfordshire, UK). For primer sequences see Table 3. For bacterial and host gene expression, qPCR was performed in 20µl reactions with 0.25mM of each of the forward and reverse primer, 2X Luminoct SYBR Green qPCR ready mix (Sigma-Aldrich, Dorset, UK), 25ng of cDNA and PCR grade water (Roche, Hertfordshire, UK). An initial denaturation cycle of 95 °C for 10min was used followed by 45 cycles of 10 s at 95 °C, 50 s at 60 °C and 1 min at 72 °C and a final extension of 10 min at 72 °C. Normalized gene expression of each gene was calculated based on the method described by Hughes *et al* 2007 [49]: Differentiation factor = (overall mean of 40 − CP value for reference gene)/(40 − CP value of reference gene of that sample); Normalised expression = (Mean

Table 3. Primers.

Target	Sequence	Gene reference	Size (bp)/efficiency (%, qPCR)	Reference
Bovine primers				
GAPDH	F = AGTTCAACGGCACAGTCAAG R = AGCAGGGATGATATTCTGGG	NM_001034034	463 bp	This study
E-cadherin	F = GGTCAAAGAGCCCTTACTGC R = TGGCTCAAGTCAAAGTCCTG	AY508164.1	105 bp	
C-met	F = TGAAGGAGGGACAACACTGA R = TAAGGTGCAGCTCTCATTGC	NM_001012999.2	112 bp	
β actin	F = GAAGGTGACAGCAGTCGGT R = TTTCGCGATATTTGGAATGA	BT030480.1	114 bp	
Listeria monocytogenes primers (qPCR)				
16srRNA	F-CTTCCGCAATGGACGAAAGT R- ACGATCCGAAAACTTCTTCATAC		95%	Werbrouck et al 2006 [57]
TufA	F- GCTGAAGCTGGCGACAACA R- CTTGACCACGTTGGATATCTTCAC		102%	
InlA	F- GAACCAGCTAAGCCIGTAAAAG R- CGCCIGTTTGGGCATCA		95%	
InlB	F- GGAAAAGCAAAAGCAIGATTC R- TCCATCAACATCATAACTTACTGTGTAAA		92%	

$(40 - CP$ value for the sample) × target primer slope)/(Differentiation factor for that sample × reference gene primer slope). Fold change = $2^{(T-C)}$, whereby T = normalised expression level of treated samples; C = s normalised expression level of control samples.

5.7. WGS and sequence analysis

DNA was extracted using the Cador Pathogen Minikit (Qiagen) following manufacturer's recommendations. High throughput sequencing was performed at MicrobesNG (Birmingham U.K.). Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol and sequenced using Illumina MiSeq. Raw reads were assembled using the A5-MiSeq pipeline [50] and contigs were uploaded to the Pasteur MLST database were they are publicly available and the MLST sequence type was determined (http://bigsdb.pasteur.fr/listeria/listeria.html).

5.8. Multi sequence alignments

All nucleotide and protein alignments were completed using secondary structure aware high throughput multi-sequence alignment DECIPHER [51] (R script available at https://github.com/ADAC-UoN/DECIPHER-Sequence-Alignment.git). Trees were calculated using maximum likelihood by Fasttree double precision (version 2.1.8) [52] and visualised in iTOL [53].

5.9. Virulence finder

Assembled genome files were uploaded to the virulence finder online *Listeria* database at the Danish Centre For Genomic Epidemiology (https://cge.cbs.dtu.dk/services/VirulenceFinder/version 1.5) [54] and searches were performed against reference isolate EDG-e, using a minimum of 90% identity along 80% of the coding sequence.

5.10. Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 6.05. To compare the growth rates of *L. monocytogenes* isolates, a one-way ANOVA was carried out followed by Dunn's multiple comparison test. To compare isolates in an infection context, a Kruskal-Wallis test was carried out, followed by Dunn's multiple comparisons test. Pearson's correlations were performed to compare data sets. *L. monocytogenes* sequence type distributions were analysed using Fisher's exact test. Significance was reported for $p < 0.05$.

Declarations

Author contribution statement

Adam M. Blanchard, Sabine Tötemeyer: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Rosemarie Billenness: Performed the experiments; Analyzed and interpreted the data.

Jessica Warren, Amy Glanvill, William Roden, Emma Drinkall, Grazieli Maboni: Performed the experiments.

Robert S Robinson, Catherine E.D. Rees: Conceived and designed the experiments; Wrote the paper.

Christiane Pfarrer: Contributed reagents, materials, analysis tools or data.

Competing interest statement

The authors declare no conflicts of interest.

Additional information

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