The magnitude of diaphragm deflection should be limited so that walls are not subjected to extreme and damaging deflections.

An appropriate level of seismic safety for existing buildings can be ensured only after careful evaluation of their ability to resist earthquake forces and strengthen them, if found deficient. The evaluation procedure follows a multi-layered approach with increasing level of complexity of analysis and investigation. At the preliminary level, the evaluation method looks for the presence and good condition of primary structural elements which are required to resist earthquake loads, alongwith some simple calculations to ensure their adequacy. A detailed evaluation includes more formal analysis coupled with assessment of present-day properties of materials for realistic estimates of member strengths. The method further includes supplementary checks for detailing issues unique to various structural systems which play a major role in seismic performance of the entire system, besides those related to strength only. The seismic evaluation methodology presented here is based on a prescriptive engineering approach similar to that in practice for new buildings; however, these may lead to more performance-oriented procedures in future, with the experience gained from its usage.

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Differential intracellular replication and virulence gene expression of *Listeria monocytogenes* isolates in human epithelial and endothelial cell lines

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***Listeria monocytogenes* isolated from a case of spontaneous abortion in a pregnant woman (placental tissue), a diseased sheep (blood) and fish were compared with respect to their capability to invade and replicate in human epithelial (Caco-2) and endothelial cells (HBMEC). Intracellular expression of various PrfA-dependent virulence genes that are of relevance for intracellular replication was determined. Our data show significant differences in the invasion and replication of *L. monocytogenes* strains in epithelial and endothelial cells in contrast to the control strain, EGD. The observations correlate with the different efficiency of intracellular expression of PrfA-regulated virulence genes in the two cell lines, as analysed by real-time PCR.**

Keywords: Cell lines, intracellular replication, *Listeria monocytogenes*, virulence genes.

LISTERIA MONOCYTOGENES is a Gram-positive, facultative, intracellular, food-borne pathogen that has evolved highly sophisticated strategies to infect humans and disseminate from the intestines to the blood and further to the brain stem and the placenta causing septicemia, encephalomeningitis, brain abscesses and abortion, especially

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in immunocompromised people[1,2]. Habitats of *L. monocytogenes* include soil, intestine of asymptomatic animals and humans. From soil and faeces they gain access to sewage and water, and can also contaminate food through food handlers and processing environments[3,4]. *Listeria* is found in marine water due to its high salt tolerance and also many types of run-offs into such an environment[5]. However, the incidence of listeriosis is reported to be low in India[6].

Recent data have shown that *L. monocytogenes* is indeed able to invade and replicate in different types of mammalian cells, which involves complex series of host–pathogen interactions[7–9]. Increasing evidence shows that many cellular genes are up- or down-regulated during infection and probably play a role in the establishment of infection and inflammation. Listerial genes, which are essential for efficient intracellular replication, have been extensively studied in the past, but our knowledge remains fragmentary[10]. Moreover, the *L. monocytogenes* EGD strain has been mainly used in detailed studies involving mammalian cell cultures. Hence, the present study was carried out to determine whether there are any strain-specific differences in the infection and replication of different *L. monocytogenes* isolates in endothelial and epithelial cell lines. For this, invasion and intracellular replication of three different *L. monocytogenes* isolates was analysed in human colon carcinoma cells (Caco-2), as a model for the epithelial intestinal barrier, and human brain microvascular endothelial cells (HBMEC), as a model for the blood–brain barrier. In addition, expression of different PrfA-regulated virulence genes of *L. monocytogenes* at the transcriptional level was analysed in these two human cell lines.

Three strains of *L. monocytogenes*, each isolated from food sample (fresh fish, labelled F56), clinical sample (human placenta from a case of intra uterine death, labelled 253), veterinary sample (blood of sheep suffering from circling disease, labelled OP7) and the reference strain EGD were used in the study. All these four strains were transformed with plasmid pLSV16-P_{actA}-gfp, which carries gene for green fluorescent protein (gfp) under the control of the listerial actA promoter, P_{actA}[11,12]. All *Listeria* strains were grown aerobically at 37°C in brain–heart infusion broth (BHI, Difco) or in BHI supplemented with 7.5 μg per ml tetracycline (for gfp transformed strains) until they reached the mid log phase (180 Klett units). The cells were harvested by centrifugation, washed twice with phosphate buffered saline (PBS) and stored in PBS with 20% (vol/vol) glycerol at –80°C until used for the infection experiments.

HBMEC were grown in complete endothelial cell culture medium on gelatine-coated plates[13,14] and Caco-2 cells were grown in RPMI 1640 supplemented with 10% foetal calf serum (Gibco) under 5% CO_2–95% air at 37°C (ref. 15). Cells were seeded in 60 mm tissue culture plates 48 h prior to infection. The cell lines were infected using infection aliquots of *L. monocytogenes* to get the multiplicity of infection (MOI) of 10 and incubated at 37°C for 1 h under 5% CO_2–95% air to allow optimal uptake of bacteria. The plates were then washed three times with Ca Mg PBS and the cells were overlaid with RPMI complete medium containing 50 μg/ml of gentamicin (Sigma, USA) to kill the extracellular bacteria and prevent reinfection. The time at which gentamicin was added was designated as 0 h post-infection.

Cell-culture plates were further incubated for 1 h at 37°C after which they were washed, lysed with distilled water and plated in various dilutions on BHI agar plates to determine the count of bacteria that infected the cells. To study the replication, RPMI containing gentamicin (50 μg/ml) was removed after 1 h post-infection and fresh medium containing gentamicin (10 μg/ml) was added. The infected mammalian cell cultures were further incubated and at defined times post-infection (2, 4, 6 and 24 h) they were washed, lysed with cold distilled water and the released bacteria were plated in various dilutions on BHI agar. All the cellular invasion and replication assays were performed in triplicate and repeated three times.

Gentamicin containing RPMI medium was removed from the infected host cells at various times after infection (2, 4, 6 and 24 h). The cells were detached from the well bottom using 0.5 ml trypsin/EDTA solution and transferred to a 5 ml tube. The plate was rinsed with 1.5 ml PBS that was then added to the same tube. The gfp fluorescence emitted by the bacteria in live infected cells was measured by flow cytometry using Beckman Coulter fluorescence assisted cell sorter (FACS). Using the 488 nm line of an argon-ion laser, gfp-based fluorescence was quantified in green light channel (525/10 nm bandpass filter), while the red light channel (630/20 nm bandpass filter) was used to exclude dead cells following staining with propidium iodide (PI; 2 μg per ml). A threshold of 0.1% was set to discriminate between gfp-positive and negative cells using control cells infected with *L. monocytogenes* without *gfp* gene. The experiment was performed in triplicate and repeated three times. Statistical analysis was done using Student's *t* test. P value $\leq$ 0.01 was considered as statistically significant. Infected cells were also analysed by epifluorescence microscopy (100× magnification), using a Leica DM IRB microscope, and photographs were taken using a MicroMAX charge-coupled device camera (Princeton Instruments), and assembled and edited using the Metamorph imaging software (Universal Imaging Media, PA).

To study the gene expression of *L. monocytogenes*, Caco-2 and HBMEC cell lines were infected according to the procedure described earlier. At the end of 6 h, the mammalian cells were washed thrice with Ca Mg PBS. The cell layers were lysed with cold distilled water and bacteria released were immediately transferred to a 50 ml tube and mammalian cells were pelleted by differential

centrifugation at 1000 rpm. The supernatant containing the bacteria was centrifuged at 5000 rpm for 15 min at 4°C. From the bacterial cell pellet, RNA was extracted using RN easy RNA extraction kit (QIAGEN) following the manufacturer's instructions. RNA was checked for purity or absence of DNA.

Next 5 μg of total RNA was mixed with 6 μg of random hexamer primer (Invitrogen), heat-denatured for 5 min at 70°C and chilled on ice for 10 min. The RT reaction master mix consisting of PCR-grade water, 1× strand buffer (Invitrogen), 0.5 mM each of dATP, dTTP, dGTP, dCTP, 1 mM dithiothreitol (DTT), 1 U ribonuclease inhibitor and 200 U Superscript TM RNase H reverse transcriptase (Invitrogen) was added. The reaction was incubated for 2 h at 42°C and then at 72°C for 15 min. At the end of the incubation, 2 μl of RNase was once again added and incubated further at 37°C for 45 min. The cDNA thus obtained was purified using QIAGEN PCR purification kit, following the manufacturer's instructions.

Two microlitres of 1 : 20 diluted cDNA was used for real-time PCR reaction. The reaction mixture included PCR-grade water, 1× PCR buffer, 3.5 mM Mg, 300 nM primers, 200 μM DNTP, 0.025 U HotGoldStar polymerase enzyme and 0.75 μl SYBR® Green. The PCR cycling conditions were: Initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 10 s and 60°C for 1 min. Four standards of *L. monocytogenes* genomic DNA (32, 3.2, 0.32 and 0.032 pg/μl) were included in each PCR reaction for relative quantitation of the genes. Real-time PCR was repeated three times for detection of virulence genes like *PrfA*, *ActA*, *PlcA*, *PlcB*, *Hly*, *Hpt* and the housekeeping gene *RpoB*. PCR reactions were carried out in PTC-200 DNA engine™ cycler (MJ Research).

Invasiveness, intracellular replication efficiency and expression of several PrfA-controlled virulence genes essential for the intracellular listerial replication cycle, were studied in three new *L. monocytogenes* isolates (OP7, F56 and 253) which were derived from different sources. While two of them (strains F56 and OP7) were of non-human origin (fish and sheep respectively), the other (strain 253) was isolated from human placenta and hence had to cross an endothelial barrier. Strains F56 and OP7 belong to serovar 4 and strain 253 to serovar 1.

Their different origins suggested that the infection capacity of these *L. monocytogenes* strains and therefore also their mode of intracellular replication and expression of the PrfA-regulated genes crucial for intracellular replication might be different. Independent studies (I. Karunasagar, pers. commun.) have shown that the known PrfA-dependent virulence genes are equally present in the three isolates.

In order to analyse the possible difference in virulence properties of the three *L. monocytogenes* isolates, we first studied uptake and intracellular replication of these isolates in the epithelial cell line Caco-2 and the microvascular endothelial cell line HBMEC.

Infection assays performed in Caco-2 cells using these isolates revealed that the fish isolate F56 was internalized and multiplied more efficiently in this cell line than the human and veterinary isolates, 253 and OP7 respectively (Figure 1 *a* and *b*). However, the same strains behaved the opposite way when infection assays were performed in the endothelial cells. In HBMEC, the human isolate 253 showed the highest internalization and replication efficiency in comparison to the other two isolates (Figure 1 *c* and *d*). In contrast, the *L. monocytogenes* strain EGD used as a control showed similar efficiency of uptake and intracellular replication in both epithelial and endothelial cells (Figure 1 *a–d*). Consistent results were obtained when the infection assays were performed in triplicate and repeated three times for each strain.

In addition, the efficiency of intracellular (cytosolic) replication of these isolates was determined by transforming the three isolates (and as a control the EGD strain) with the plasmid pLSV16-P_{actA}-gfp and measuring the expression of GFP in the cytosolically replicating bacteria. *L. monocytogenes* which carries *gfp* cDNA under the control of P_{actA} expresses enough GFP to yield fluorescent bacteria only when the bacteria reach the cytosol of the host cells, but not when they are growing in rich medium or residing in the phagosome of the host cells[11,16]. GFP-mediated fluorescence of the strains multiplying in Caco-2 and HBMEC was determined at different time points (0, 2, 4, 6 and 24 h) post-infection by FACS analysis (Figure 2 *a* and *b*) and microscopic examination (Figure 3 *a* and *b*). Photographs were taken and assembled using the metamorph imaging software (Universal Imaging Media, PA). Microscopic findings (Figure 3 *a* and *b*) and the FACS results (Figure 2 *a* and *b*) were in good agreement with the results obtained in the infection assays when measuring the viable bacterial counts (Figure 1 *a–d*).

Next we studied the expression of the PrfA-dependent virulence genes, *prfA*, *plcA*, *hly*, *actA*, *plcB* and *hpt* in the isolates after growth in the two host-cell lines to determine whether the different intracellular replication efficiencies observed with the three isolates could be due to differences in the expression of these virulence genes which are essential for the intracellular replication cycle. Real-time PCR was performed with RNA isolated from each of the *L. monocytogenes* strains after growth for 6 h in the endothelial and epithelial cell lines. Upon lysis of the infected host cells, the bacteria were separated by differential centrifugation from most of the host cell debris. RT–PCR showed that *prfA* and the PrfA-regulated genes, *actA*, *hly*, *plcA*, *plcB* and *hpt* were expressed best in the human isolate 253 when grown in HBMEC cells (Figure 4 *b*). On the contrary, these PrfA-regulated genes were poorly expressed by 253 in Caco-2 cells (Figure 4 *a*). The food and veterinary isolates (F56 and OP7) showed the opposite, i.e. efficient expression of the PrfA-regulated genes in Caco-2 cells and rather poor expression in HBMEC. The control strain EGD expressed these PrfA-

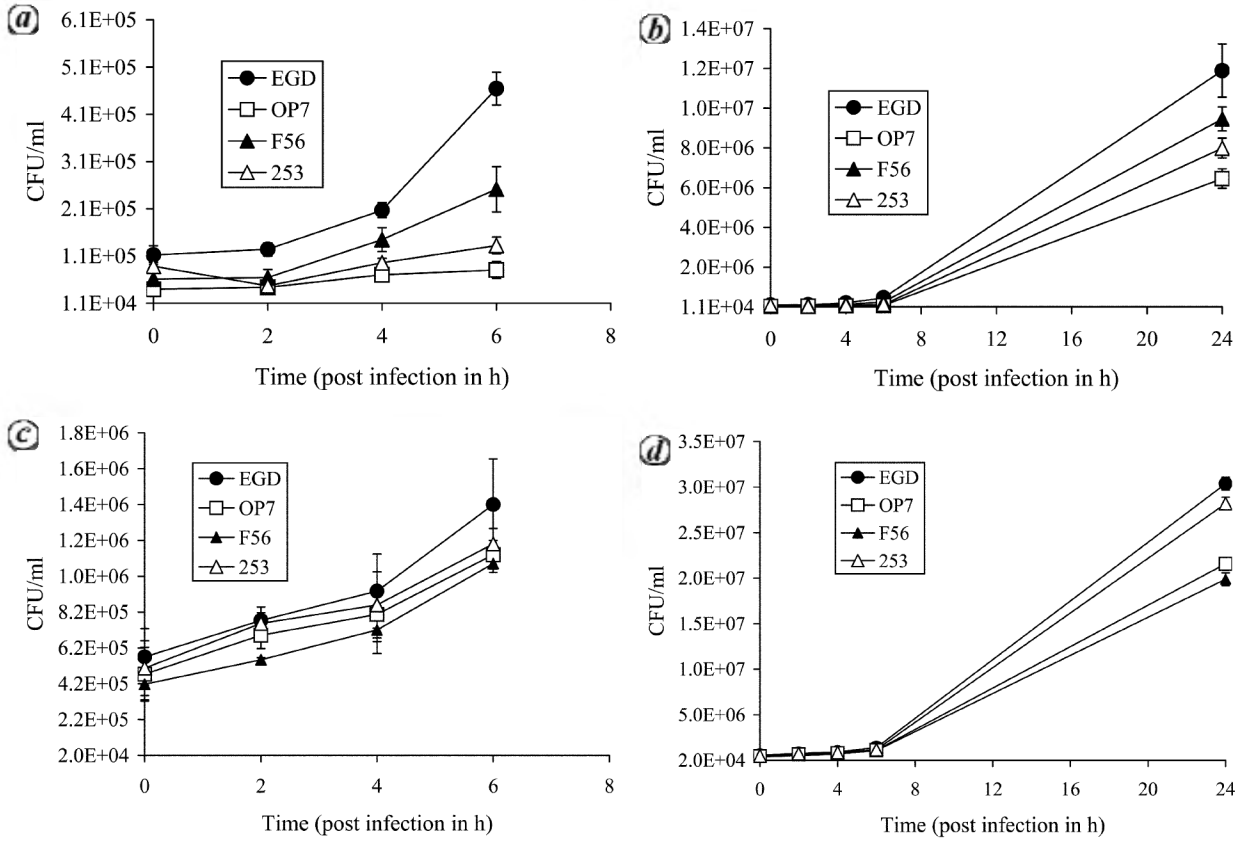

Figure 1. Invasion and replication of *Listeria monocytogenes* strains in epithelial and endothelial cells. The number of intracellular bacteria was determined by plating the lysed infected host cells at different time points. Data are mean ± standard deviation from three separate experiments. ***a***, ***b***, Number of intracellular bacteria in infected Caco-2 cells at (***a***) time points 0, 2, 4 and 6 h post-infection and (***b***) time points 0, 2, 4, 6 and 24 h. ***c***, ***d***, Number of intracellular bacteria in infected HBMEC at (***c***) time points 0, 2, 4 and 6 h and (***d***) time points 0, 2, 4, 6 and 24 h.

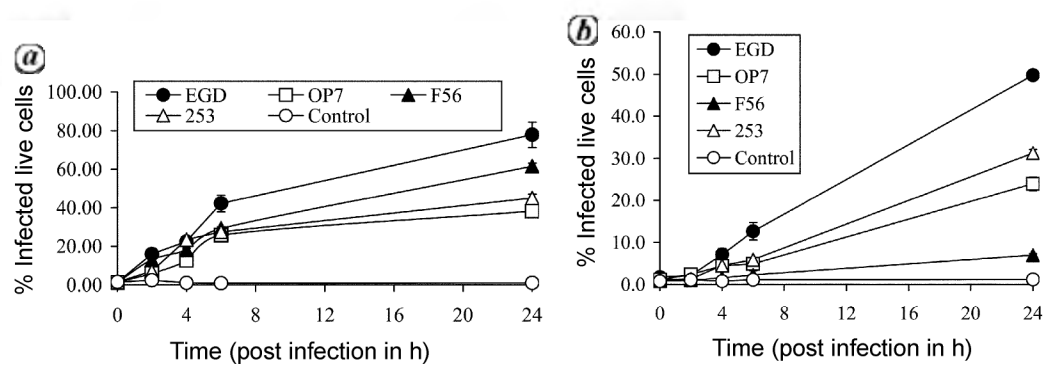

Figure 2. Invasion and replication of transformed *L. monocytogenes* strains expressing GFP in epithelial and endothelial cell lines. Fluorescence intensities of live infected cells were determined at various time points post-infection by flow cytometry. Data are the mean ± standard deviation from three separate experiments. ***a***, ***b***, Fluorescence intensities of intracellular bacteria in (***a***) Caco-2 cells at time points 0, 2, 4, 6 and 24 h and (***b***) HBMEC at time points 0, 2, 4, 6 and 24 h.

controlled genes equally well in both cell lines (Figure 4 *a* and *b*).

Thus the expression of the analysed PrfA-regulated genes that are of relevance for intracellular replication proved to be also cell type-specific and correlated well with the differential growth behaviour of the three *L. monocytogenes* strains in the two host-cell lines. No such differential expression of these genes was observed with

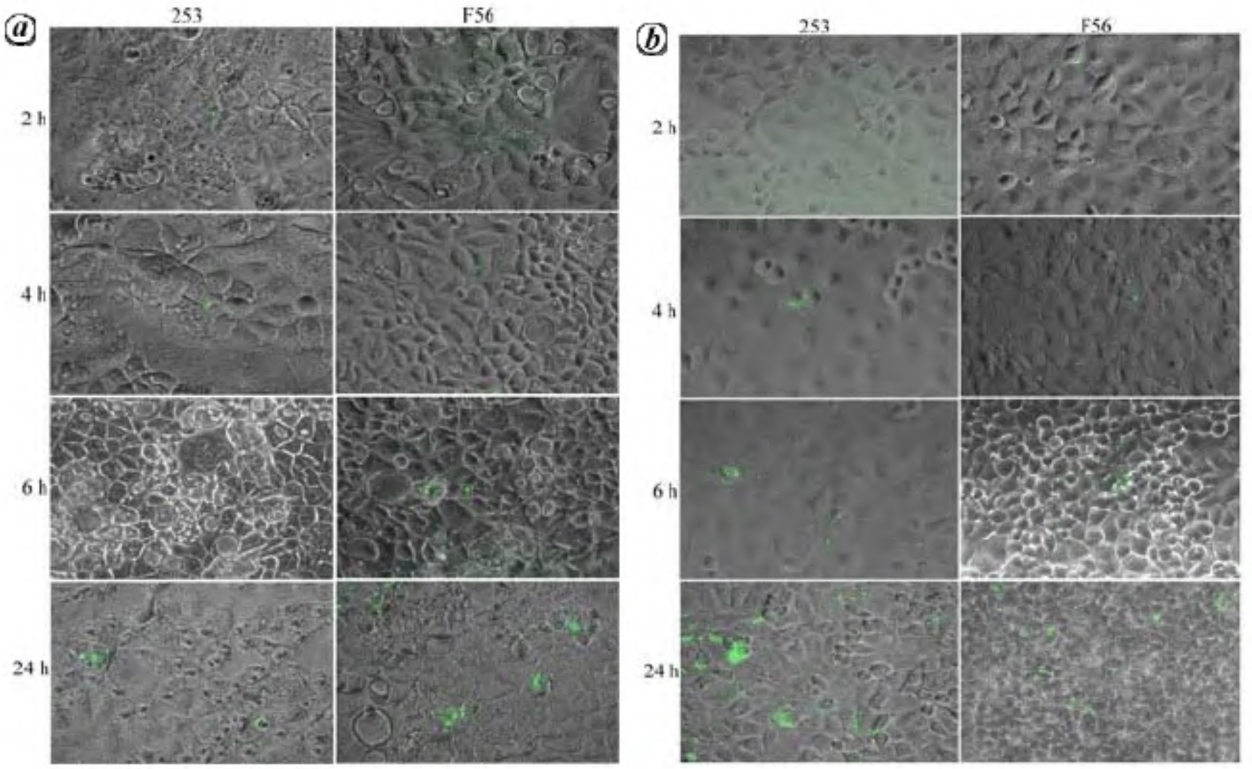

Figure 3. Microscopic images of intracellular, replicating, GFP-transformed *L. monocytogenes* (F56 – food and 253 – human) strains in epithelial and endothelial cells as observed under 100× magnification. ***a***, ***b***, Intracellular replication of GFP expressing *L. monocytogenes* in (***a***) Caco-2 cells at time points 2, 4, 6 and 24 h and (***b***) HBMEC at time points 2, 4, 6 and 24 h.

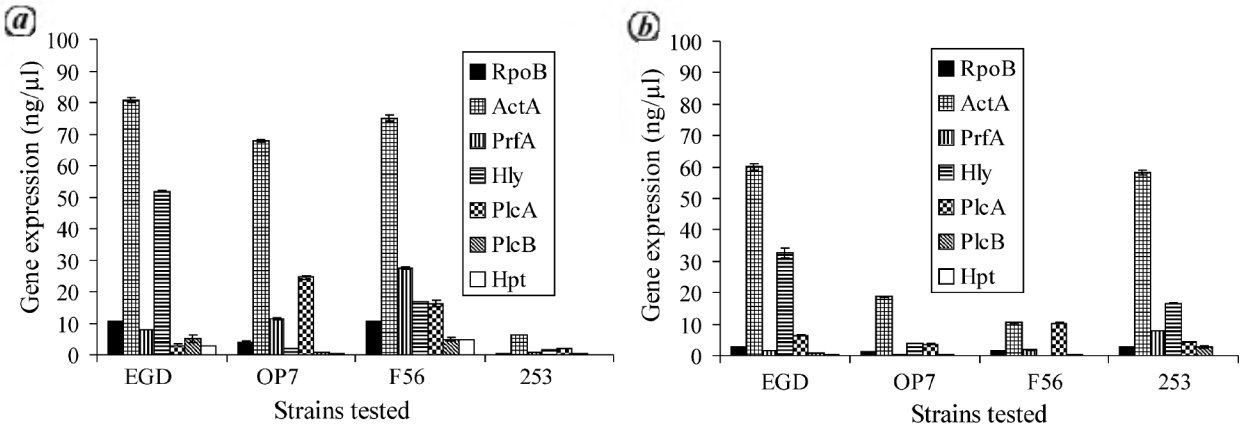

Figure 4. Expression of different PrfA-regulated virulence genes of *L. monocytogenes* strains during replication in Caco-2 cells (***a***) and HBMEC (***b***). Cell lines were infected with different strains of *L. monocytogenes* and gene expression was determined by real-time PCR using specific primers for virulence genes. Data are mean of the normalized value of gene expression ± standard deviation from three separate experiments.

the EGD strain in the two cell lines, which is again in agreement with earlier reports that showed no significant difference in the intracellular gene expression between this EGD strain and strain NCTC 7973 in different mammalian cell lines when assessed by RT–PCR[11].

To the best of our knowledge this is the first report showing strain-specific differential intracellular growth and expression of PrfA-dependent virulence genes in epithelial and endothelial cells which seem to correlate with the infection route taken by these *L. monocytogenes* isolates. Further studies are presently being performed to better understand the molecular basis of this host cell type-specific replication of the *L. monocytogenes* isolates.

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Mating success is not correlated with fluctuating asymmetry in *Drosophila ananassae*

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Sexual selection results from differential mating success among individuals within a population. In the present study, the relationship between the mating success, and size and asymmetry of different morphological traits, viz. thorax length, wing length, sternopleural bristle number, wing-to-thorax ratio, sex-comb tooth number and ovariole number was investigated in two geographical strains of *Drosophila ananassae*. Mating success was scored in an Elens–Wattiaux mating chamber for 60 min. After 1 h of observation, mated and unmated flies of both sexes were kept separately and different traits were measured. The size of all the morphological traits was higher in mated than unmated flies. The level of fluctuating asymmetry was similar in mated and unmated flies for all the traits, except sternopleural bristle number in females. Positional fluctuating asymmetry, a sensitive measure of developmental instability was also significant for bristle number in females. When fluctuating asymmetry (FA) across all traits was combined into a single composite index (CFA), the magnitude of CFA was similar in mated and unmated flies. Interestingly, there is significant difference in males and females for CFA values in both mated and unmated flies. Males show higher FA in comparison to females, suggesting that males are more prone to developmental perturbations. The results suggest that the size of the sexual trait is a more reliable indicator of individual quality in sexual selection rather than fluctuating asymmetry in *D. ananassae* and thereby weakens the hypothesis that FA is a cue of overall 'genetic quality'.

Keywords: Developmental stability, *Drosophila ananassae*, fluctuating asymmetry, morphological traits, sexual selection.

SEXUAL selection arises due to variation in mating success among individuals of a population due to competition for mates[1]. Two distinct processes that are responsible for this variation are intrasexual and intersexual selection[2]. The former is contest interaction between members of the same sex for access to mate, e.g. male–male competition to gain access to reproductive females. The latter is mate selection by individuals of one sex among members of the opposite sex, e.g. females prefer to mate with potential mating preferences in order to get fitness benefits from being choosy through direct (for themselves) and

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