

Original Research Article

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Virulence Potential and Intercellular Spread of *Listeria monocytogenes*

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ABSTRACT

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Listeria monocytogenes is a gram positive, rod shaped intracellular pathogen. Its the most virulent foodborne pathogen and is responsible for human listeriosis on a large scale. In this study, *Listeria monocytogenes* bacterium was isolated from varied sources including dairy food materials like cheese and other milk products and human infections. These isolates were cultured and allowed to proliferate in brain heart infusion broth. Isolates were screened for their proliferation and ability to infect other cells by assessing their intercellular spread and plaque formation by cell plaque assay in a murine macrophage cell line and virulence potential was determined by ANOVA methodology. Among all isolates, human origin *Listeria* strains demonstrated highest virulence potential.

Introduction

Listeria monocytogenes is a gram positive, facultative anaerobic, non spore forming, rod shaped bacterium. Since they are tolerant to extremes of pH, temperature and salt conditions⁶, *Listeria spp.* is present ubiquitously in varied environment including food, soil and water, sewage, vegetables, milk, milk product, even in fish and fishery products. It causes septicemia, encephalitis, meningitis and gastroenteritis particularly in children, immunocompromised individuals and also causes miscarriage in pregnant women and in animals.

Studies suggested that plaque forming assay could be used as a preliminary test to evaluate the virulence of *Listeria* strains in order to reduce the cost of testing of strains by in vivo methods⁵. Indrawattana *et al.*, (2011) reported that *Listeria monocytogenes* isolates positive for virulence genes readily invade CaCO₂ cells. Reports suggest that strains isolated from human listeriosis were more invasive than those isolated from meat and milk sources, regardless of serovars¹⁴.

Predominantly pathogenic *Listeria monocytogenes* were able to multiply rapidly in CaCO₂ cells with listeriolysin O being the

major factor responsible for intracellular growth. Studies showed that *Listeria monocytogenes* are able to multiply rapidly in murine hepatocyte cell line ATCC TIB3⁵. Evaluation of monolayers stained with wrights stain showed plaques, with large number of intracellular *Listeriae* visible within hepatocytes that lined the margins of these plaques. Reports suggest the in vitro cytopathic plaque formation assay of *Listeria monocytogenes* of food origin isolated from eastern china Zhejiang province⁶.

The pathogenesis of *Listeria monocytogenes* involves phagocytosis of bacterium by host macrophages. Mackaness was the first to demonstrate that *Listeria. monocytogenes* is able to survive and multiply in macrophages⁹.

Listeria monocytogenes are intracellular pathogens that are highly invasive in nature, internalizing cells that are normally phagocytic. The bacterium gradually sinks into dip-like structures of host cell surface until its engulfed in a zipper like fashion. The cell receptors used by *Listeria spp.* for engulfment include the transmembrane glycoprotein E- cadherin, the C1q complement fraction receptor, the Met receptor for hepatocyte growth factor (HGF), and components of the extracellular matrix (ECM) such as heparan sulfate proteoglycans (HSPG) and fibronectin. The bacterial ligands identified to date are all surface proteins, such as the internalins InlA and InlB, the actin polymerizing protein Act A, and p60¹⁴.

The intracytoplasmic bacterium have an actin tail which is composed of two populations of cross linked actin filaments, This polar arrangement helps in propulsion of bacterium in cytoplasm. This random movement helps some bacteria reach periphery, leading to formation of finger like processes with bacterium at tip at cell periphery. These processes penetrate uninfected cells,

dissolving its cell membrane and initiating a new round of intracellular proliferation and cell to cell spread in the process¹. The pore-forming protein listeriolysin O (LLO) helps in escape from the vacuole; strains lacking LLO do not escape from a vacuole and may survive in tissue culture cell lines, but do not grow¹⁴. In cell culture, this is reflected by plaque formation.

Despite being pathogenic at species level, *Listeria monocytogenes* is made of varied strains with differing pathogenicity, some being highly virulent and others causing no harm. In vitro cell culture techniques are better cost effective methods for assessing *Listeria monocytogenes* pathogenicity.

The aim of the present study is to demonstrate the cell to cell interepithelial spread of *Listeria monocytogenes* in vitro which is directly attributed to their virulence potential.

Materials and Methods

Preparation of murine macrophage cell line Raw 264.7

The macrophage cell line was grown in fresh 5 ml DMEM [Dulbecco's Modified Essential Media, ThermoFisher Scientific] medium supplemented with 10% FBS[Fetal Bovine Serum, ThermoFisher Scientific]. The media was renewed every 2-3 days till confluence of 80% was achieved. The cells were then detached by scraping and plated into each well of 6 well tissue culture plate and incubated the plate at 37°C in 5% CO₂ incubator. The plates were monitored daily for formation of monolayer.

Preparation of *Listeria monocytogenes* culture

Test strain of *Listeria monocytogenes* isolated from varied sources was inoculated in BHI

(Brain heart infusion) broth and allowed to grow overnight at 37°C in 5% CO₂ incubator. Turbidity value was adjusted to 0.1 by subsequent dilution with millipore distilled water and recorded absorbance at 600 nanometer in spectrophotometer (Eppendorf Biospectrometer). The bacterial culture was then centrifuged at 12000rpm for 3 mins and the pellet was then resuspended in 4ml DMEM.

Infection of cells

The cell culture plates (6 well tissue culture plate, Costar 3516, Corning, NY, USA) were washed twice with DMEM and then 125 microlitre of bacterial suspension was added along with DMEM in each well in duplicates. One positive well was kept to check for contamination containing only media.

The plates were incubated at 37 °C for 2 hours in 5%CO₂ incubator. After incubation, the cells were washed twice with DMEM and then 1 millilitre of DMEM with 50 microgram/millilitre gentamicin was added to each well to prevent contamination of wells.

The plate was incubated at 37°C in 5% CO₂ incubator for 30 mins and then washed twice with DMEM. Wells were then overlaid with DMEM growth medium (1.2%) [1millilitre per well] and incubated for 3 days at 37°C in 5% CO₂ incubator.

Staining

Post incubation 1 millilitre of 0.04% of neutral red mixed in 10 millilitre of DMEM was added to each well and incubated at 37°C

for 3 hrs. After staining, neutral red stain was pipetted out and washed with DMEM. Plaques were visualized under inverted microscope using SCOPETEK software. Plaques size was measured using one way ANOVA.

Results and Discussion

The mean size of plaque produced by standard strain of *Listeria monocytogenes* (MTCC) was 16500 sq. micron metre. The isolate of human origin (560) produced significantly larger plaque (27600) as shown in Fig. 1.

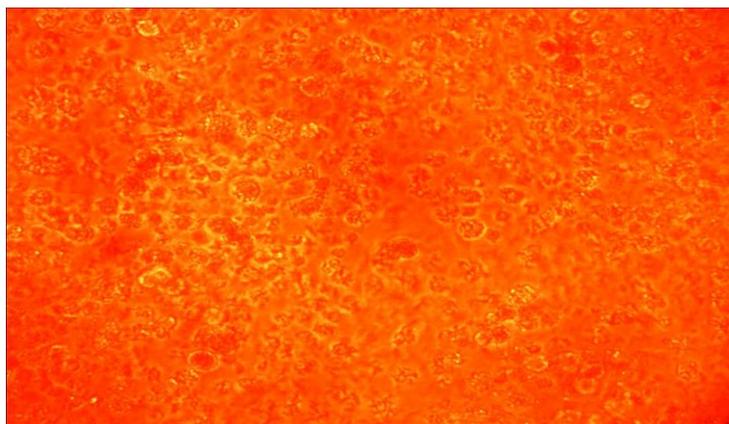
The isolates from dairy sources produced plaques in the range between 4000 to 7200 sq. micronmetre respectively. The observations are in congruance with the reports¹⁴. The plaque sizes are given in Table 1.

In conclusion the present study was aimed to establish the intercellular spread of *Listeria monocytogenes* bacterium in murine macrophage cell line and determine their virulence potential. Plaque formation assay was carried out and the mean plaque size produced by standard strain was used to compare with that of test isolates. It was observed that the plaque produced by human origin strains was higher than that of dairy sources. Thus it establishes that in vitro plaque formation assay is suitable to assess the invasive potential of *Listeria monocytogenes*. Also, isolates obtained from human origin were highly invasive compared to dairy origin isolates.

Table.1 Mean plaque size produced by strains of *Listeria monocytogenes* in Raw 264.7 cell line

| <i>L. monocytogenes</i> isolate No. | 1144 MTCC | 491 | 560 | 175 |
|-------------------------------------|-----------|------|-------|------|
| Mean plaque area sq. micron | 16500 | 4000 | 27600 | 5505 |
| SE | 2077 | 590 | 3360 | 1180 |

Fig.1 Human isolate showing larger plaque in murine macrophage cell line Raw 264.7



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