

## Invasion and Interaction Studies of *Salmonella typhimurium* Sub Sp *Enteritis* in Vero and MDCK Cell Lines

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**Abstract:** Vero cells and Madin - Darby Canin Kidney cells were exposed to *Salmonella typhimurium* sub spp. enteritis to study the interaction and invasion. The monolayer cell line bottles were selected and sub cultured. The sub cultured bottles were incubated at 37°C in 5% CO<sub>2</sub> atmosphere for the formation of monolayer. After the complete confluence of cells, the bottles were selected for the inoculation of bacteria. The mid logarithmic phase bacterial culture (10<sup>5</sup> CFU/ml) was used. These infected cell monolayer were incubated at 37°C in 5 % CO<sub>2</sub> environment. After half an hour of bacterial inoculation, the monolayer was observed under the inverted microscope. The shape of the cells became changed. Before inoculation, the shape of the Vero cells was sickle cell shape. The cells become round shape. After 1 hour of bacterial inoculation, the cells were fully changed into round shape and the cells became swollen. The experiment was performed to test the invasion ability of *Salmonella* on Vero cells and Madin Darby Canin Kidney cells.

**Key words:** *Salmonella typhimurium* sub sp enteritis • Vero cell line • MDCK cell line

### INTRODUCTION

*Salmonella* is one of the most extensively characterized bacterial pathogens and is a leading cause of bacterial gastroenteritis. It is also most extensively studied bacterial species in terms of its physiology, genetics, cell structure and development. *Salmonella* are motile, gram negative, rod-shaped bacteria belonging to the enterobacteriaceae family; the species are closely related to *Escherichia coli*. *Salmonella* was first cultured in 1884 by Gaffky [1]. Most of the serotypes infect a broad range of warm blooded animals and are capable of causing disease in humans. The majority of serovars that cause disease in humans belong to serogroup 1 [2-3].

*Salmonella* is capable of causing a variety of disease enteric fever, bacteriemia, enterocolitis and focal infections. Enteric fever (typhoid fever) is caused primarily by *Salmonella typhi* and *Salmonella paratyphi* and occasionally by other serotypes. Approximately 2000 serotypes of *Salmonella* have been associated with

enterocolitis. The incubation period is typically 6-48 hours and is followed by headache, abdominal pain, diarrhoea and vomiting. Transmission of *Salmonella* to humans is usually by consumption of contaminated food, but human to human transmission and animal to human transmission may also occur. The most common source of *Salmonella* is water, beef, poultry, fish and eggs.

The first step in the disease process is transmission to a susceptible host. It is estimated from volunteer studies that 10<sup>5</sup> to 10<sup>10</sup> bacteria are required to initiate an infection. It is generally believed that a large inoculum is required to overcome the acidity and to compete with the normal flora of the intestinal tract. Many intracellular parasites are capable of penetrating host epithelial barriers. To study this process in more detail, we examined the interactions between *Salmonella* spp. and monolayers of Vero cells and Madin Darby Canine Kidney cells. Tissue culture was first devised at the beginning as a method for studying the behavior of animal cells free of systemic variations that might arise in the animal both

during normal homeostasis and under the stress of an experiment [4]. In addition to cancer research and virology, other areas of research have come to depend heavily on tissue culture techniques. Tissue culture has contributed greatly, via the monoclonal antibody technique to the study of immunology already dependent on cell culture for array techniques and the cell lines.

## MATERIALS AND METHODS

**Sample Collection:** The cultures *Salmonella typhimurium* sub spp. *enteritis* was collected from K.S.R College of Arts and Science Microbiology lab. The culture was further identified on selective medium Wilson Blair agar plate. The plates are incubated at 37°C for 24 hours. After the growth has obtained in the selective media the organism is further confirmed with biochemical test.

**Layout of Tissue Culture Lab:** The major requirement that distinguishes tissue culture most other laboratory techniques is the need to maintain asepsis. Six main functions need to be accommodated in the laboratory: Sterile handling, incubation, preparation, wash up, sterilization and storage. Sterile work should be located in a quiet part of the laboratory, should be restricted to tissue culture and there should be no through traffic or other disturbance.

**Cleaning of Tissue Culture Wares:** Washing of tissue culture ware is undertaken to remove dirt, organic materials and toxic material. Detergents are used for washing glassware as they are surface acting agents decrease surface tension, increase wetting, free from Calcium and Magnesium and work very well in hard water and are freely removed on rinsing.

**Aseptic Technique:** Contamination by microorganisms remains a major problem in tissue culture. Bacteria, mycoplasma, yeast and fungal spores may be introduced via the operator, the atmosphere, solutions and many other sources. Proper aseptic technique seeks to eliminate such contaminants. All material that will come in to direct contact with the culture must be sterile and manipulations designed such that there is no direct link between the culture and its non sterile surroundings. Wash hands, wrists and arms with a disinfectant soap before and after all tissue culture work. Work cleanly, cleanup any spill immediately with that bacteria and fungi can grow on spilled media and act as sources of contamination.

Swab down the work surface before and during work. When working on an open bench flame the necks of bottles and screw caps before and after opening a bottle and before and after closing it.

**Preparation of Tissue Culture Media:** The sigma MEM powder was dissolved in 1000ml of sterilized triple guard distilled water. Adjust the PH of the medium using a NaHCO<sub>3</sub> (8.8%) for slight alkaline (slight pink). The medium is sterilized by passing them in 0.22µm or 0.45µm pour sized membrane filter. After filtration antibiotics are added.

|                      |             |
|----------------------|-------------|
| Benzyl penicillin    | -100 µ/ml   |
| Streptomycin sulfate | - 100 mg/ml |
| Amphotericin B       | - 100 mg/ml |

**Preparation of Growth Medium and Maintenance Medium:** In the filter sterilized MEM medium 10ml of foetal bovine serum is added in the 190ml to prepare growth medium. For maintenance medium the foetal bovine serum is not added, the MEM medium is used as a maintenance medium.

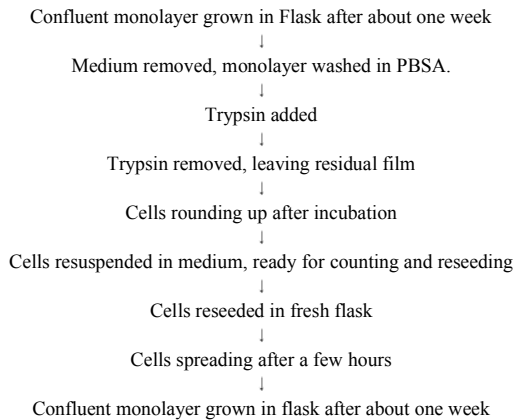
**Sterility Checking Medium:** 1ml of MEM medium is inoculated in various sterility checking medium for bacteria and fungi.

**Culturing of MDCK and VERO Cells:** Fully monolayer of these cell line bottles is bought from Madras Veterinary College and they are subculture and maintained.

**Procedure of Sub Culturing:** Culture was taken to a sterile work area. Then Phosphate buffer saline was added and Pre washed (0.2ml/cm<sup>2</sup>) to the side of the flask opposite the cells. The cells were rinsed. After that Trypsin Versin Glucose (0.1ml/cm<sup>2</sup>) was added to the side of the flask opposite the cells. It is amust to ensure that the monolayer is completely covered. The flask was kept at a stationary phase for 15-30 seconds and then withdrew all. With the flask lying flat, it was incubated until the cells round up and detached from the surface of the bottle. Medium (0.1-0.2 ml/cm<sup>2</sup>) was added and dispersed the cells by repeated pipetting over the surface bearing the monolayer. Count of the cells was done by using hemocytometer. Then dilution of the suspension to the appropriate seeding concentration (5×10<sup>4</sup> cells /bottle) or desired split up ratio 1:4. Then the distribution of the cell suspension to culture bottles, with required growth medium was performed. Incubation of the cultures was done at 5% CO<sub>2</sub> and 37°C until confluent cell monolayer is

formed. The mid logarithmic phase *Salmonella* culture is diluted with the sterile saline up to maximum of  $10^5$  CFU/ml. 1ml of *Salmonella* culture is inoculated into the Vero cell monolayer and Madin Darby Canin Kidney cell monolayer.

### Steps Involved in Sub Culturing:



### RESULTS AND DISCUSSION

Salmonellosis is one of the major food borne diseases caused by *Salmonella*. *Salmonella* is capable of causing a variety of disease syndromes: enteric fever, bacteremia, enterocolites and focal infections. Approximately 2,000 serotypes of *Salmonella* have been associated with enterocolites. We focus on the interaction of enterocolites causing *Salmonella* with the Madin-Darby Canin Kidney cells and Vero cells. In vitro cell culture models for the interactions of these bacteria with the cell lines are discussed.

The assay described by Finlay and Falkow [5] was used to examine the cellular invasion under in vitro condition in cell lines such as Vero cells and Madin-Darby Canine Kidney mono layers. The number of eukaryotic cells seeded into each bottle was determined by quantification in a counting chamber. The number of eukaryotic cells seeded into each bottle was  $5 \times 10^4$ . The average number of days required for confluence was 1 and 5 days for Vero and Madin-Darby Canine Kidney cells respectively. After one hour of bacterial inoculation, the mono layers were observed under the inverted microscope. The shape of the cells became changed. Before inoculation, the shape of the Vero cells is sickle cell shape (Plate no. 1). The cells start to become round shape after half an hour of *Salmonella* infection. After 1 hour of bacterial inoculation, the cells completely become round shape and the cells are became swollen (Plate no. 2).

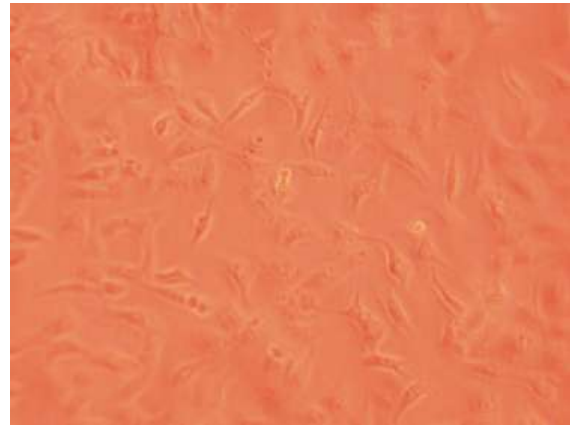


Plate 1: Vero cells (Before inoculation)



Plate 2: Vero Cells (After inoculation)

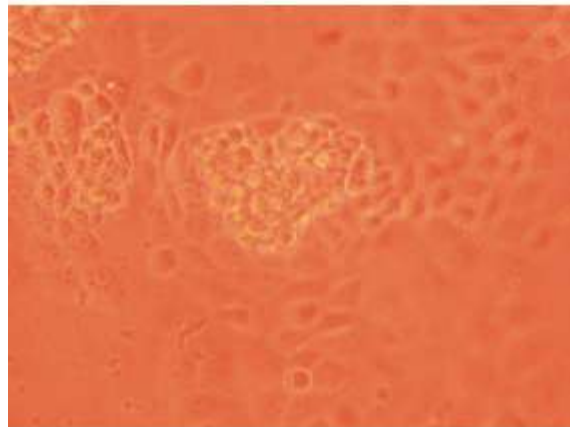


Plate 3: MDCK cells (Control)

Similar results were found when the bacteria are inoculated in to the Madin Darby Canin Kidney cell lines. Before inoculation, the cells were healthier (Plate no. 3). After half an hour of inoculation, the cells became round and after 1 hour of inoculation, the cells were fully swollen

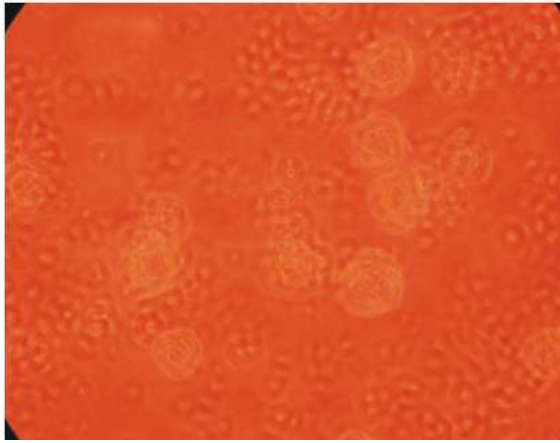


Plate 4: MDCK cells (After inoculation)

and damaged (Plate no. 4). Earlier study done by Adam Griffith *et al.* demonstrates that during invasion, CaCO<sub>2</sub> cells was highly variable and it was seemed to correlate with the amount of time elapsed after confluence was achieved. Cells typically grew to confluence after 5 to 6 day in culture and were used up to 1 week after confluence was achieved. Because, CaCO<sub>2</sub> cell polarization and differentiation have been identified as factors in invasion by several bacterial species, a series of experiments was performed to test the effect of time in culture of CaCO<sub>2</sub> cells on invasion by *Salmonella* to represent active growth (day 4), confluence (day 7), polarization (day 11) and differentiation (day 14 to 17).

Possible reasons for the marked difference between the results of Madin-Darby Canin Kidney cell invasion and Vero cell invasion studies reported here and those of Adam Griffith *et al.* that include difference in the degree of differentiation, a well described property of eukaryotic cells, which can impact invasion, regard to technique, Adam Griffith *et al.* used both a lower inoculum and a longer incubation period for their studies. Both polarization and differentiation of Vero cells and Madin-Darby Canine Kidney cells are important for invasion by different bacterial species bacteria that requires polarization of monolayer. Further, experiments will be necessary for the identification of invasion mutants in vitro and testing their ability to invade in vivo. Ultimately these kinds of experiment will be required for the complete understanding of *Salmonella* pathogenesis.

1 to 5 % of infections with *Salmonella* are reported [6], thus there are actually 2to 4 million cases in the US each year with an estimated annual cost of over \$2 billion [7]. The incidence of Salmonellosis in the United States has steadily increased since World War II [8]. Eggs can

be contaminated through cracks in the shell or transovarally from an infected ovary or oviduct to the yolk prior to deposition of the shell [9]. The infectious dose decrease when *Salmonella* is consumed with food that traverse the stomach rapidly (i.e., liquids) or with food that neutralizes the stomach acidity (i.e., cheese, milk).

Individuals with high gastric pH, such as the elderly, are more susceptible to infection [10]. It has also been shown that pretreatment of mice with Streptomycin which reduces the amount of the normal flora, decreases the dose of *Salmonella* required to infect 50 % of the mice [11]. Similar effects of antibiotic treatment have been observed in humans [12]. To get information about the progression of infection relevant to human situation, infected rhesus monkeys and examined the colonization and histopathology of various organs at several time points (1, 2, 4 and 7 days) post infection. The symptoms that developed in these monkeys were similar to what is seen in humans [13].

Experiments using the gentamicin protection assay to quantify invasiveness T24 was the only cell lines found to be invade. Epithelial cells that have been investigated include human bladder (t24), Madin Darby Canine Kidney (MDCK), Opossum Kidney (OK) and human Colonic Carcinoma (CaCO<sub>2</sub>) cell lines [14].

Cells typically grew to confluence after 5 to 6 day in culture and were used up to 1 week after confluence was achieved. Because CaCO<sub>2</sub> cell proliferation and differentiation have been identified as factors in invasion by several bacterial species [15] a series of experiments was performed to test the effect of time in culture of CaCO<sub>2</sub> cells on invasion by *E. coli* K1 strain, RS218 [16]. Tissue culture was first devised at the beginning as a method for studying the behavior of animal cells free of systemic variations that might arise in the animal both during normal homeostatis and under the stress of an experiment [4].

Most cells from solid tissue grow as adherent monolayer and unless they have transformed and became anchorage independent. Normal cells can divide only for a limited number of times; hence cell lines derived from normal tissue will die out after a fixed number of population doublings. This is genetically determined event, known as senescence [17]. The demonstration that human tumors could also give rise to continuous cell lines encouraged interest in human tissue, helped later, by the classical studies of Lwonerd Hayflick on the finite life span of cells in culture [18] and the requirements of Virologists and Molecular Geneticists to work with human material.

A transcytosis assay was also used to examine the ability of *E. coli* K1 to invade polarized monolayer of T84 or CaCO<sub>2</sub> cell. For these studies 1×10<sup>4</sup> eukaryotic cells in 0.2 ml of tissue culture medium were seeded onto the apical side of 0.6 cm<sup>2</sup> polycarbonate transwell membranes, pore size 3 µm. Both gentamicin protection assays and transcytosis assays using CaCO<sub>2</sub> cells were performed at different times (4, 7, 11, 14 and 17 day) to assay the effects of polarization and differentiation upon invasion. Brush border enzymes were harvested from CaCo -2 cells at each of these times according to the method of Rousset *et al.* [19].

Invasion by RS218 was demonstrated at day 11 with a dramatic decrease at the later time points. Maximum enzyme activity for both sucrose and alkaline phosphatase appeared to peak at day 11 to 14 suggesting that at this time cells were maximally differentiated [16]. *Salmonella* enteritica serovar typhimurium is a well-characterized model system for the study of host - pathogen interactions. It possesses two functionally distinct type III secretion systems, both of which are encoded by pathogenicity islands.

*Salmonella* pathogenicity islands 1 (SPI-1) is required for invasion of epithelial cells, as it secretes effector proteins that induce cytoskeleton polymerization and membrane ruffling in the target host cell. SPI -2 is required for intracellular replication and systemic disease in mice [20]. SPI -2, located at 63 min on the *Salmonella* serovar typhimurium chromosome map is a 40- Kb segment that encodes a type III secretion system. Proteins secreted by SPI-1 are involved in cell invasion and in the induction of apoptosis in murine macrophages [21].

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