The Behaviour of Both *Listeria monocytogenes* and Rat Ciliated Ependymal Cells Is Altered during Their Co-Culture

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**Abstract**

**Background:** Ciliated ependymal cells line the cerebral ventricles and aqueducts separating the infected CSF from the brain parenchyma in meningitis.

**Principal Findings:** Investigation of the interaction of *Listeria monocytogenes* with cultured rat brain ependymal cells showed that certain strains reduced the beat frequency of the cilia but all the strains studied significantly reduced the ciliary beat amplitude (the linear distance travelled by the tip of each cilium per beat cycle).

**Conclusion:** The presence of the ependyma caused aggregation of some listeria strains and in some cases extracellular material also was seen in association with bacterial aggregates. These observations were dependent on the expression of genes required for invasion, intracellular survival and listerial cell to cell spread that are regulated by the transcriptional activator, positive regulatory factor A (PrfA).

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**Introduction**

*Listeria monocytogenes* is a food-borne pathogen that, if ingested, has the potential to enter the systemic circulation and move from the blood to penetrate the blood-brain barrier to cause meningitis and meningoencephalitis, especially in the unborn and newborn infant [1,2]. The risk of listeriosis is also markedly increased in immunocompromised patients, such as those with AIDS [3].

In meningitis, bacteria in the cerebrospinal fluid are separated from the neuronal tissue adjacent to the ventricular system and aqueducts, by the ependyma. The ependyma is a single, uninterrupted, layer of ciliated cells that lines the cerebral ventricles, cerebral aqueducts and the central canal of the spinal cord. Each ependymal cell has approximately 40 cilia that beat continuously at a frequency of around 40 Hz, moving the CSF close to the ventricular wall, in the direction of CSF flow [4]. In addition, the ependymal cilia are thought to act as a barrier to pathogen infection of the underlying neuronal tissue [5]. Prior to the entry of listeria into the cells of the brain the bacteria need to overcome this mechanical beating of the cilia. Abnormal movement of the ependymal cilia has been strongly linked to the development of hydrocephalus [6,7,8].

During meningitis caused by *L. monocytogenes*, bacteria have been shown to be present inside ependymal [9] and neuronal [10] cells, as well as in the cerebrospinal fluid [9,11,12]. However, the details of *L. monocytogenes* attachment to ciliated ependymal cells and subsequent invasion are poorly understood.

The expression of genes required for invasion, intracellular survival and listerial cell to cell spread is dependent upon a transcriptional activator known as PrfA (positive regulatory factor A) [13,14,15]. PrfA was first identified as a regulatory factor required for listeriolyisin-O transcription and has since been shown to regulate the expression of a large number of bacterial gene products directly associated with virulence [16]. To date, only 10 of the 2,853 coding sequences of the *L. monocytogenes* EGDe genome [17] have been confirmed to be directly regulated by PrfA [18,19].

It is thought that PrfA serves as a switch enabling *L. monocytogenes* transition from the outside environment into an animal host [20]. The absolute requirement of PrfA for *L. monocytogenes* virulence has been demonstrated, as has the requirement for several genes within the PrfA regulon [21,22]. Strains with mutations within the PrfA gene failed to replicate within the cytosol of host cells or failed to spread into adjacent cells [15,21]. These mutants also were severely attenuated for virulence in murine models of listeriosis [21].

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**Materials and Methods**

**Preparation of bacterial suspensions**

*Listeria monocytogenes* strains used in this study are listed in Table 1. *Listeria* were grown in 200 ml tryptose soya broth overnight at...
Table 1. Listeria monocytogenes strains.

| Strain   | Description               | Reference |
|----------|---------------------------|-----------|
| 10403S  | L. monocytogenes wild-type strain | [22]      |
| C52      | L. monocytogenes wild-type strain | [40]      |
| EGDe     | L. monocytogenes wild-type strain | [41]      |
| 10403SΔprfA | prfA gene disrupted by integration of Tn 917 | [42]      |
| EGDeΔprfA | prfA gene disrupted by integration of pAUL51-10 | [22]      |

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37°C with shaking at 200 rpm. The bacteria were then sedimented (4,000g for 10 min) and re-suspended in 10 ml medium 199 (Invitrogen, UK) containing 10% v/v glycerol and stored at −70°C until required. Before use, frozen stocks were thawed at room temperature. Numbers of viable bacteria were determined by colony counting on tryptose soya broth agar plates. For growth of EGDe ApfA and 10403S ApfA, 5 mg/ml erythromycin was added to the medium. For use, listeria were diluted to 2×10^6 cfu/ml in tissue culture medium 199.

Ependymal cell culture

The research using new born rat brains was conducted according to national guidelines under the UK, Animals (Scientific Procedures) Act 1986 and all animals were born in the University of Leicester Biomedical Sciences Unit. The research on the ex-vivo tissue was approved by the University of Leicester ethics committee.

An adaptation of a previously described method [23] was used to grow the ependymal cells. Eight-well rectangular (35 mm) tissue culture trays (Fisher, UK) were coated with bovine fibronectin (35 μg/cm²) and were incubated at 37°C in 5% CO₂ for 2 hours before use. Following cerebral dislocation, the brains of newborn (1 to 2 day old) Wistar rats were removed. The cerebellum was removed, as were small (3 mm) edge regions of the frontal cortex and the left and right cortical hemispheres. The remaining brain regions (containing ependymal cells and ventricles) were mechanically dissociated in 2 ml of tissue culture medium 199 [24]. Dissociated tissue from each brain was seeded (500 μl/well) into the wells of 25×35 mm tissue culture trays; each well contained 2 ml of medium. The medium was serum-free minimum essential medium (Gibco Life Technologies, Paisley, UK) containing penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml), bovine serum albumin (5 μg/ml), insulin (5 μg/ml), transferrin (10 μg/ml), selenium (5 μg/ml) [Invitrogen, UK] and from day 3 onward, thrombin (0.5 IU/ml) (Sigma-Aldrich, UK) [24]. The medium was replaced on day 3 after seeding. Thereafter, the adherent ependymal cells were fed by the replacement of 2 ml of medium, three times a week [24]. The ependymal cells grew as colonies within the wells and the beating cilia were used to identify them at day 5. The ciliated cells were used for experiments when aggregates were seen, five readings were taken from cilia covered area (4800 cells, each 10 μm in diameter, −0.48 mm²×2.3 mm height of media above the colony = 1.1 mm³). The total volume added was 12.6 mm³, therefore, the cells will be in contact with 2³ ×12.6/1.1/4800 approximately equal to 3654 bacteria per cell. Before use the MEM medium was completely removed from each well and the cells were washed 5 times with PBS and 1 ml of medium 199 was added to each well. For infection, 1 ml of L. monocytogenes suspension was added, to give a final concentration of 1×10⁷ cfu/ml.

Measurement of ciliary beat frequency

Ciliary beat frequency was measured as previously described [24,25]. Briefly, trays of ependymal cells were placed in a humidified (80 to 90% humidity) thermostatically controlled (37°C) incubation chamber on a light microscope stage (Dihot; Nikon). All measurements were taken with the solution temperature between 36.5 and 37.5°C and the pH between 7.35 and 7.45. Beating cilia were recorded (32x objective) using a troubleshooter 1000 high speed video camera [Lake Image Systems Ltd, UK] at 500 frames per second. All video files were created using the AVI video format. For viewing the AVI files MiDAS 4.0 player software (http://www.xcitex.com/html/downloads.php) was used. Video sequences were played back either at reduced frame rates or frame by frame and ciliary beat frequency was determined by timing a pre-selected number of individual ciliary beat cycles. At each time point, measurements of twenty individual cilia from the same colony of cells were made and averaged. This was repeated in 5–11 independent experiments (i.e. A total of 100–220 CBF readings were made).

Measurement of ciliary beat amplitude

Still photographs were obtained from various stages of the experiment. Each photograph was equivalent to 62.5 μm×140 μm of ciliated ependyma were chosen at random and from each area five measurements of ciliary beat amplitude were made. If listeria aggregates were seen, five readings were taken from cilia covered by these aggregates and five readings from cilia outside these areas. The amplitude of the ciliary beat in the control that was not exposed to listeria was defined as 100%.

Measurement of bacterial aggregation

Scanning electron microscopy

Ependymal cells were fixed with 4% v/v glutaraldehyde in Sorensen’s buffer (pH 7.4) [24] for 48 hours and then rinsed 3 times in Sorensen’s buffer. The cells were post-fixed in 1% w/v osmium tetroxide for 1 hour and rinsed in Sorensen’s buffer. The samples were then dehydrated through a graded ethanol series and immersed in hexamethyldisilazane (HMDS). The HMDS was allowed to evaporate, leaving the cells fixed to aluminium scanning electron microscope stubs. These were then sputter-coated with gold prior to examination [24].

Statistical analysis

All data are expressed as the mean ± standard deviation of 5 to 11 independent experiments. Data were analysed by one way
were strain specific; the study was repeated with two other wild-
incubation with C52, in contrast to 10403S there was a significant
amplitude of antibiotic (penicillin, ampicillin or gentamicin), these were
a 64% reduction in ciliary amplitude (P<0.001, Table 2). The reduction was not significantly different (P>0.05) from the wild-types (Table 3).

Discussion

In this study we investigated the effect of L. monocytogenes on
ependymal cilia to improve our understanding of the pathophys-
ology of listerial meningitis. Three aspects of the results are
noteworthy: the strain-specific effect of listeria on the ependyma,
the presence of aggregates of listeria on the cilia and the
dissociation of ciliary beat frequency from beat amplitude.

The introduction of high speed digital video imaging has
allowed us to measure the amplitude of beating cilia in an
ependymal culture system and to simultaneously determine their
ciliary beat frequency [26,27]. At a concentration seen in the rat
brain during listerial meningitis [9,11,12], three of the wild-type L.
monocytogenes strains studied were capable of decreasing the ciliary
amplitude. However only strain C52 significantly reduced ciliary
beat frequency. A different strain-specific pattern was seen with
aggregation around cilia, with EGDe showing the greatest
aggregation but no aggregation of C52. Aggregates were most
often seen in the presence of extracellular matrix. The reasons for
these strain differences are not clear. Inter-strain variation in
listeria has been previously reported, for example, in adhesion to
abiotic surfaces [28] and in response to sodium chloride and low
pH [29]. Those results and the data from this study indicate that
careful must be observed when extrapolating from results with a
single strain of L. monocytogenes.

Adherence of pathogens to host cells is pre-requisite for cell
invasion [30,31]. In this study we showed that listeria attached to
ependymal cells but an unexpected observation was the extensive
aggregation of the listeria attached to cilia, but no attachment to
other parts of the ependymal cells. The rapid adhesion of listeria to
ependymal cilia and the marked aggregation of listeria with the
formation of extracellular material may help to explain why CSF
takes samples taken clinically from patients with listerial meningitis
contain relatively small numbers of listeria. Interaction between
the listeria and cilia is necessary for aggregate formation but direct
interactions between individual bacteria cells also is occurring.
Significantly, aggregates were only seen physically attached to
the cilia with no aggregates in the culture medium or in spent
culture medium. Incubation of each bacterial strain and spent
supernatant (supernatant from a previous experiment) did not
result in bacterial aggregate formation, indicating that bacterial
aggregates and extracellular material formed as a result of contact
between the ependymal cells and the bacteria. Although it remains
to be determined if this material is secreted by the ependymal cells or the bacteria. It seems that signalling events are occurring, presumably after initial cilium/listeria attachment, resulting in a change in the surface phenotype of the bound listeria, which leads to more bacteria binding. In some instances extracellular material was visible and where present it may contribute to the formation of the aggregate but this material is not a prerequisite for aggregation because aggregates are found in the absence of this material. The formation of these bacterial aggregates may benefit the listeria by stabilising contact with the ependyma and by slowing CSF flow.

Figure 1. Scanning electron microscope images of Listeria on ciliated ependymal cells. (A) low magnification of rat ciliated ependymal cells in vitro. Scale bar represents 70 μm, (B) high magnification of a rat ciliated ependymal cell in vitro. Scale bar represents 5 μm, (C) L. monocytogenes strain 10403S on ciliated ependymal cell. The arrow shows areas of extracellular material. Scale bar represents 4 μm, (D) L. monocytogenes strain 10403S aggregate (arrow) on ciliated ependymal cells. No extracellular material is visible. Scale bar represents 2 μm, (E) presence of a thick layer of extracellular material on ciliated ependymal cells after incubation with L. monocytogenes strain EGDe. The arrow is pointing to an area with extracellular material. Scale bar represents 5 μm, (F) L. monocytogenes EGDe aggregate in the presence of extracellular material. The arrow is pointing to an area of extracellular material. Scale bar represents 5 μm.

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movement through interference with ciliary functioning, thereby facilitating invasion and decreasing clearance by CSF movement.

Interestingly, we observed a dissociation of ciliary beat frequency from beat amplitude, in that amplitude could be reduced without an effect on frequency. To our knowledge such dissociation has not been reported before. Therefore, in order to determine effects on overall ciliary function, measurement of ciliary beat frequency and pattern should be used. For cilia associated with listerial aggregates, a ‘drag’ effect due to the attached aggregates is likely to contribute to decreased amplitude. However, in the case of C52 no aggregates were formed thus a ‘non-mechanical’ mechanism is possible. It is also possible that the viscosity of the medium surrounding the cilia is increased. However, these points do not explain the maintenance of beat frequency and pattern should be used. For cilia associated with listerial aggregates, a ‘drag’ effect due to the attached aggregates is likely to contribute to decreased amplitude.

Table 2. Ependymal ciliary beat frequency and percentage of tissue covered with bacterial aggregates in the presence of extracellular material.

| L. monocytogenes strain | ciliary beat frequency (Hz) | % of tissue covered by bacterial aggregates in the presence of extracellular material |
|-------------------------|-----------------------------|----------------------------------------------------------------------------------|
| Control                 | 41±2                        | 0±0                                                                              |
| 10403S                  | 44±5                        | 11±3\(^a\)                                                                      |
| C52                     | 16±2\(^a\)                  | 0±0                                                                              |
| EGDe                    | 46±5                        | 62±10\(^b\)                                                                    |
| 10403Saprfa             | 42±4                        | 0±0                                                                              |
| EGDeaprfa              | 43±5                        | 0±0                                                                              |

Rat ciliated ependymal cells incubated with L. monocytogenes strains in vitro. At 3 hours post-infection ciliary beat frequency was measured. Data are the mean ± standard deviation of 5 to 11 experiments (taken from a total of 100–220 CBF readings).

\(^a\)Significantly different from the control (non-infected ependymal cells) p<0.05.

\(^b\)Significant difference from the C52 (p<0.05) or wild type EGDe (p<0.001) and indicates significant difference from 10403S and C52 (p<0.001).

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Table 3. Ependymal ciliary beat amplitude inside and outside the listerial aggregates.

| L. monocytogenes strain | ciliary beat amplitude outside the listerial aggregates | ciliary beat amplitude inside the listerial aggregates |
|-------------------------|--------------------------------------------------------|------------------------------------------------------|
| Control                 | 100±0                                                   | NLA                                                  |
| 10403S                  | 88±6\(^a\)                                             | 36±4\(^a\)                                           |
| C52                     | 67±4\(^b\)                                             | NLA                                                  |
| EGDe                    | 81±6\(^b\)                                             | 36±3\(^b\)                                           |
| 10403Saprfa             | 80±6\(^b\)                                             | NLA                                                  |
| EGDeaprfa              | 77±2\(^b\)                                             | NLA                                                  |

Data are the mean ± standard deviation of 4 to 5 experiments.

\(^a\)Ciliary beat amplitude is the percentage of the amplitude in the absence of bacteria.

\(^b\)Significantly different from the control ciliary beat amplitude outside the listerial aggregates. There were no significant differences in the ciliary beat amplitude outside the listerial aggregates in the presence of all listeria strains. NLA: no listerial aggregates.

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Figure 2. Scanning electron microscope image of listeria and extracellular material. L. monocytogenes strain 10403S was embedded within the extracellular material. The arrow is pointing to a strand of extracellular material between the bacteria. Scale bar represents 1 μm.

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suggests there is a fixed timing of intra-cilia events between ciliary strokes. Future investigations are required to decipher these mechanisms. However, before we can deduce any pneumococcal mechanism of interference, what can be said is that the successful invasion of ependymal cells by listeria does not appear to play a role in this, because the majority of cells were not infected during the 3 hour time course of this study.

We recently studied theeffect of viscosity on ependymal cells [32] and found an increase in viscous loading was followed by a rapid decrease in the frequency of ependymal cilia to a level that was maintained while the increased viscous load was present. The ciliary beat amplitude was not affected with low viscous loads (1–40 cP) but was reduced at higher viscosities (60 cP). These results are similar to the effects of increasing viscosity respiratory and oviductal ciliary beat frequency [33]. Johnson and colleagues [33] found no significant reduction in beat amplitude over a viscosity range of 1–150 cP. Of interest they found that at levels above 180 cP ciliary activity became very unpredictable. Cilia either stopped beating, just slowed down or seemed to beat more quickly with a short amplitude. Insight into the mechanism of autoregulation of ciliary beat frequency following exposure to changes in viscosity was provided by Andrade and colleagues [34]. They found that changes in mucous viscosity activate vanilloid 4-like (TRPV4) channels that elevate intracellular Ca\(^{2+}\). They also found channel opening requires the activity of phospholipase A\(_2\). This occurs at high viscous loads allowing cilia to adapt to a wide range of viscosities.

Bacterial pathogens, including L. monocytogenes, are known to form structured populations of microorganisms, adhered and embedded in an extracellular matrix consisting mainly of exopolysaccharides (biofilms) [35]. It is thought that these biofilm structures protect the bacteria from host immune responses, as well as antimicrobials [36]. Bacterial biofilms have been shown to occur in a sequential process generally involving, 1) initial attachment of individual cells to the surface 2) formation of aggregates 3) further cell proliferation and biofilm maturation with polysaccharide formation [37]. It is possible that the aggregates seen in this study are due to the same processes that are important
for biofilm formation. The observation that the formation of aggregates and extracellular material was abolished in *ΔprfA* mutant indicates that genes within the regulon of the central virulence regulator, PrfA, are involved. To our knowledge there are no PrfA regulatory genes that have been shown to be involved in aggregate formation. The inhibition of ciliary beat frequency by C52, a strain in which PrfA is in a constitutively activated state [38,39], also indicates that expression of the PrfA regulon prior to exposure to the ependymal cells affects ciliary functioning. The absolute requirement of PrfA for *L. monocytogenes* virulence has been demonstrated before, as has the requirement for several genes within the PrfA regulon [21,22], but the explanation of how the regulon determines the effects reported here remains to be determined.

Our future studies will focus on elucidation of the molecular determinants of the events reported.

**Supporting Information**

**Video S1** Rat brain ciliated ependymal cells after incubation in tissue culture medium 199 for three hours (negative control). A slow motion video of beating cilia on ependymal cells. Note the distance travelled by the tip of each cilium; this is the ciliary amplitude. For all videos the beating cilia were recorded (32x objective) using a troubleshooter 1000 high speed video camera (Lake Image Systems Ltd, UK) at 500 frames per second. Video sequences were played back at a reduced frame rate. For viewing the AVI files, MiDAS 4.0 player software should be used (http://www.xcitex.com/html/downloads.php).

**Video S2** Rat brain ciliated ependymal cells after incubation with wild type *L. monocytogenes* strain 10403S for three hours. This slow motion video shows the listerial aggregates attached to and moving at the same frequency as the cilia. Compared to the control, the ciliary amplitude is markedly reduced where associated with the bacterial aggregates.

Found at: doi:10.1371/journal.pone.0010450.s002 (8.76 MB AVI)

**Video S3** Rat brain ciliated ependymal cells after incubation with wild type *L. monocytogenes* strain EGDe for two hours. This slow motion video shows long chains of the EGDe bacterial strain attached to and moving at the same frequency as the cilia. The bacteria appear to form a network over the underlying cilia. The ciliary beat frequency is normal, however the amplitude of the ciliary beat is reduced compared to the control.

Found at: doi:10.1371/journal.pone.0010450.s003 (10.07 MB AVI)

**Video S4** Rat brain ciliated ependymal cells after incubation with wild type *L. monocytogenes* strain EGDe for 3 hours. This slow motion video shows large areas of bacteria aggregated to extracellular material and covering the cilia on ependymal cells. The bacteria are moving at the same frequency as the cilia. The ciliary beat frequency is normal but the ciliary beat amplitude is dramatically reduced compared to the control.

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**Author Contributions**

Conceived and designed the experiments: RAH CO PA. Performed the experiments: MJFS AR ISR PA. Wrote the paper: MJFS CO PA.

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