MODELS OF INFECTION

Effect of multiplicity of infection on *Listeria monocytogenes* pathogenicity for HeLa and Caco-2 cell lines

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The significance of multiplicity of infection (moi) for invasiveness and intracellular multiplication of *Listeria monocytogenes* in Caco-2 and HeLa cell monolayers was investigated. A low moi (1:1) resulted in recovery of significantly more *L. monocytogenes* when these bacteria were used to infect either cell line. At high moi (100:1), the percentage recovery of bacteria was comparatively low, even after extensive invasion and intracellular multiplication. Microscopic analysis of Giemsa- and immunofluorescent-stained infected monolayers revealed extensive cell disruption and exposure of the internalised bacteria to the bactericidal effect of gentamicin. By contrast, a low moi resulted in minimal cytopathic effects and evidence of cell to cell spread by *L. monocytogenes* was consistently observed in HeLa and J774, but not in Caco-2 cell lines. Nevertheless, the use of HeLa and Caco-2 cell monolayers enabled a clear distinction to be made between invasive (*L. monocytogenes*) and non-invasive *Listeria* spp. (*L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. grayi*, *L. welshimeri* and *L. monocytogenes* LLO19). The use of a low moi with HeLa cell monolayers provided a reliable tissue-culture model of infection for *L. monocytogenes*.

Introduction

Listeria monocytogenes is a ubiquitous gram-positive organism. It is commonly isolated from soil, decaying vegetation, silage, water and sewage [1], and is known to be responsible for perinatal infections, septicaemia and meningoencephalitis in man [2]. Elderly patients, pregnant women or individuals with predisposing conditions that compromise their immunity, such as transplants and malignancies such as lymphomas, are at particular risk [3]. Increased use of immunosuppressive drugs in the treatment of malignancies and organ transplantations, in addition to the current epidemic of AIDS, has considerably increased the population at risk to listerial infection. Epidemiological investigation of outbreaks of listeriosis has also demonstrated that infection results from ingestion of food contaminated with *L. monocytogenes* [4]. For these reasons, worldwide attention has been focused on this micro-organism.

L. monocytogenes has long been used as a model pathogen for the study of cell-mediated immunity. *L. monocytogenes* evades antibody and complement-mediated lysis and killing by professional macrophages by entering, multiplying and persisting within host cells [5]. More recently, tissue-culture techniques have been applied to study the interactions between pathogen and host. This led to the development of in-vitro models of infection with *L. monocytogenes* involving tissue-culture monolayers. Gaillard *et al.* [6] first established an in-vitro model of penetration and intracellular growth of *L. monocytogenes* with the human enterocyte-like cell line Caco-2. *L. monocytogenes* was able to initiate entry into cells by inducing phagocytosis, followed by a period of bacterial intracellular multiplication. The use of this model for distinguishing between *Listeria* spp. on the basis of virulence was also described. Caco-2 cells display typical enterocyte differentiation under standard culture conditions, such as the development of a brush border which contains some enzymes that are normally located in the small intestine [7, 8]. For these reasons Caco-2 cells were considered to be a suitable cell line for the analysis of *L. monocytogenes* virulence.

Subsequent investigation showed that a wide variety of cell types are able to support the growth of *L. monocytogenes*, including macrophages, fibroblasts, epithelial cells, enterocytes and hepatocytes. The combined use of tissue-culture monolayer models of infection and electron and immunofluorescence microscopy provided insight into the cell biology of *L. monocytogenes* infections and facilitated the analysis of the bacterial genetic determinants responsible for pathogenesis [6, 9–15].

The Caco-2 cell line has become the most popular model for the analysis of *L. monocytogenes* virulence, primarily because of the typical enterocyte differentiation, and appears to be most susceptible to *L. monocytogenes* infection [6, 16, 17]. Pine *et al.* [18], in a comparison of the ability of *L. monocytogenes* to invade 12 different mammalian tissue-culture cell lines, observed that all were able to internalise *L. monocytogenes*, albeit to varying degrees, but Caco-2 cells were at least 10-fold more efficient at mediating internalisation. This unusually high efficiency of bacterial invasion has been suggested to be the result of enterocyte-like cells being the most probable initial site of entry in patients with foodborne listeriosis. Nevertheless, all comparative studies to date have neglected to consider the effect that multiplicity of infection (moi) has on the efficiency of *L. monocytogenes* to invade various cell types. In this study, the effect of moi on *L. monocytogenes* infection was investigated and the effectiveness of the HeLa epithelial cell line the Caco-2 enterocyte-like cell line as *in vitro* models for studying the virulence of *Listeria* spp. was compared.

Materials and methods

Bacterial strains, media and chemicals

L. monocytogenes strain SLCC5764 (1/2a) was a gift from H. Hof (University of Heidelberg, Mannheim, Germany). *L. monocytogenes* strain DRDC8 was an isolate from milk obtained from New South Wales Dairy Corporation Laboratories. *L. monocytogenes* LLO19 was a non-haemolytic phenotypic mutant of strain DRDC8 containing a Tn917-*lacZ*-*cat86* insertion mediated by pTV53 [19], in the *prfA* gene encoding the positive regulatory factor of listeriolysin O [20, 21]. All other *Listeria* spp. used in this study were acquired from the Institute of Medical and Veterinary Research, Adelaide, South Australia. Bacteria were cultivated routinely in Brain Heart Infusion (BHI) Agar or BHI Broth (Difco) at 37°C with aeration. *L. monocytogenes* LLO19 was cultivated in media containing erythromycin 0.01 g/L and lincomycin 0.025 g/L. Unless otherwise stated, all chemicals used in this study were purchased from either Ajax Chemicals, BDH Chemicals or Sigma Chemical Co.

Tissue cultures and growth media

Tissue-culture cell lines used in this study were the human colon adenocarcinoma enterocyte-like cell line Caco-2 (ATCC HTB 37), the human epithelial cell line HeLa (ATCC CCL 2.1) and the murine macrophage-like cell line J774 [22]. Caco-2 cells, used between passages 20 and 45, were cultured in Eagle's Minimal Essential Medium (EMEM) (Gibco Laboratories) supplemented with fetal bovine serum (Gibco) 10% v/v, glutamine 0.29 g/L, NaHCO₃ 1.8 g/L, penicillin 0.1 g/L and streptomycin 0.01 g/L. HeLa and J774 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with fetal bovine serum 5% v/v, glutamine 0.29 g/L, NaHCO₃ 3.6 g/L, penicillin 0.1 g/L and streptomycin 0.01 g/L. Cells were cultured routinely in 75-ml plastic tissue-culture flasks (Corning) at 37°C in a humidified atmosphere of CO₂ 5% v/v in air. Semi-confluent monolayers (c. 5×10^6 cells/ml) for invasion assays were prepared by subculture of cell monolayers into 24-well tissue-culture trays (Corning), containing acid-washed 12-mm coverslips, to a final concentration of about 1×10^6 cells/ml for Caco-2 cells and 5×10^5 cells/ml for HeLa and J774 cells, and incubating overnight.

Invasion of tissue culture monolayers

Cell monolayers were washed twice in the appropriate culture medium free of antibiotics. Cultures (10 ml) of logarithmic-phase bacteria were washed once in saline (NaCl 8.5 g/L) and resuspended in 2 ml of antibiotic-free culture medium. Bacteria (100 µl) diluted in antibiotic-free culture medium (5×10^8 , 5×10^7 , 5×10^6 bacteria/ml), were overlayed on to the monolayers to achieve multiplicity of infection (moi) of 100:1, 10:1 and 1:1, respectively. Analysis of the colony forming units (cfu) applied to the monolayers was confirmed by plating suitable dilutions on BHI agar. Monolayers were incubated at 37°C in CO₂ 5% v/v in air for 2 h, followed by three washes in antibiotic-free culture medium. A final incubation at 37°C in CO₂ 5% v/v in air for 2 h in culture medium supplemented with gentamicin (Schering Corporation, USA) 0.04 g/L was carried out to kill extracellular bacteria. Monolayers were washed twice in Hanks's Balanced Salts Solution. In addition, after the HeLa cell monolayers were infected with bacteria, they were centrifuged in a GPR centrifuge (Beckman) at 2500 rpm for 15 min at room temperature (RT) to sediment the bacteria on to the monolayers. Furthermore, J774 monolayers were infected with bacteria at a moi of 1:1 and were incubated at 37°C in CO₂ 5% v/v in air for 30 min, followed by incubation for 30 min in medium containing gentamicin. Monolayers were finally washed in antibiotic-free DMEM.

Intracellular bacteria were recovered by lysis of the monolayers in 1 ml of ice-cold Triton X-100 (0.1% v/v in sterile saline). The number of cfu recovered

from cell lysates was determined by plating appropriate dilutions on BHI agar. The degree of bacterial invasion is represented by calculating the percentage of the original inoculum recovered after lysis of infected tissue-culture monolayers.

Immunofluorescence microscopy

Tissue-culture monolayers grown on coverslips were fixed in formaldehyde 3.7% in phosphate-buffered saline (PBS) (NaCl 8 g/L, KCl 0.2 g/L, KH_2PO_4 0.2 g/L and NaHPO_4 (anhydrous) 1.5 g/L) for at least 1 h at 4°C. Monolayers were washed once in PBS and the cell membranes were permeabilised by the addition of Triton X-100 0.1% v/v in PBS for 1 min. Monolayers were washed six times with PBS, after which 10 μl of diluted rabbit anti-*Listeria* O antiserum polyvalent serotypes 1, 4 (Difco) was overlayed on to the monolayers and incubated at RT for 1 h in a humid chamber to prevent drying. Antisera was diluted in fetal bovine serum 10% v/v in PBS. Monolayers were washed another six times with PBS. To the monolayers, 10 μl of diluted goat anti-rabbit IgGAM (H+L) fluorescein isothiocyanate (FITC) conjugate (The Binding Site Ltd, Birmingham) and 10 μl of phalloidin

rhodamine (TRITC) conjugate (Sigma) were applied and incubated at RT for 1 h in a humid chamber, followed by six washes in PBS. The coverslips were removed, air-dried, mounted face down on to microscope slides with 3 μl of Moviol mounting medium and sealed with clear nail polish. Monolayers were examined with an Olympus BH-S microscope with a BH2-RFC UV fluorescent attachment and photographs were taken with Kodak Tmax 400 film.

Giemsa staining for light microscopy

Monolayers were fixed in cold (−20°C) methanol for at least 5 min. The coverslips were air-dried then stained in filter-sterilised Giemsa stain (BDH) for at least 10 min at RT. Coverslips were washed in Giemsa buffer (Na_2HPO_4 (anhydrous) 0.545 g/L, KH_2PO_4 0.475 g/L) until the desired intensity of stain was achieved. Coverslips were air-dried, mounted face down on to microscope slides with DPX neutral mounting medium (Koch-Light Laboratories). Monolayers were viewed with an Olympus BH-2 light microscope and photographs were taken with Kodak Tmax 100 film.

Statistical analysis

Two-way analysis of variance was performed to compare the effect of moi on the potential of *Listeria* spp. to invade tissue-culture cell lines.

Results

Effect of moi on recovery of *L. monocytogenes* from Caco-2 and HeLa cell monolayers

The effects of inoculum size of *L. monocytogenes* on ability to invade and multiply in tissue-culture monolayers was investigated by infecting Caco-2 and HeLa cells with bacteria at moi of 100:1 (100 bacteria to 1 mammalian cell), 10:1 and 1:1. Numbers of bacteria recovered after infection were expressed as a percentage of the original inoculum and taken as an indication of the level of bacterial invasion.

When Caco-2 and HeLa cells were infected with *L. monocytogenes* strains DRDC8 and SLCC 5764, significantly more bacteria were recovered from monolayers when low moi were used (100:1 < 10:1 < 1:1) ($p < 0.001$) (Fig. 1). In the absence of a short centrifugation step, recovery of bacteria from infected HeLa cells was reproducibly low (data not shown). In addition, when the non-haemolytic *L. monocytogenes* strain LLO19, and the non-haemolytic non-pathogenic *L. innocua* were used to infect either cell line, consistently low recovery of bacteria was obtained. On the basis of these data, an moi of 1:1 was used in further experiments to compare the ability of Caco-2 and HeLa cell lines to discriminate between invasive and non-invasive isolates on the basis of the percentage of bacteria recovered from cell lysates.

Fig. 1. Effect of moi (■1:1; ▨10:1; □100:1) on percentage recovery of *Listeria* spp. from cell lysates after infection of Caco-2 (Panel a) and HeLa (Panel b) cell monolayers. **a**, *L. monocytogenes* DRDC8; **b**, *L. monocytogenes* SLCC5764; **c**, *L. monocytogenes* LLO19; **d**, *L. innocua*. Each column represents the mean of at least four independent experiments and bars indicate the SEM.

When monolayers were infected with either *L. monocytogenes* strain DRDC8 or SLCC 5764 at a moi of 1:1, these bacteria invaded at a similar level irrespective of the cell line used (Fig. 2A). Furthermore, similar numbers of bacteria/infected cell at 2 h post-infection were also obtained, independent of the cell line (Fig. 2B). However, neither cell line could reliably discriminate the invasive potential of strains DRDC8 or SLCC 5764 on the basis of bacterial recovery from infected monolayers. Nevertheless, both cell lines were able to discriminate between invasive and non-invasive isolates of *Listeria* (e.g., *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. innocua* and *L. monocytogenes* strain LLO19) ($p < 0.001$).

Microscopic analysis of Caco-2 and HeLa cell monolayers after infection with *L. monocytogenes*

Although studies of invasiveness of isolates may provide an indication of the pathogenicity of that

Fig. 2. Comparison of the extent of intracellular location of *Listeria* spp. in Caco-2 (■) and HeLa (▨) cell monolayers after infection with a moi of 1 bacterium to 1 cell. Panel a: percentage bacterial recovery from cell lysates; Panel b: number of bacteria/epithelial cell determined by light microscopy of Giemsa-stained, infected monolayers. a, *L. monocytogenes* DRDC8; b, *L. monocytogenes* SLCC5764; c, *L. ivanovii*; d, *L. seeligeri*; e, *L. welshimeri*; f, *L. grayi*; g, *L. innocua*; h, *L. monocytogenes* LLO19. Each column represents the mean of at least four independent experiments and bars indicate the SEM.

isolate, the latter does not always follow. Consequently, light microscopy was used to examine the ability of invasive isolates to induce cytopathic effects characteristic of virulent infections.

Giemsa-stained preparations of cells infected with either strain DRDC8 or SLCC 5764 at an moi of 100:1 showed that complete disruption of the cell monolayers had occurred. Few Caco-2 cells were identified and these showed extensive cytopathic changes when a moi of 10:1 was used (data not shown). However, at a moi of 1:1, intact cells that contained bacteria were detected. The morphology of these cells was similar to that of uninfected cells or cells infected with non-invasive organisms (Figs. 3A and B). By contrast, HeLa cell monolayers infected with either strain DRDC8 or strain SLCC 5764 at an moi of 10:1 showed marked loss of confluence and viable cells were heavily infected with bacteria and showed a rounded morphology with indistinct membranes (Fig. 3C). At a moi of 1:1, HeLa cell monolayers retained confluence with no apparent loss of viability, as observed with control cell monolayers (Figs. 3D and E). Furthermore, pseudopod structures used to facilitate bacterial cell to cell spread [10, 14] were readily identified.

Cell-to-cell spread during bacterial infection was confirmed by immunofluorescence techniques [10, 23, 24]. Caco-2, J774 and HeLa cell lines infected at an moi of 1:1 with *L. monocytogenes* strains DRDC8 or SLCC 5764, demonstrated classical features indicative of bacterial-induced actin polymerisation and cell to cell spread (Fig. 4). Moreover, these features were more evident in the J774 and HeLa cells, and suggested more extensive bacterial parasitism within these cell lines. However, HeLa cells also revealed distinct patterns of filamentous actin, a feature not observed with either Caco-2 or J774 cells. Non-invasive strains of *Listeria* spp. and *L. monocytogenes* LLO19 were unable to multiply extensively within cells or polymerise actin or engage in cell-to-cell spread. These data suggested that the HeLa cell line is equally suitable for distinguishing between virulent and avirulent *Listeria* spp. based on its ability to support invasion and intracellular parasitism.

Discussion

Over the last decade, major advances have been made in understanding the role of virulence factors involved in the pathogenesis of *Listeria*. Development of tissue-culture models of infection provided the catalyst for these rapid advances. In particular, the Caco-2 colon carcinoma cell line has been used routinely in studies associated with *L. monocytogenes* infection [6, 11, 12, 17, 25]. Caco-2 cells are considered to be significantly more sensitive to *L. monocytogenes* invasion

Fig. 3. Light photomicrographs ($\times 1000$) of Giemsa-stained Caco-2 and HeLa cells at 4 h after infection with *Listeria* spp. **a**, Caco-2 cells infected at a moi of 1 bacterium to 1 cell (1:1) with *L. monocytogenes* DRDC8; **b**, Caco-2 cells infected with *L. monocytogenes* LLO19; **c**, HeLa cells infected at a moi of 10:1 with *L. monocytogenes* DRDC8; **d**, HeLa cells infected at a moi of 1:1 with *L. monocytogenes* DRDC8; **e**, HeLa cells infected at a moi of 10:1 with *L. monocytogenes* LLO19. Arrows indicate intracellular bacteria. Arrowheads indicate pseudopod-like structures indicative of cell to cell spread of *L. monocytogenes*. Micrographs shown are representative of the degree of monolayer confluence of tissue culture cells.

than other human epithelial cell lines including HeLa, Henle 407 and Hep-2 [18, 26]. This increased sensitivity is assumed to be due to features unique to Caco-2 morphology [7, 8].

This study showed that permissiveness of *L. monocytogenes* to invade either HeLa or Caco-2 cell lines is directly dependent upon the assay conditions. To establish significant bacterial invasion of the HeLa cell line, a centrifugation step was incorporated into the infection protocol. Under these conditions it was

possible to assess the effect of multiplicity of infection on invasion and multiplication of *L. monocytogenes* in HeLa and Caco-2 cells. In particular, significantly more bacteria were recovered from infected monolayers when low moi were used, irrespective of the cell line. This finding was reflected by the extent of cell lysis of monolayers infected at high moi, observed by microscopy of Giemsa-stained preparations. Infection at high moi resulted in extensive invasion and intracellular multiplication, ending in cell lysis. Intracellular bacteria were then exposed to inhibitory

Fig. 4. Photomicrographs ($\times 1000$) of phalloidin rhodamine conjugate-stained J774, Caco-2 and HeLa cells after infection with *Listeria* spp. **a**, J774 cells infected at a moi of 1 bacterium to 1 cell (1:1) with *L. monocytogenes* DRDC8; **b**, J774 cells infected at a moi of 1:1 with *L. monocytogenes* LLO19; **c**, Caco-2 cells infected at a moi of 1:1 with *L. monocytogenes* DRDC8; **d**, Caco-2 cells infected at a moi of 1:1 with *L. monocytogenes* LLO19; **e**, HeLa cells infected at a moi of 10:1 with *L. monocytogenes* DRDC8; **f**, HeLa cells infected at a moi of 1:1 with *L. monocytogenes* DRDC8; **g**, HeLa cells infected at a moi of 1:1 with *L. monocytogenes* LLO19. Arrowheads indicate pseudopod-like structures indicative of cell-to-cell spread of *L. monocytogenes*.

concentrations of gentamicin contained in the culture medium that prevented recovery of viable bacteria from cell lysates. Gentamicin is unable to permeate through cell membranes, and is, therefore, used routinely in tissue-culture models of infection to kill extracellular bacteria [27–30]. This aspect has been overlooked in earlier studies that described recovery and intracellular multiplication of *L. monocytogenes* in Caco-2 monolayers after infection with high moi

[6, 24, 31]. Heavily infected cells probably lyse due to excessive structural stress imposed by high numbers of intracellular bacteria. In contrast, lytic activity induced by bacterial enzymes is unlikely to result in cell membrane disruption. For example, cytolytic activity of listeriolysin O, a pore-forming haemolysin responsible for membrane disruption [32] is restricted to the phagolysosome, where an internal pH of 5.5 is necessary for optimal activity [33].

The significant monolayer disruption noted after prolonged exposure (16–24 h) of monolayers to high moi of *L. monocytogenes* [6, 18] was overcome by routinely limiting the incubation period to 4 h with a moi of 1:1. Under these conditions, a direct correlation between low moi and high bacterial recoveries from infected monolayers was observed. In our hands, at least, careful selection of moi is essential to provide a more appropriate method of determining the ability of an organism to invade and multiply within tissue-culture cells. Furthermore, HeLa and Caco-2 cells were equally efficient at discriminating between invasive (*L. monocytogenes*) and non-invasive *Listeria* spp. (*L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. innocua* and *L. monocytogenes* LLO19), a feature observed in other tissue-culture models of infection [6, 18, 34, 35]. While the results of the present study agree with previous suggestions that Caco-2 cells are more susceptible to invasion by *L. monocytogenes* [6, 17, 18, 26], we do not agree with the view that this cell line is the most suitable for the analysis of listerial invasion. Any advantages of Caco-2 cells over HeLa cells can be overcome by employing a combination of a centrifugation step, short incubation periods, and low moi. With this modified infection process, the utility of the HeLa cell line for studying *L. monocytogenes* infection has been clearly demonstrated.

Invasiveness of an organism is not the only requirement for establishing an infection. Virulence of *L. monocytogenes* also depends on ability to spread from cell to cell. Consequently, the utility of HeLa cells as a suitable tissue culture model of infection was confirmed by assessment of pathogenicity of *Listeria* spp. with immunofluorescence microscopy to compare the ability of *Listeria* spp. to engage in cell-to-cell spread. In addition to Caco-2 and HeLa cells, the murine macrophage cell line J774 was included in this study. These cells have been used extensively to study characteristics of *L. monocytogenes* infection [14, 24, 36–38]. As expected, *L. monocytogenes* contained within J774 cells displayed a high degree of filamentous actin polymerisation and cell to cell spread in comparison to infected Caco-2 and HeLa cell lines. Nevertheless, *L. monocytogenes*-induced filamentous actin polymerisation and pseudopodia-like structures were more distinctive in HeLa cells than in Caco-2 cells, suggesting a greater parasitism of pathogenic *L. monocytogenes* for the HeLa cell line. This effect may result from a greater rate of growth in HeLa cells.

Variations in bacterial growth rates within different cell lines have been described previously. In particular, an intracellular doubling time of *c.* 90 min has been reported for *L. monocytogenes* in Caco-2 cells [6]. However, a doubling time of *c.* 60 min was observed in various cell lines including the epithelial cell line Henle 407 [29]. The ability of *Listeria* strains to

multiply intracellularly is used routinely as a measure of bacterial virulence [39]. It follows that enhanced intracellular multiplication of an organism is most likely reflected by an increase in intracellular parasitism. This study did not seek to determine bacterial growth rates within HeLa and Caco-2 cells. However, these data could help in understanding why *L. monocytogenes* shows greater parasitism for HeLa cells. While Caco-2 cells may be more efficient at mediating invasion [6, 17, 18, 26], HeLa cells may promote rapid intracellular growth of *L. monocytogenes*.

The isogenic mutant *L. monocytogenes* strain LLO19 was included as a negative control. This strain contains a Tn917 insertion in the gene encoding the positive regulatory factor PrfA, which is responsible for expression of a number of necessary virulence determinants of *L. monocytogenes* [20, 21, 31] and is essential for the establishment of infection [40]. As expected, this mutant demonstrated significantly reduced invasive potential and was unable to engage in cell to cell spread. Furthermore, the viability of the cell monolayers was not compromised by infection with strain LLO19. This suggests that *L. monocytogenes* does not shed toxic by-products during in-vitro growth that may contribute to cytopathic effects on tissue-culture cells. Interestingly, the animal pathogen *L. ivanovii* was unable to polymerise host cell actin and was significantly less invasive than *L. monocytogenes*. Similarly, *L. ivanovii* is unable to invade the mouse embryo fibroblast cell line 3T6 [35]. No evidence of cytopathogenicity for hybridoma and myeloma cell lines has been observed, even after prolonged infection [34]. However, other studies have reported invasion of cell lines and polymerisation of host cell actin [6, 16]. Although we have no explanation for these apparent anomalies, strain variation among *L. ivanovii* isolates may contribute to the different responses observed.

In conclusion, the results of this study show the importance of moi in tissue-culture models of infection. Indeed, under the conditions described, HeLa and Caco-2 cells were equally capable of discriminating between invasive and non-invasive *Listeria* spp. Furthermore, HeLa cells provided an easier means to distinguish between pathogenic and non-pathogenic *Listeria* spp. by immunofluorescence microscopy. In contrast to Caco-2 cells, certain features of HeLa cells, including flat morphology, large cytoplasmic area and ease of culture, make this the preferred cell line for analysis of pathogenicity of *Listeria* spp. by tissue-culture models of infection.

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