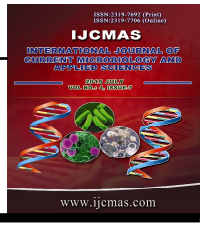
Int.J.Curr.Microbiol.App.Sci (2015) 4(8): 825-831

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 8 (2015) pp. 825-831**
http://www.ijcmas.com



Original Research Article

Molecular analysis of Virulence of field isolates of *Salmonella typhimurium* isolated from poultry in Tarai region (Uttarakhand State) of India

B. H. Jagadish and M. K. Saxena*

College of Veterinary and Animal Sciences, G.B. Pant University of A & T, Pantnagar, (U.A), India
**Corresponding author*

A B S T R A C T

In the pathogenic life cycle of a number of microbes invasion of eukaryotic cells is an essential step. In case of enteric bacteria invasion is the critical step for systemic infection. For *Salmonella typhimurium* invasion decides whether it is less or more pathogenic. We here report variation in the virulence of *Salmonella* typhimurium isolates in terms of their ability to invade the chick embryo fibroblast CEF. Further the role of plasmid in invasion and the presence of *vir* gene and its value in detection of virulent strains of *Salmonella typhimurium*. We also report that, CEF can be very well used as an *in vitro* model for the invasion studies.

Keywords

Plasmid, Salmonella, *vir* gene

Introduction

Virulence is defined as the relative capacity of microbes to cause damage in host. Virulence is dependent on different independent variables that includes the microbial aggressiveness, invasiveness, infectivity, toxigenecity and communicability (Lipsitch and Moxon, 1997). Many of the pathogens' virulence exclusively depend on single most critical virulent factor. In case of *Salmonella typhimurium* invasion of gastrointestinal mucosa is an essential and crucial step for pathogenesis and strains unable to invade animal cells are avirulent (Giannella *et al.*, 1973).

Characterization of virulence of different isolates of *Salmonella typhimurium* in terms of their ability to invade eukaryotic cells is one approach that may enable a better understanding of their pathogenecity. Cultured eukaryotic cell lines are commonly used to study bacterial invasion since they provide reproducible and uncomplicated infection models (Tang *et al.*, 1993)

In the present study, nine isolates of *Salmonella typhimurium* which were isolated from poultry farm of Tarai region were studied for their virulence status.

These isolates were screened for plasmid and their invasion in chicken embryo fibroblast cells. Gene was amplified and findings were correlated between invasion rate, plasmid profile and "vir" gene amplification.

Materials and Methods

Bacterial isolates

Nine isolates of *Salmonella typhimurium* used in this study are maintained at the Animal biotechnology Centre, Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, G.B. Pant University of Agri. & Tech. Pantnagar,(U.A), India. These isolates were isolated from poultry farms of Tarai region of Uttrakhand state. The isolates were antigenically characterized at National *Salmonella* Centre, Division of Bacteriology, IVRI, Izatnagar, UP, India.

The isolates were grown overnight at 37^0C in Luria Bertani broth (LB broth) and were sub cultured into shaking L.B broth culture until the late-logarithmic phase of growth (Wavelength 600nm).

Preparation of Chicken Embryo Fibroblast (CEF) culture

Monolayer required for bacterial invasion study were prepared from 10-11 day old chicken embryos procured from flocks free of *Salmonella* from Instructional Poultry Farm (IPF), GBPUA&T, Pantnagar. Primary CEF culture was prepared as per the method described by Tang *et al.,* 1993 with some modifications. The embryos were collected aseptically and placed in Hank's Balances Salt Solution (HBSS, Himedia, India). After removal of head, extremities and visceral organs, the remaining tissue was washed and minced using a sterile scissors. Then the tissue was digested with 0.25% trypsin solution (Himedia, India) for 20 min. with constant stirring. The cells were filtered through sterilized muslin cloth and centrifuged at 800 g for 5min. at 4^oC. The cells were then washed in HBSS and finally in minimum essential medium (MEM, Himedia, India). The washed cells were re-suspended and counted using Neubar's chamber under a microscope. Finally the cell concentration was adjusted to 10^7 cells per ml in MEM containing 10% fetal calf serum (Himedia, India) and distributed in to 6-Well culture plates.

Bacterial infection of fibroblast

Infection of chicken embryo fibroblasts with *Salmonella typhimurium* isolates was carried out according to the protocol of Cano *et al.,* 2001 with some modifications. Briefly, CEF were grown in MEM medium containing 10% fetal calf serum to 30% confluence at the time of infection. Bacteria were grown overnight in the LB broth at 37^oC under static conditions without shaking. The multiplicity of infection was 10 bacteria per fibroblast cell, and the infection time and pH were standardized for maximal invasion. Infection time were 15min, 30min, 45min, 1hr 15min and 2hr, and the pH selected were 4.5, 6.5, 7.5 and 8.5. Infected cells were washed three times with phosphate buffer saline (PBS pH 7.4) and incubated in fresh medium containing 100µg of Gentamicin/ml. The antibiotic concentration was decreased to 10µg/ml at 2 h post infection. After 2 h the media was removed and the monolayer was washed with PBS and was exposed to 1% Triton-X to lyse the cells and to recover the bacteria that were in side the cells, the lysates were poured on BGA plates for finding the viable count which gave the estimate of invasion of given isolate.

Isolation of genomic DNA and '*vir*' PCR

For isolation of genomic DNA, the bacteria were grown overnight at 37^oC for 18hr in 25 ml LB Broth with constant shaking in shaker cum incubator. Genomic DNA was isolated by the C-TAB method (Wilson (1987)).The

protocol for '*vir'* PCR was carried out as per the method of Rexach *et al.* (1994) and Saxena *et al*. (2004) with some modifications.

Bacterial DNA was amplified with a programmable Thermal cycler (Biometra). Taq. polymerase (1.5U) was added to 25µl of a reaction mix containing 1-X assay buffer (1.5mM MgCl2). 200µM of each dNTPs, 20 pico mole of each primer and 40ng template of target DNA. PCR was performed with initial denaturation at 94^{o}C for five minutes, followed by 30 cycles of denaturation for one minute at 94^{o}C, annealing at 65^{o}C for 1min, polymerization at 72^{o}C for 1min, and final polymerization for 5min. Negative control reaction was set up in each replica, containing all reagents except for the template DNA.

Plasmid isolation

The plasmid DNA was isolated and electrophoresed in 0.8% agarose gel as described in Sambrook *et al*. (1990).

The molecular weight of plasmid was calculated by comparing with the Marker plasmid (*E. coli* MTCCV517).

Results and Discussion

The invasiveness of *S. typhimurium* in CEF

For this experiment ML36, a representative isolate of *Salmonella typhimurium* was taken and the invasiveness was checked at different time of exposure, *viz*, 15min, 30min, 45min and 1hr 15min (Table 1). The results of this experiment indicate that *Salmonella typhimurium* need 45min for maximum invasion.

For determining the optimum pH for invasion time was kept at 45 min and pH was varied (Table 2). It indicates that pH 6.5is optimum for invasion of CEF by *Salmonella typhimurium.*

Invasiveness among the isolates of *Salmonella typhimurium* varies significantly:

In present study we have differentiated different isolates of *Salmonella typhimurium* based on their ability to invade CEF (Table 3).

Comparative study of invasiveness was made for nine different isolates of *Salmonella typhimurium* (Table 3). As per the observations, the isolate ML7 showed highest invasion and ML25 least invasion. The degree of invasiveness between least and maximum invading isolates was about 200 times.

Plasmid isolation

The plasmid of (32.2kD) was isolated from six isolates ML4, ML6, ML7, ML14, ML23, and ML36. ML11 has small plasmid of approximately 2 kDa. In remaining two cultures there was no plasmid.

***'vir'* gene PCR**

All the nine isolates were tested for the presence of '*vir*' gene by PCR which on amplification with specific primers produced a product of approximately 530bp in 7 of the 9 isolates. The isolates ML5 and ML25 were not positive for '*vir*' gene PCR (Fig. 1).

Invasion of eukaryotic cells is an essential step in the pathogenic life cycle of a number of microbial pathogens. Different organism has evolved different strategies to enter and survive inside the eukaryotic host cells (Falkow, 1991). A number of enteric

bacteria including *Salmonella* have developed the ability to penetrate non phagocytic cells. This ability of *Salmonella* constitutes a very important pathogenic /virulent property. The optimal pH and time of ph 6.5 and 45 min obtained for maximum invasiveness in this study is in the agreement with earlier reported findings. Similar study in bovine suggested bovine halt of bacterial interaction with follicle-associated epithelium by 60 minutes. Time dependence invasiveness has greater relevance for deciding the incubation period of a pathogen in host organism. *Salmonella* invasion is receptor mediated; therefore a fixed number of receptor on membrane allows a maximum number of organisms to enter in to host cells at a given point of time

The first major stressful environment that *Salmonella* encounters after an oral infection is exposure to acidic gastric contents. Acid tolerance may contribute to virulence of *S. typhimurium* (Humphrey *et al.,* 1996).Adaptation to specific pH range is likely to be an important variable in *Salmonella* pathogenicity, since pH has been identified as a regulator of virulence gene expression in animal pathogens. In slightly different study was also carried out in *S.* Enteritidis in which different isolates were tested for acid tolerance at pH 4 and they found wide variation in their ability to tolerate this pH and they also reported that the isolates with higher acid tolerance were more virulent than those with acid intolerance isolates (Lu *et al.,* 1999). In *S* Typhi invasion at pH of 5 and 6.5 in human intestinal epithelial cell culture reveal that the *S.* Typhi has higher invasion rate at 6.5 pH than at pH 5. This variation is mainly because, the expression of gene responsible for invasion are pH dependent. For example, the expression of the invF, pagC, prgH, prgK, orgA, sspA, and sspC genes of *S. typhimurium* (Bajaj *et al.,* 1996) and the inv gene of *Y. enterocolitica* (Pepe *et al.*, 1994) are regulated by the pH of the immediate environment. Our study supports these findings.

Invasion of epithelial cells is a characteristic of *Salmonella* associated with virulence. The comparative study of invasiveness of different isolates of *S. typhimurium* in CEF monolayer revealed that, within a given serovar there is great variation in the invasiveness. We found almost 200 times difference in degree of invasiveness between lowest and highest invasiveness obtained. Similar experiment performed earlier in *S. typhimurium* reported variance in invasiveness from 51.80 to 36.80 percent with the different isolates in CEF cells. Difference in invasion between different isolates was also found in *S.* Cholerasuis in MDCK epithelial cells monolayer and *S.* Typhi in HeLa cell monolayer. Similarly the variation in the invasion ability of different isolates was also reported by Lu *et al.,* 1999 in *Salmonella* Enteritides. The variation in the ability to invade the cultured mammalian cells is accounted for the presence of virulence plasmid (Galan *et al.*, 1989) and SPI1. An operon (spvRABCD), containing five genes, is present on plasmids commonly associated with some serovars. The spv genes possibly have the ability to increase the severity of enteritis and allow infection and persistence at extra-intestinal sites (Libby *et al.,* 2000). The chromosomally located invasion gene invA is thought to trigger the invasion of *Salmonellae* in to cultured epithelial cells. The locus of this gene is in SPI1. Hence the presence or absence of plasmid and SPI1 is considered responsible for the variation found in the invasion rate. In our study we found the isolates without the large plasmid had low invasion rate was found less compared to the isolates with the plasmid. The earlier findings also support these observation viz.

ability of virulent *Salmonella typhimurium* strains to adhere to and invade HeLa cells, and to kill mice following oral administration, depends on the presence of 92kbp large plasmid (Jones *et al.*, 1982).

PCR has been used for detection of pathogen (Shivchandra *et al.*, 2006, Shivshankar *et al.*, 2001) for molecular epidemiology (Saxena *et al.*, 2006) and for detection of virulence (Saxena *et al.*, 2006) in bacteria. Virulence associated gene in the plasmid is detected by the amplification of approximately 530bp product with vir gene specific primers. The presence of gene linked to virulence in the isolates of *Salmonella typhimurium*. All the isolates were tested for the presence of '*vir*' gene, out of 9 isolates 7 isolates (ML4, ML11, ML6, ML7, ML14, ML23, ML36) showed amplification of a single product of approximately 530 bp. The amplification was observed only in those isolates in which plasmid was present. Virulence associated gene has been reported in Indian isolates of *S.* Gallinarum and British isolates of *S. typhimurium* (Jones *et al.*, 1982). Similarly, plasmids encoding '*vir'* gene in isolates of *Salmonella* was also reported (Saxena *et al.*, 2004). However, it has been reported that every plasmid bearing serovars are not necessarily the carriers of virulence gene (Boyd *et al.*, 1998). In ML 11 plasmid of low molecular weight was obtained but "vir" gene was present. This isolate also exhibit low invasion, so we conclude that there is a positive correlation between large plasmid and invasion rate.

Table.1 The invasiveness of *S. typhimurium* in CEF at different times of infection

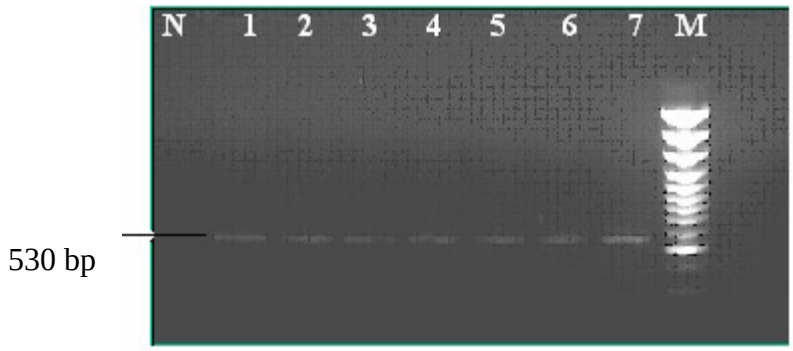
Isolate	Number of CEF cells.	Number of bacteria inoculated	Recovered intracellular viable bacteria at different exposure period			
			15min	30min	45min	75min
ML-36	4×10^7	40×10^7	1.3×10^3	9.3×10^3	2.1×10^4	1.4×10^4

Table.2 The effect of pH on the invasion rate of *S. typhimurium* in CEF

Isolate	Number of CEF cells	NO, of bacteria inoculated	Colonies on BGA (colonies/100µl of lysate)			
			4.5	6.5	7.5	8.5
ML36	4×10^7	40×10^7	$1.8x10^2$	2.19×10^3	7.5×10^2	1.6×10^3

Table.3 Comparative invasion rate of different isolates of *S. typhimurium* in CEF

Isolate	Number of CEF cells.	Number of bacteria infected.	Recovered intracellular viable bacteria(CFU/100µl)	Recovered intracellular viable bacteria (CFU/ml)
ML4	4×10^7	40×10^7	500	5.0×10^3
ML6	4×10^7	40×10^7	350	3.5×10^3
ML5	4×10^7	40×10^7	30	3.0×10^2
ML7	4×10^7	40×10^7	3300	3.3×10^4
ML11	4×10^7	40×10^7	20	2.0×10^2
ML14	4×10^7	40×10^7	1200	1.2×10^4
ML23	4×10^7	40×10^7	440	4.4×10^3
ML25	4×10^7	40×10^7	18	1.8×10^2
ML36	4×10^7	40×10^7	2100	2.1×10^4
Control	4×10^7	40×10^7	No Colonies	No Colonies



N-Negative, 1-ML4, 2-ML36, 3-ML6, 4-ML7, 5-ML11, 6-ML14, 7-ML23,
M- marker 100 bp plus (Fermentas)

Fig.1

References

Bajaj. V., R. L. Lucas, C. Hwang, and Lee, C. A. 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of hilA expression. Molecular Microbiology 22: 703-714.

Boyd, F., and Harti, D. J. 1998. *Salmonella* virulence plasmid: Modular acquisition of the spv virulence region by an F-plasmid in salmonella enterica subspecies I and insertion into chromosome of subspecies II, IIIa, IV and VII isolates. Genetics 149: 1183-1190.

Cano, D. A., M. Martinez, M. Moya, E. Pucciarelli, J. Groisman and Garcia F. 2001. *Salmonella* enterica serovars Typhimurium response involved in attenuation of pathogen intracellular proliferation. Infection and Immunity 69:6463-6474.

Falkow, S. 1991. Bacterial entry into eukaryotic cells. Cell 65: 1099-1102.

Frost, A., A. Bland and Wallis, T. 1997. The early dynamic response of the calf ileal epithelium to *Salmonella typhimurium*. Veterinary Pathology 34: 369-386.

Galan, J., and Curtiss, R. 1989. Cloning and molecular characterization of genes whose product allow *Salmonella typhimurium* to penetrate tissue culture cells. Proceeding of National Academy of Science USA 86: 6383-6387.

Giannella, A., O.Washington, P. Gemski and Formal, S. 1973. Invasion of HeLa cells by *Salmonella typhimurium*: a model for study of invasiveness of *Salmonella*. Journal of Infectious Diseases 128: 69-75.

Humphrey, T., K. Williams, M. McAlpin, J. Lever, P.Guard and Cox, M. 1996. Isolates of *Salmonella enterica* Enteritidis PT4 with enhanced heat and acid tolerance are more virulent in mice and more invasive in chickens. Infection and Epidemiology 117: 79-88.

Jones, G., D. Rabert and Whitfield, H. 1982. Association of adhesive, invasive and virulent phenotypes of *Salmonella typhimurium* with autonomous 60 megadalton plasmid.

Infection& Immunity 38:476-486.

Libby, S., M. Lesnick, P. Hasegawa, E. Weidenhammer and Guiney D. 2000. The *Salmonella* virulence plasmid spv genes are required for cytopathology in human monocyte-derived macrophages. Microbiology 2:49-58.

Lipsitch, M., and Moxon, E. 1997. Virulence and transmissibility of pathogens:
what is the relationship? Trends in Microbiology 5: 31-37

Pepe, J., J. Badger and Miller, V. 1994. Growth phase and low pH affect the thermal regulation of the *Yersinia enterocolitica* inv gene. Molecular Microbiology 11: 123-135.

Rexach, L., F. Dilasser and Fach, P. 1994. Polymerase chain reaction for *Salmonella* virulence- associated plasmid genes detection: a new tool in *Salmonella* epidemiology. Infection & Epidemiology 112: 33-34.

Sambrook, J., E Fritsch and Maniatis, T. 1980. Molecular Cloning: A laboratory manual (Vol 1, 2, and 3) Cold Spring Harbor Laboratory, New York

Sangwei, L., R. Amee, Y. Manges, C. Ferric and Lee, W.1999. Analysis of Virulence of Clinical Isolates of *Salmonella* Enteritidis *In Vivo* and *In Vitro*. Infection and Immunity 67: 5651-5657.

Saxena, M. K., V.P. Singh, A. Saxena, B.D. Lakhchaura and Sharma, B. 2004. Detection of virulence in Indian isolates of *Salmonella* by Polymerase Chain Reaction. Indian Journal of Biotechnology 3:37-40.

Saxena, M. K., Singh, V. P., Kumar, A.A., Chaudhri, P., Singh V.P., and Sharma B. 2006. REP-PCR analysis of *Pasteurella multocida* isolated from wild and domestic animals in India. Veterinary Research Communication 30:851-861.

Shivshankara, N., M.K. Saxena and Singh, V. P. 2001. Rapid Diagnosis of haemorrhagic septicemia by PCR assay. Indian Veterinary Journal 78:101-103.

Shivachandra, S.B., A.A. Kumar, R. Gautam, S. Joseph, P. Chaudhuri, M.K. Saxena, S.K. Srivastava and Singh, N. 2006. Detection of *Pasteurella multocida* in experimentally infected embryonated chicken eggs by PCR. Indian Journal of Experimental biology 44: 321-322

Tang, P., V. Foubister, M. Pucciarelli and Finlay B. 1993 Methods to study bacterial invasion. Journal of Microbiological Methods 18: 227-240.

Wilson K. 1987. Preparation of genomic DNA from bacteria, Current Protocols in Molecular Biology: Unit 2. 4.1. New York: Wiley.