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A new bovine conjunctiva model shows that *Listeria monocytogenes* invasion is associated with lysozyme resistance

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ABSTRACT

Listerial keratoconjunctivitis ('silage eye') is a wide spread problem in ruminants causing economic losses to farmers and impacts negatively on animal welfare. It results from direct entry of *Listeria monocytogenes* into the eye, often following consumption of contaminated silage. An isolation protocol for bovine conjunctival swabbing was developed and used to sample both infected and healthy eyes bovine eyes ($n = 46$). *L. monocytogenes* was only isolated from one healthy eye sample, and suggests that this organism can be present without causing disease. To initiate a study of this disease, an infection model was developed using isolated conjunctiva explants obtained from cattle eyes post slaughter. Conjunctiva were cultured and infected for 20 h with a range of *L. monocytogenes* isolates ($n = 11$), including the healthy bovine eye isolate and also strains isolated from other bovine sources, such as milk or clinical infections. Two *L. monocytogenes* isolates (one from a healthy eye and one from a cattle abortion) were markedly less able to invade conjunctiva explants, but one of those was able to efficiently infect Caco2 cells indicating that it was fully virulent. These two isolates were also significantly more sensitive to lysozyme compared to most other isolates tested, suggesting that lysozyme resistance is an important factor when infecting bovine conjunctiva. In conclusion, we present the first bovine conjunctiva explant model for infection studies and demonstrate that clinical *L. monocytogenes* isolates from cases of bovine keratoconjunctivitis are able to infect these tissues.

1. Introduction

Listeria monocytogenes is an intracellular pathogen which has been extensively studied as a cause of human food borne infections associated with a high mortality rate (McLauchlin et al., 2014). In both man and animals *L. monocytogenes* is able to infect a wide range of tissues including the spleen, liver and brain and can cross the placenta to cause abortions (Cossart and Toledo-Arana, 2008). It is found widely in the farm environment and its ability to use plant sugars as part of its saprophytic lifestyle means that it can reach levels as high as 10^8 cfu g^{-1} wet weight in some poorly fermented silages when low pH does not suppress growth of the organism (Wiedmann et al.,

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1996). Listerial keratoconjunctivitis and uveitis ('silage eye') are common problems in ruminants in the UK, which negatively impact on animal welfare and causes economic losses to farmers (Erdogan, 2010). A strong correlation has been established between silage eye and the use of big bale silage and silage feeding in ring feeders and these infections are believed to occur when the organism directly enters the eye, possibly facilitated by corneal abrasions (Erdogan, 2010; Revathi et al., 2011). Treatment of listerial keratoconjunctivitis includes parenteral and/or topical use of antibiotics generally resulting in recovery within two weeks, however, antibiotic resistance has been noted as an increasing problem (Erdogan, 2010).

There have been increasing field reports of silage eye in ruminants since the late 1980s (Erdogan et al., 2001) but very little research to try and understand the route of transmission, carrier state in the eye/conjunctiva or pathology of the disease. Currently the lack of understanding of silage eye infection routes and epidemiology reduces the development of treatment and preventative measures (Erdogan, 2010). One of the main defence mechanisms in bovine tears is lysozyme with concentrations in bovine tears of up to 580 $\mu g\, ml^{-1}$ reported in the literature (Gionfriddo et al., 2000). Similar levels have been reported in sheep's eyes (600 $\mu g\, ml^{-1}$; Gionfriddo et al., 2000) but can be much higher in humans, and is reported to be up to 2.0 $mg\, ml^{-1}$ (Sherman et al., 1994). Like many bacterial pathogens, *L. monocytogenes* is known to be naturally resistant to lysozyme by modifying its peptidoglycan (PG) structure so that it cannot be degraded by the enzyme. Three PG modifying enzymes involved in lysozyme resistance, peptidoglycan deacetylase PgdA, putative carboxypeptidase PbpX and acetyltransferase OatA, have been identified (Aubry et al., 2011; Boneca et al., 2007; Burke et al., 2014) and also two regulators of gene expression, DegU (response regulator) and Rli31 (long non-coding RNA) which are believed to up-regulate the expression of these genes (Burke et al., 2014). The PG modifications carried out by PgdA and OatA also result in reduced host immune response by suppressing NOD1-dependent and toll-like receptor 2 (TLR2) IL-6 and interferon-β secretion as well as IL1β and IL12 mRNA expression (Aubry et al., 2011, 2012; Rae et al., 2011). Hence the high level of lysozyme resistance seen in many clinical isolates may be linked to a selective advantage conferred by an ability survive host lysozyme challenge and evade the host immune response.

The aim of this study was to develop a conjunctiva explant infection model to determine whether *L. monocytogenes* isolated from bovine keratoconjunctivitis cases have properties that allow better infection of conjunctiva in comparison to isolates from healthy cattle eyes, milk or other bovine clinical conditions.

2. Materials and methods

2.1. Bacterial culture

L. monocytogenes strains used in this study are listed in Table 1. Bacteria were cultured overnight (approximately 17 h) at 37 °C in Heart infusion (HI) broth (Oxoid) or on HI agar plates. Optical density (600 nm) was used as an estimate of cell number and then cfu ml^{-1} ascertained by viable count. Haemolytic activity was assessed by growth on sheep blood agar plates (Oxoid) after incubation at 37 °C overnight.

2.2. Sample collection

Clinical samples were collected by veterinary practitioners and ethical approval was obtained from the ethics committee of the School of Veterinary Medicine and Science, University of Nottingham. The swabs (Culture Swab Transport System, VWR International) were placed into the conjunctival sac and moved in a lateral and medial direction. Eye swabs were also taken from cattle heads which had been decapitated and facial muscles and skin removed in the slaughter process at abattoir or after transport to School of Veterinary Medicine and Science (SVMS). In those cases, the eye surface was also sampled using the same swab. Only bovine eyes that had a minimum area of skin of approximately 3 cm around the eye left were swabbed to reduce the microbial contamination through damage of the eyelids or conjunctiva during carcass processing. All swabs were stored at 4 °C overnight before culturing.

Table 1
Listeria monocytogenes isolates used in this study.

Strain number	Source[a]	PCR serotype[b]	PCR lineage[c]	Source/reference
10403S	Skin Lesion	1/2a	II	Bishop and Hinrichs (1987)
AR008	Healthy eye	1/2a, 3a	II	This study
C00938	Silage eye	1/2a, 3a	II	APHA
R06262	Silage eye	1/2b, 3b	I	APHA
C02118	Silage eye	4b	I	APHA
LM7644	Abortion	1/2a, 3a	II	APHA
C08389	Abortion	1/2a, 3a	II	APHA
G03652	Meningitis	1/2b, 3b	I	APHA
LM4	Milk	1/2b, 3b	I	Lawrence et al. (1995)
LM6	Milk	4b	I	Lawrence et al. (1995)
LM7	Milk	4b	I	Lawrence et al. (1995)

APHA, Animal and Plant Health Agency.
[a] All isolates are from bovine sources except for the human isolate 10403S.
[b] 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.
[c] Lineages were determined using the PCR-based method of Ward et al. (2004).

2.3. Listeria monocytogenes isolation from eye swabs

For direct plating, swabs were streaked onto Oxford agar (Oxoid) and plates incubated at 37 °C for 24–48 h. Any black/brown colonies were purified by streaking onto Brilliance Listeria agar (Oxoid) and incubated at 37 °C for 24–48 h. To detect low number of cells and adaptation of the ISO 112090-1 food sampling protocol was used. The same swabs used for direct plating were placed in 20 ml of Fraser broth (Oxoid) and vortexed for 30 s. After 1 h static incubation at 37 °C, the sample was divided into two portions and incubated at either 37 °C for 24–48 h or 4 °C for 10 d. Samples (100 μl) from any Fraser broth cultures displaying a colour change to brown/black after incubation were plated onto Brilliance Listeria agar. Gram stain was performed on all isolates giving the characteristic blue/green colonies surrounded by a zone of clearing. All Gram-positive rod shaped bacteria were further grown on to 5% sheep blood agar plates to identify weak β-haemolysis.

2.4. 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* (Doumith et al., 2004; Ward et al., 2004). To prepare template DNA, three to six colonies were picked from Heart Infusion (HI) agar plates and mixed into 1 ml of sterile water, incubated at 90 °C for 10 min and then chilled on ice for 10 min and 1 μl of this was added to each PCR reaction.

2.5. Bovine conjunctiva explant culture

Conjunctiva samples were taken from cattle heads obtained from a commercial abattoir. Conjunctiva were dissected by cutting in the medial canthus area towards the orbit and from there a cut was made from cranial to caudal. The conjunctiva was then cut along the lateral canthus to meet with the previous cut. The palpebral tissue was then removed leaving a rectangular tissue section. Tissue sections were placed in phosphate buffered saline (PBS, Oxoid) and fat tissues were removed prior to using an 8 mm punch biopsy tool (Valu Biopsy Punch 8 mm 135963; National Veterinary Services, Stoke-on-Trent) to cut tissue into discs. Tissue disks were placed into basic medium (Dulbecco's Modified Eagle's Medium/Harris Nutrient Medium F12 (1:1 DME/F12 Modified, Sigma Aldrich) containing 5 $\mu g\, ml^{-1}$ each of penicillin and streptomycin (P/S, Gibco Invitrogen, Paisley, England) and 0.63 $\mu g\, ml^{-1}$ fungisome (Lonza). Before inoculation, tissue disks were placed in individual wells of a 24 well plate and washed with pre-warmed basic medium (37 °C) three times to remove any P/S and fungisome. Basic medium supplemented with 10% (v/v) foetal bovine serum and 2 mM L-glutamine (Gibco Invitrogen, England) was added to each well so that samples were just covered in medium.

2.6. Explant infection

L. monocytogenes cultures were grown overnight in HI broth 37 °C and inoculum of 10^9 cfu ml^{-1} was prepared. A sample (10 μl) of the inoculum was placed onto the epithelial surface of each tissue disc (MOI approx. 100; Infection density of 10^7 *Listeria* cells per 50 mm^2 and each explant was estimated to contain 3×10^5 conjunctival epithelial cells, based on an average cell size of 160 μm^2; Doughty and Hagan, 2013). Tissue discs were incubated in 5% CO_2 at 37 °C for 20 h, then the medium was replaced with basic medium containing gentamycin (100 $\mu g\, ml^{-1}$) to inactivate any remaining bacteria that had not entered the tissue. After 1 h samples were washed three times with PBS before being homogenised in PBS using stainless steel beads (5 mm, Qiagen Gmbh, Germany) and Bead Mill MM 301 (Retsch Gmbh, Germany) for 4 min at 30 movements s^{-1}. Bacteria were enumerated by plating onto HI agar plates.

2.7. Caco2 infections

The Caco2 infection assay was performed as described by Gaillard et al. (1987) with the following modification: Caco2 cells were infected with a multiplicity of infection (MOI) of 25 and 200 for 1 h, followed by incubation with gentamycin containing medium (100 $\mu g\, ml^{-1}$) for 1 h. Cells were washed with pre-warmed PBS and lysed with ice cold 0.5%, v/v Triton for 20 min prior to serial dilutions and plating on HI agar.

2.8. Lysozyme sensitivity assays

Bacteria were grown overnight, then resuspended in DME/F12 Modified medium and diluted to 10^5 cells ml^{-1} in 1:1 DME/F12 Modified medium supplemented with different concentrations of chicken egg Lysozyme (Sigma Aldrich; 1 $\mu g\, ml^{-1}$ to 1 $mg\, ml^{-1}$). Samples were incubated for 24 h at 37 °C in a static incubator. Minimal inhibitory concentration (MIC) for lysozyme was determined by the dilution at which no visible growth was apparent. To identify the minimal bactericidal concentration (MBC), samples from wells where there was no visible growth were plated onto HI agar to determine if any viable bacterial cells remained.

For the lysozyme survival assay, a fixed concentration of lysozyme was used (580 $\mu g\, \mu l^{-1}$). The bacterial cells were prepared as before, but incubated in the presence or absence of lysozyme for 24 h in a 5% CO_2 incubator at 37 °C before the number of surviving bacteria was determined by viable count.

2.9. Statistical analysis

One way ANOVA (95% confidence interval) followed by Tukey's multiple comparison test was performed using GraphPad Prism6 software package.

3. Results

3.1. Development of swabbing and isolation protocol from bovine conjunctiva

The limit of detection of the swabbing and isolation protocol was validated by using eyes in bovine heads collected post-slaughter. Conjunctiva were inoculated with a 30 μl inoculum containing between 1 and 10^6 *L. monocytogenes* 10403S. The eyes were immediately swabbed as described previously, and then samples directly plated on to Oxford agar or inoculated into Fraser broth for enrichment and detection. The detection limit of the swabbing method was found to be 10^3 *L. monocytogenes* cells per conjunctiva.

3.2. Isolation of L. monocytogenes from bovine eyes without clinical signs of silage eye/keratoconjunctivitis

From 13 swabs from cattle with 'weepy' eyes obtained by veterinary practitioners, no *Listeria* were isolated. Based on colony morphology on Oxford and Listeria Brilliance agars, Gram stain reaction and weak haemolysis on sheep blood agar, two out of 33 swabs taken from clinically healthy eyes post-slaughter at an abattoir were identified as *L. monocytogenes*. However only one of these (AR008) survived freezing and could therefore be used for further experiments. This isolate, and all the clinical isolates obtained from the Animal and Plant Health Agency (APHA) culture collection, were serotyped using a multiplex PCR method (Doumith et al., 2004; Table 1). The strains used included representatives of serotypes 1/2a, 1/2b and 4b which cause 90% of human infections and are also commonly associated with animal infections (Bundrant et al., 2011).

3.3. Infectivity in conjunctiva explant model

Conjunctiva explants were infected with 10 different *L. monocytogenes* isolates, including the well characterised strain 10403S which was used as a reference strain. As the conjunctiva were taken from abattoir samples, a negative control set was also included to demonstrate that the eyes were not naturally infected with *L. monocytogenes* and no *Listeria* was recovered from these samples (Fig. 1). The other strains tested included the healthy eye isolate AR008, three isolates from cases of bovine conjunctivitis, one cattle abortion strain and three isolates from bovine milk (Table 1). The explants were inoculated with approximately 10^7 bacteria per sample. After incubation with the conjunctiva explants for 20 h, all *L. monocytogenes* isolates used were found to be able to invade the tissue, as indicated by the fact they were protected against a 1 h treatment with gentamicin (Kuhbacher et al., 2014). The level of bacteria recovered were 3–5 $\log_{10}$ cfu per explant (Fig. 1) but significant differences could be seen in the ability of the different *L. monocytogenes* to infect the explants. In particular two isolates, AR008 (healthy bovine eye) and LM7644 (bovine abortion) produced significantly lower levels of recovered intracellular bacteria compared to the other strains tested (Fig. 1).

3.4. Further investigation of strain characteristics

Haemolysin is a key enzyme required for the efficient release of internalised *L. monocytogenes* cells from the vacuole (Portnoy et al., 1992). To determine if variation in the ability of strains to infect the conjunctiva was

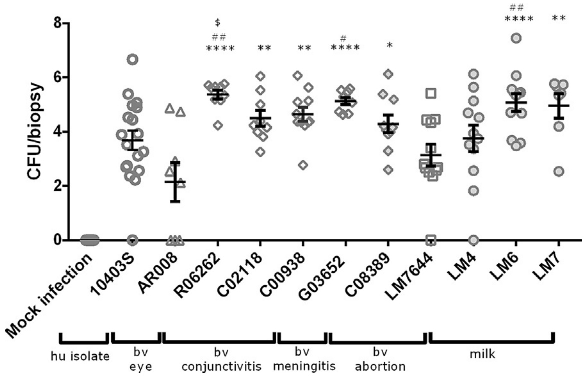

Fig. 1. Infection of conjunctiva explants. Conjunctiva explants were inoculated with 10^7 bacteria per disc (50 mm^2) and incubated for 20 h at 37 °C. For each strain a minimum of 7 independent experiments were performed; Mock infection $n = 18$, 10403S $n = 18$, LM4 $n = 12$, LM6 $n = 12$, LM7 $n = 7$, LM23 $n = 12$, LM7644 $n = 12$, AR008 $n = 8$, R06262 $n = 9$, C02118 $n = 9$, C00938 $n = 11$, G03652 $n = 9$, C08389 $n = 10$. All data points and mean ± SEM are shown. Explants infected with any of the *L. monocytogenes* strains showed significant differences to mock infection with $p < 0.0001$, except for AR008 with $p < 0.01$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$ compared to AR008; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ compared to LM7644; $^{\$}p < 0.05$ compared to 10403S (One way ANOVA & Tukey's multiple comparison test).

associated with the level of production of haemolysin, all strains were tested by plating on sheep blood agar and were confirmed to be haemolytic, with all strains showing the same level of weak haemolysis as the control strain 10403S, none of the strains were either ahaemolytic or hyper-haemolytic (Table 2).

To determine if these strains were generally less able to infect human cells, the well-established *L. monocytogenes* Caco2 infection model was also used. Using this model AR008 showed the same ability to infect Caco2 cells as the control strain 10403S (Fig. 2). In contrast the abortion strain LM7644 and the milk isolates LM4 were both impaired in their ability to infect this cell type, with significantly lower number of bacteria recovered irrespective of the initial level of bacterial challenge (MOI = 25 or 200; Fig. 2).

3.5. Sensitivity to lysozyme

Since lysozyme is one of the main antibacterial defence mechanisms in the eye, we performed MIC/MBC assays to determine the lysozyme sensitivity of our isolates. The healthy eye isolate AR008 and the abortion strain LM7644, which were both less able to infect the conjunctiva explants, showed the lowest resistance towards lysozyme treatment (MIC = 78 μg ml^{-1}, Table 2). While the MBC value for LM7644 was also low (625 μg ml^{-1}), that for AR008 was in the same range as the lysozyme-resistant strains (Table 2).

A survival assay was also performed using the highest concentration of lysozyme reported in the literature in bovine eyes (580 μg ml^{-1}) (Gionfriddo et al., 2000). This experiment confirmed that both AR008 and LM7644 were the most sensitive to lysozyme challenge and that they were significantly more sensitive than all the bovine

Table 2
Haemolytic activity and lysozyme sensitivity.

Strain number	Source	Haemolysin production[a]	MIC[b] μg ml^{-1} lysozyme	MBC[c] μg ml^{-1} lysozyme
10403S	Skin Lesion	+	2500	3750
AR008	Healthy eye	+	78	2500
C00938	Silage eye	+	2500	2500
R06262	Silage eye	+	1250	5000
C02118	Silage eye	+	2500	2500
LM7644	Abortion	+	78	625
C08389	Abortion	+	1250	7500
G03652	Meningitis	+	1875	1875
LM4	Milk	+	1875	6250
LM6	Milk	+	1875	2500
LM7	Milk	+	1875	5000

[a] All strains showed equivalent levels of weak haemolysis on sheep blood agar.
[b] MIC = minimum inhibitory concentration.
[c] MBC = minimum bactericidal concentration.

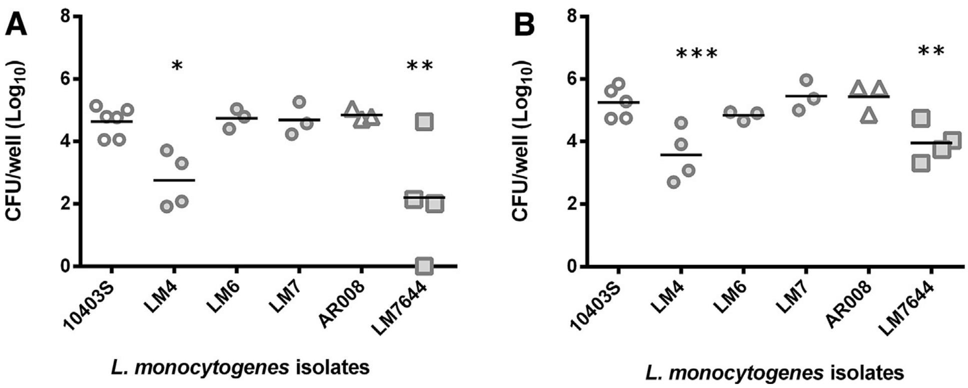

Fig. 2. Infection of Caco2 cells. Caco2 cells were infected with an MOI = 25 (A) or 200 (B) for 2 h. For each strain a minimum of 3 independent experiments were performed. 10403S $n = 6$, LM4 $n = 4$, LM6 $n = 3$, LM7 $n = 3$, AR008 $n = 3$, LM7644 $n = 4$. All data points and mean are shown. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to 10403S (One way ANOVA & Tukey's multiple comparison test).

conjunctivitis isolates (Fig. 3). Two other strains, the abortion isolate C08389 and the meningitis isolate G03652, also showed lower levels of lysozyme resistance than the majority of the isolates tested (Fig. 3).

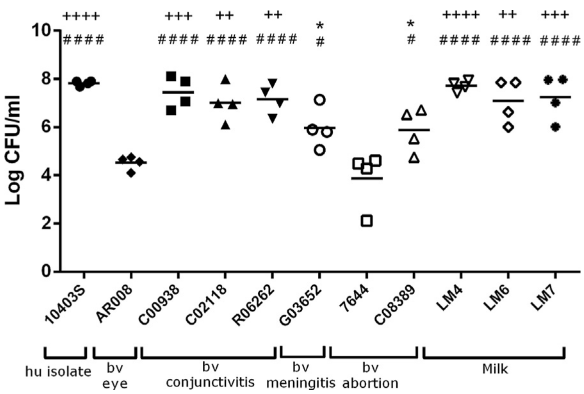

Fig. 3. Lysozyme sensitivity of *L. monocytogenes* isolates. Bacteria were incubated with 580 μg ml^{-1} lysozyme for 24 h at 37 °C ($n = 4$). All data points and mean are shown. $^{*}p < 0.05$; $^{****}p < 0.0001$ compared to LM10403S; $^{++}p < 0.01$; $^{+++}p < 0.001$, $^{++++}p < 0.0001$compared to AR008; $^{\#}p < 0.05$; $^{\#\#\#\#}p < 0.0001$ compared to LM7644 (One way ANOVA and Tukey's multiple comparison test).

4. Discussion

From a total of 46 eye swabs, either from cattle with 'weepy' eyes or clinically healthy eyes post-slaughter, *L. monocytogenes* was only recovered from two samples, neither of which had any sign of clinical infection. Given the limit of detection of the swabbing technique was determine in the laboratory to be 10^3 cells per eye, this suggests that *L. monocytogenes* can be found at reasonably high levels in cattle eyes without causing infection.

The two strains that were least able to infect the conjunctiva were also the stains that were most sensitive to lysozyme treatment, indicating that perhaps the ability of *L. monocytogenes* isolates to infect bovine conjunctiva is associated with lysozyme resistance. While reports of lysozyme concentrations found within the bovine eye are variable (0–580 μg ml^{-1}) (Gionfriddo et al., 2000; Prieur, 1986), our results using the upper range of these values suggest that there is a relationship between the ability to infect explanted conjunctiva tissues and natural levels of resistance to lysozyme.

In *Listeria*, modification of the peptidoglycan (PG) by PdgA and OatA has been shown to make the cells resistant to lysozyme by modifying the sugar backbone so that it is a poor substrate for the enzyme (Bera et al., 2005; Vollmer and Tomasz, 2000). These modifications are known to be important for survival inside the mammalian host, and *pgdA* and *oatA* mutants are also both impaired in their ability to survive in macrophages (Boneca et al., 2007; Rae et al., 2011). Recently it has been suggested that natural levels of lysozyme resistance of *L. monocytogenes* are due to the up-regulation of PG modifying-enzymes rather than acquisition of novel PG modifications, through the actions of the orphan response regulator DegU and an abundant non-coding RNA encoded by *rli31* (Burke et al., 2014). Mutations in both of these regulatory elements resulted in lysozyme sensitivity and reduced virulence in mice. In contrast the invasion of Caco2 cells, a non-phagocytic epithelial cell line, was not impaired in *L. monocytogenes* lacking PgdA, suggesting that this peptidoglycan modification which confers lysozyme resistance is not essential to be able to enter and survive within non-phagocytic cells (Boneca et al., 2007). This is consistent with our observation that the lysozyme-sensitive strain AR008 showed no impairment in its ability to infect Caco2 cells. However, the bovine abortion strain LM7644 was less able to infect Caco2 cells, suggesting a different basis for the lysozyme sensitivity of this strain.

Here we present the first bovine conjunctiva explant model for infection studies and demonstrate that clinical *L. monocytogenes* isolates from cases of bovine keratoconjunctivitis are able to infect these tissues recovered from animals slaughtered for commercial use. We have used this model to show that lysozyme resistance may be a key property to establish eye infections, and that cattle eyes may be colonised, but not infected by, strains of *Listeria* with naturally lower levels of lysozyme resistance. This model will allow us to study the host pathogen interactions that occur during the establishment of eye infections without the requirement for the use of live animals which is in keeping with the drive towards reduced animal usage in studies of infection. In addition, this model could be used to study other infectious bacteria which commonly cause conjunctivitis in cattle, such as *Moraxella* spp. (Henson and Grumbles, 1960).

Conflicts of interest

Authors declare that they have no conflicts of interest.

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