Iranian Biomedical Journal 11 (1): 47-52 (January 2007)

Comparing Invasive and Non-Invasive of Isolated *Shigella flexneri* by Electron Microscopy of Cell Culture, SDS-PAGE and Congo Red Method

Mojdeh Hakemi Vala*[1], Jamileh Nowroozi[1], Farideh Ghazi[1], Parvaneh Nabavi Tabatabai[2] and Saeed Haghighi[3]

[1]*Dept. of Medical Microbiology, Medical School, Iran University of Medical Sciences;* [2]*Dept. of Electron Microscopy, Medical School, Iran University of Medical Sciences;* [3] *Dept. of Microbiology, Pasture Institute of Iran, Tehran, Iran*

Received 12 November 2005; revised 24 June 2006; accepted 9 August 2006

ABSTRACT

Background: The aim of this study was to compare invasive and non-invasive strains of *Shigella flexneri* isolated from Tehran by a 120 kDa protein band by SDS-PAGE, electron microscopy of cell culture and Congo red dye methods. **Methods:** *S. flexneri* strains were isolated by standard bacterial methods from fecal specimens of children attending to the 3 children's hospitals. Phenotype analysis for screening virulent of strains of *S. flexneri* was done on a plate of tryptic soy agar contained 0.003% Congo red dye. Whole membrane protein preparations were used to examine the protein profiles of the inner and outer membrane of these Gram-negative bacteria. The protein mixture was electrophoresed through a polyacrylamide gel. The gel was stained with Coomassie brilliant blue R250 and destained with ethanol and acetic acid. HeLa cell culture was done by two-step preparations: one for light microscopy and the other for electron microscopy. **Results:** Some of *S. flexneri* (46%) were Congo red positive colonies. *S. flexneri* with negative Congo red phenotype could not enter the HeLa cell culture. A 120 kDa protein band was found in 46% of these bacteria which could enter into HeLa cell culture. Pseudopod structures which facilitate bacterial cell-to-cell spread were readily identified by electron microscopy. **Discussion:** Since the existence of 120-kDa protein band was corresponded to enter of *S. flexneri* into the HeLa cell culture and correlated with Congo red dye positive, for identification of invasive and non-invasive *S. flexneri* strains, the use of a 120-kDa protein band by SDS-PAGE or a simple, rapid and very cheap Congo red dye method is recommended. Because, there are some deaths due to *Shigella sp.* in our country, notification on the isolation of these bacteria in both children hospitals laboratories and private clinical laboratories is important. *Iran. Biomed. J. 11 (1): 47-52, 2007*

Keywords: *Shigella flexneri*, HeLa cell culture, Electron microscopy, Congo red dye

INTRODUCTION

Among pathogenic microorganisms invasive bacteria have the ability to penetrate mammalian epithelial cells both *in vivo* and *in vitro* [1]. *Shigella flexneri*, which is responsible for a dysenteric syndrome in human, belongs to the invasive group of pathogens [2-4]. It is known that the ability of *S. flexneri* to penetrate epithelial cells is encoded by a 20-kilo base portion of a 220-kilo base plasmid [1, 4, 5]. This is unexpected for a non-motile microorganism which has been related to icsA inter cellular spread, vir G, a gene encoding a 120-KDa outer membrane protein which allows interaction with microfilaments [1, 6]. The entry of *S. flexneri* into epithelial cells is achieved through internalization of the bacterium into a membrane bound vacuole derived from the host cell plasma membrane [1]. *Shigella* species remain within human intestinal epithelial cells where they cause the destruction of enterocytes and induce an inflammatory response [2]. *S. flexneri* requires both adhesive and invasive phenotype to efficiently colonize follicle-associated epithelium (FAE) [7]. Recent studies on the enter invasive pathogen, *S. flexneri,* have shown that, in addition to allowing

*Corresponding Author;

intracellular growth [8], lysis of the phagocyte vacuole also allows bacteria to spread intracellular and infect adjacent cells [8, 3]. Cell cultures are commonly used to assess the ability of intracellular bacteria to invade susceptible eukaryotic cells [9].

In the years of 1980, there were a lot of studies on identification and the intracellular existence of *S. flexneri*. *B*ut in recent years, because of health improvement in conditions in developed countries, search for isolation of this bacteria decreased but in developing countries, such as Iran and Taiwan, many children still lose their life for infection of *S. flexneri* [10, 11]. So, the aim of this study was to compare the invasive and non-invasive properties of isolated *S. flexneri* by electron microscopy (EM) of cell culture, SDS-PAGE and Congo red.

MATERIALS AND METHODS

Bacterial strains. *S. flexneri* strains were isolated from fecal specimens of children attending to the 3 children's hospitals (Markaze-Tebbi Kodakan, Aliasghar and Mofid Hospitals) from January 2001 to December 2003. In this study, 350 *Shigella sp*. were isolated and identified by standard methods. After that, 100 *S. flexneri* strains were randomly chosen and stored in peptone and glycerol at -70°C.

Bacterial suspensions. Bacteria were harvested in tryptic soy broth in the exponential phase, washed in PBS [NaCl (8.8 gL^{-1}); Na_2HPO_4. $2H_2O$ (2.250 gL^{-1}); NaH_2PO_4. H_2O (0.257 gL^{-1}): pH 7.4] and suspended at the appropriate density of 2×10^6 cfu ml^{-1} in MEM.

Congo red binding. Phenotype analysis for screening virulent of strains of *S. flexneri* was done by Congo red dye. A colony of fresh culture of isolated bacteria was inoculated on a plate contained TSA (tryptic soy agar) and Congo red solution at final concentration of 0.003% to detect red pigmentation colony [12, 13].

Protein preparation and SDS-PAGE. Whole membrane preparations were used to examine the protein profiles of the inner and outer membrane of Gram-negative bacteria. In this process, pellet of the cells were suspended in 30 mM Tris-HCl (pH 8.1) and resuspended in 20% sucrose/30 mM Tris-HCl (pH 8.1) plus lysozyme. Then, 3 mM EDTA (pH 7.3) was added. Terminal pellets were suspended in l × LUG buffer [Tris-HCl (pH 6.8), 50 ml; SDS 25 ml, 0.25 M; glycerol, 2g; beta-mercaptoethanol, 5 ml; bromophenol blue, 2 ml; of 1%, distilled water, 100 ml] [14]. Following certain preparative steps, the protein mixture was electrophoresed through a polyacrylamide 8% gel. The gel was stained with Coomassie brilliant blue R250 and continuously destained with mixture of ethanol and acetic acid. The 120-kDa band was compared with protein ladder (protein ladder, Page Ruler^{tm} # Smo661, Fermentas, Lituani).

HeLa cell cultures and EM. To proceed for experiment, two-step preparations were done: one for light microscopy and the other, for EM. HeLa cell was obtained from the Public Health Medical School of Tehran University (Tehran, Iran). New cultures were prepared in tissue culture (trays) wells consisting of cover slip (22 × 22 mm) and concentration of 2×10^5 cell/ml using 0.5 ml MEM (Minimal Essential Medium) with 5% FCS. Then, bacterial suspensions with MOI (multiplicity of infection) 10 bacteria/cells were added to each well. Process of bacterial suspension preparation was as explained below:

After gentle mixing, the trays were inoculated in the CO_2 incubator at 37°C for 1 h and then washed three times and fresh tissue culture medium (MEM) with 5% FCS containing gentamicin at a final concentration of 40 mg/ml was added to each well and were incubated in the CO_2 incubator at 37°C for another 2 h. The cover slips were fixed with methanol at 4°C overnight and then stained with Giemsa stain and washed with Giemsa buffer for light microscopy examination.

For EM investigation, HeLa cell monolayer was seeded in 100 ml culture flasks and after washing for three times, the bacterial suspensions were added. All flasks scraped off and centrifuged. Fixation with glutaraldehyde 2% was done on pellets of cells for 2 hours. Continuously, a centrifuge step was done and melted agar 2% was added to each cell pellet, mixed well and post fixed for 1 h with Osmium tetroxide, and then, 4 concentrations of aceton were added for dehydration and samples were embedded in resin spur (R1032, Agar Scientific, UK). Gold sections were taken and stained with saturated uranyl acetate and lead citrate [15]. A gride of cell culture without infection with *S. flexneri* was used as control.

RESULTS

Congo red binding. All 100 *S. flexneri* isolated were tested for binding to Congo red dye. Then, isolated bacteria were identified as positive invasive phenotype with red colonies and non-invasive phenotype identified with white colonies. In this study, 46 (46%) isolated *S. flexneri* were Congo red positive colonies on TSA contained 0.003% Congo red dye. All the Congo red positives (100%) strains produced β haemolysin on blood agar.

SDS-PAGE. From 100 isolated *S. flexneri*, a 120-kDa band was detected in 46 isolates (46%). The range of protein bands was from 30 kDa to 150 kDa. The protein profiles of some strains are shown in Table 1 and Figure 1. On the bases of protein bands, these were 12 distinct groups. Protein bands with the same size were present in several strains, for example, most of strains (20.7%) were in group I.

HeLa cell culture and EM. As it was described above, all isolates were tested for binding to Congo red dye; so, invasive and non-invasive strains were separated. Bacterial suspensions from *S. flexneri 2a* strain (Reference Strain, RS) with Congo red positive and probably IcsA positive, *S. flexneri* strains with Congo red positive and probably IcsA positive and *S. flexneri* strains with Congo red negative and probably IcsA negative isolated from patients were used to infect HeLa cell line. HeLa cell cultures which were infected with either RS or *S. flexneri* strains with Congo red positive and probably IcsA positive at MOI 10:1, showed marked loss of confluence and viable cells were heavily infected with bacteria and showed morphology with indistinct membrane (46%). According to pictures, invasive *shigella* strains penetrated into these cells (Fig. 2). HeLa cells infected with *S. flexneri* strains with Congo red positive and probably IcsA positive, exhibited structures similar to those of non-infected cells. However, *S. flexneri* strains with Congo red positive and probably IcsA positive adhered and occasionally entered to HeLa cells. Furthermore, pseudopod structures used to facilitate bacterial cell-to-cell spread were readily identified.

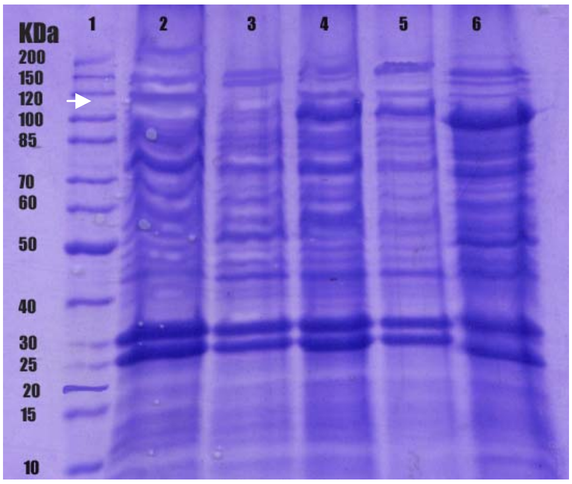

Fig. 1. SDS-PAGE of some strains of *S. flexneri*. Lane1, ladder; lane 2, *S. flexneri* 2a standard had a 120-kDa band; lane 3, negative sample had not a 120-kDa band; lanes 4-6, positive samples; all had a120-kDa band.

Table 1. Protein profiles of *S. flexneri* isolated.

	Protein bands (kDa)									Bacteria (%)
Group	30	40	50	60	70	85	100	120	150	
I.	+	+	+	+	+	+	+	+	-	20.6
II.	+	+	+	-	-	-	-	-	-	12.1
III.	+	+	+	+	+	+	+	-	-	7.3
IV.	+	+	+	+	+	+	-	+	-	6.0
V.	+	+	+	+	-	-	-	-	-	2.3
VI.	+	+	+	+	+	+	+	-	-	17.0
VII.	+	+	+	+	+	-	-	-	-	1.2
VIII.	+	+	-	-	-	-	-	+	-	2.4
IX.	-	-	-	-	-	-	-	+	-	12.3
X.	+	+	+	+	-	-	-	+	-	1.2
XI.	+	-	+	+	+	+	-	+	-	3.0
XII.	+	+	+	+	+	-	+	-	-	14.6

+, exist; -, not exist .

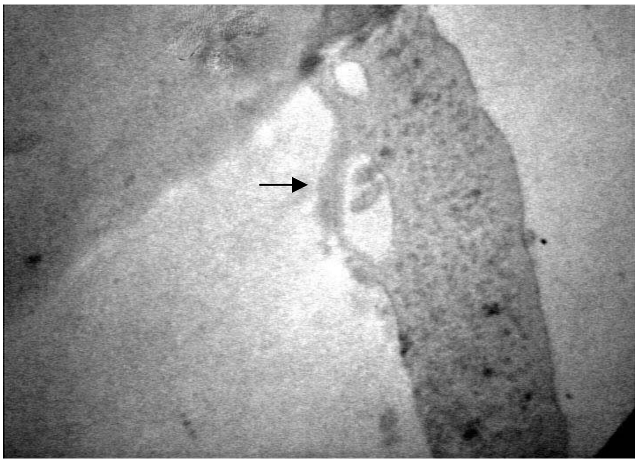

Fig. 2. Penetration of invasive *S. flexneri* (vacuole formation) which had a 120-kDa band (presumptive IcsA protein) and Congo red positive.

Electron microscopy. *S. flexneri 2a* (RS) with Congo red positive and probably icsA positive and patient's isolated *S. flexneri* strains with Congo red positive and patient's isolated *S. flexneri* strains with Congo red negative and probably icsA negative which infected HeLa cell cultures were used for EM investigation. Internalization, existence of bacteria into cells and cell disruption of both Congo red positive and probably IcsA positive *S. flexneri* strains and *S. flexneri 2a* (RS) were detected. Existence of pseudopod filaments were confirmed by EM (Fig. 3).

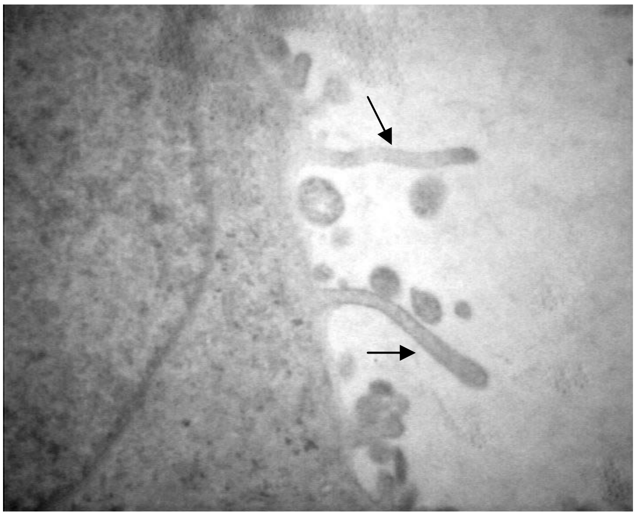

Fig. 3. Pseudopod filaments formation in infected HeLa cell with *S. flexneri* strain which had a 120-kDa band and Congo red positive.

DISCUSSION

In this study, 350 *Shigella spp.* from fecal of patients were isolated and 142 (40.57%) were *S. flexneri*. One hundred of *S. flexneri* strains which recovered from stool specimens of children's were chosen randomly for presumptive determination of IcsA protein (120 kDa band) and Congo red binding, then, infect HeLa cell line as noted above. Forty six (46%) of S. flexneri strains were Congo red positive colonies on TSA plate contained 0.003% Congo red dye. Relation between the virulence of *S. flexneri 2a* and its ability to absorb Congo red was examined in Mounier study [16]. This property is correlated to invasiveness phenotype of *S. flexneri* in cell culture [17]. Present results showed, all Congo red positive isolates had adhesion and invasion properties to HeLa cells.

These data were agreed with those of other studies [1, 9, 18-22]. Francis and Thomas showed that, infected Caco-2 or HeLa cell cultures by *L. morocytogenes* at high MOI, had extensive invasion, intra-cellular multiplication and finally cell lysis [10, 21].

As it was noted above, in 46 (46%) of isolated *S. flexneri* strains , a 120 kDa band was detected in SDS- PAGE method , that might be correlated to IcsA protein. Presence of a 120 kDa band and phenotype Congo red positive were detected in 46 (46%) of isolates in the same time. In this study, both *S. flexneri 2a* RS and *S. flexneri* strains with Congo red positive and probably IcsA Positive were isolated from patients, showed similar internalization, cell existence and cell disruption. In addition, pseudopod filaments were confirmed with EM.

In contrast, most of *S. flexneri* strains probably IcsA negative could not enter HeLa cells culture. Moreover, a few of these bacteria could adhere and enter to the HeLa cells, but cell disruption was not detected. These bacteria produce necrosis later, but can not be detected after 3 h incubation. Unlike the Hly (Haemolysin) positive strains of *L. monocytogenes*, none of Hly negative bacteria had spread inter-cellular after 2 h of incubation and only a few number of them had spread after 4 h of incubation [18].

In other study, interaction of *Salmonella typhimurium*, *Listeria monocytogenes* with murin's M cells were compared. Tissue infected with the lower dose of organisms did not show significant M cell disruption at the various times examined and had the same appearance as the FAE. In contrast, at the higher dose, the interactions between *S. flexneri* strains and the epithelium of peyer's patches were similar to those observed for *L. monocytogenes*. Destroyed regions revealed, membrane blebs and a denuded epithelial surface that closely resembled to those observed for *Listeria*. These data demonstrated that *L. monocytogenes* and *S. flexneri* possess the ability to induce massive destruction of FAE when inoculated into intestinal loops at inoculums of 4×10^9 cfu per ml [5].

In this study, the 120 kDa protein band which detected by SDS-PAGE has been correlated with IcsA protein (1), the detection of this protein band may be a useful tool in epidemiological studies for searching prevalence sources.

Results showed, the existence of the 120 kDa protein band was corresponded to enter of *S. flexneri* into the HeLa cell culture, demonstrated by EM and also, this band protein was found in all *S. flexneri* strains with Congo red dye positive. In the other hand, our results detected by different methods (SDS-PAGE, EM and Congo red) could confirm each other. Moreover, the ability of *S. flexneri* to enter the host cells, spread to adjacent cells and intra cellular growth could damage and lyse the host cells, which indicated invasiveness property (8). So, for identification of invasive and non-invasive *S. flexneri* strains, detection of the 120 kDa protein band by SDS-PAGE or a simple, rapid and very cheap Congo red dye method is recommended.

Because, there are some deaths particularly in children due to *Shigella spp* in our country [12] notification on the isolation of this bacteria in both children hospitals laboratories and private clinical laboratories is important.

ACKNOWLEDGEMENTS

We gratefully acknowledge from Dr. H. Monavari in Hazrate Rasol Hospital Laboratory (Tehran, Iran) who helped us for cell culture. Moreover, we thank Miss Nafisi, Mrs. Abedini from Microbiology Laboratory of Mofid and Tebbi Kodakan Hospitals respectivly.

REFERENCES

1. Clerc, P.L., Berthon, B., Claret, M. and Sansonetti P.J. (1989) Internalization of *Shigella flexneri* into HeLa cells occur without an increase in cytosolic Ca^{2+} concentration. *Infect. Immune. 57 (9): 2919-2922*.
2. Behrana, J.V., Harty J.T and Jones B.D. (1998) Interaction of invasive pathogens *Salmonella typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri* with M cells and murine peyer's patches. Infect. Immune. 66(8): 3758-3766.
3. La Brec, E.H., Schneider, H., Magnani, T.J. and Formal S.B. (1964) Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. *J. Bacteriol. 88: 1503-1518*.
4. Wing, H.J., Yan, A.W., Goldman, S.R. and Goldberg, M.B. (2004) Regulation of IcsP, the outer membrane protease of the *shigella actin* tail assembly protein IcsA, by virulence plasmid regulators virF and virB. *J. Bacteriol. 186: 699-705*.
5. Clark, M.A., Jepson, M.A., Simmons, N.L and Hirst, B.H. (1994) Differential surface characteristics of M cells from mouse intestinal peyer's and Caecal patches. *Histochem. J. 271-280*.
6. Wing, H.J., Goldman, S.R., Ally, S. and Goldberg, M.B. (2005) Modulation of a protease contributes to the virulence defect of *Shigella flexneri* strains carrying a mutation in the virK locus. *Infect. Immun. 73 (2): 1217-1220*.
7. Sansonetti, P.J., Arondel, J., Cantey, J.R., Provost, M.C. and Huerre, M. (1996) Infection of rabbit peyer's patches by *Shigella flexneri*: effect of adhesive or invasive bacterial phenotypes on follicle-associated epithelium. *Infect. Immun. 2752-2764*.
8. Suzuki, T. and Sasakawa, C. (2001) Molecular basis of intracellular spreading of *shigella*. *Infect. Immun. 69 (10): 5959- 5966*.
9. Clerc, P. and Sansonetti, P.J. (1987) Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. *Infect. Immun., 55: 2681-2688*.
10. Chen, J.H., Chiou, C.S., Chen, P.C., Liao, T.L., Li, J.M. and Hsu, W.B. (2003) Molecular epidemiology of *shigella* in a Taiwan during 1996 to 2000. *J. Clin. Microbiol. 41 (6): 3078-3088*.
11. Ranjbar, R., Soltan Dalal, M.M., Pourshafie, M.R., Aslani, M.M., Majdzadeh, R. and Khoramzade, M.R. (2004) Serogroup distribution of *shigella* in Tehran, Iran. *J. Pub. Health 33: 32-35*.
12. Sun, A.N., Camelli, A. and Porthy, D.A. (1990) Isolation of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. *Infect. Immune. 58 (11): 3770-3778*.
13. Goldberg, M.B. and Therist J.A. (1995) *Shigella flexneri* surface protein IcsA is sufficient to direct action-based motility. *Proc. Natl. Acad. Sci. USA. 92: 6572-6576*.
14. Morona, R., VanDen Bosch, L. and Manning P.A. (1995) Molecular genetic and topologic

characterization of O-antigen chain length regulation in *Shigella flexneri*. *J. Bacteriol. 177:1059-1068*.

15. Hoppert, M. and Holzenburg, A. (1998) Electron microscopy in microbiology. 1st ed. BIOS. Scientific Publishers Ltd.
16. Monier, J., Ryter, A. and Sansonetti, P.J. (1990) Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interactions with F-actin in the enterocyte like cell line Caco-2. *Infect. Immun. 65 (2): 1048-1058*.
17. Maurelli, A.T., Blackman, B. and Curtiss III, R. (1987) Loss of pigmentation in *S. flexneri 2a* is correlated with loss of virulence and virulence associated plasmid. *Infect. Immun. 44 (1): 397-401*.
18. Sechi, A.S., Wehland, J. and Small, V. (1997) The isolated comet tail pseudopodium of *Listeria monocytogenes*, A tail of 2 actin filament population, long and axial and short and random. *J. Cell Biol. 137: 3867-3871*.
19. Daskaleros, D. and Payne, M. (1987) Congo red binding phenotype is associated with hemin binding and increased infectivity of *Shigella flexneri* in the HeLa cell model. *Infect. Immun. 55 (6): 1393-1398*.
20. Bernardini, M., Mounier, J. and Sansonetti, P.J. (1989) Identification of icsA, a plasmid locus of *Shigella flexneri* that governs bacterial intra and intercellular spread through interaction with F-actin. *Proc. Natl. Acad. Sci. USA. 86: 3867-3871*.
21. Francis, M.S. and Thomas, C.J. (1996) Effect of multiplicity of infection on *Listeria monocytogenes* pathogenicity for HeLa and Caco-2 cell line. *J. Med. Microbiol. 45: 323-330*.
22. Mounier, J., Ryter, A.T., Coquis-Rondon, M. and Sansonetti, P.J. (1990) Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocyte like cell line Caco-2. Infect. *Immun. 58 (4): 1048-1058*.