

Determination of Distribution of *icsA* gene and IcsA Protein Bands between *Shigella flexneri* Isolated from 3 Hospitals in Tehran

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Abstract

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Introduction: *Shigella* is a facultative intracellular pathogen that uses the host actin cytoskeleton protein for intra- and intercellular spread. The aim of this study was to determine the distribution of *icsA* gene and IcsA expressed protein bands among *Shigella flexneri* strains isolated from 3 clinical centers in Tehran.

Material and Methods: Two hundred and seventy five isolated *Shigella flexneri* strains were identified by standard microbiological and biochemical methods. DNA isolation was performed using sodium perchlorate method. Hot start-PCR was done with 2 pairs of primers and the products were separated through agarose gel (0.8%) in TAE buffer. DNA fragments were visualized by ethidium bromide staining under UV illumination. Whole membrane preparation was used to examine the protein profiles and identification of probable IcsA (120-kDa) protein band by SDS-PAGE.

Results: From 100 isolated *Shigella flexneri* strains, both bands of 1600 bp and 1709 bp were detected in 46 isolates (46%). A 120 kDa band which seems to be related to IcsA protein was detected in 46 isolates (46%). The protein bands varied between 30 and 150 kDa.

Discussion: IcsA is both necessary and sufficient for actin assembly in *Shigella flexneri*. Since *icsA* gene and IcsA protein band were not found in all *Shigella* strains, it seems that not all strains have the same pathogenesis. On the other hand, since the demonstration of *icsA* gene by PCR in all *Shigella* strains (46%) corresponded to the presence of a 120 kDa protein band by SDS-PAGE (46%), it seems that both tests may confirm each other. However, the PCR may be more accurate than SDS-PAGE.

Keywords: *Shigella flexneri*, *icsA* gene, IcsA protein, PCR, SDS-PAGE

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Introduction

Shigella is a facultative intracellular pathogen that uses the host actin cytoskeleton for intra- and intercellular spread (1). At the bacterial old pole, IcsA, a surface protein of *Shigella*, mediates the actin polymerization in the host cells to produce actin tails which generate sufficient force for bacterial distribution within the cell cytoplasm and into adjacent cells (2). IcsA protein consists of two domains, α and β ; α domain consists of 706 amino acids and β domain is a 344 amino acid region (3, 4). The expression of IcsA on wild-type *Shigella* is maximal during exponential growth and

markedly reduced during stationary phase (5). IcsA protein is encoded by the large virulence plasmid of *Shigella* spp. (6, 7).

IcsA has been classified as a type V secreted protein. Unlike many type V family members, cleavage of IcsA at the junction of α and β domains is not autocatalytic, but rather is mediated by the protease IcsP (SopA) (8, 9, 10).

Furthermore, the aim of this study was to determine the distribution of *icsA* gene and protein bands between *Shigella flexneri* strains isolated from 3 clinical centers in Tehran.

Material and Methods

Bacterial strains

275 *Shigella flexneri* strains were isolated from fecal specimens or rectal swabs of children attending to three children hospitals (Markaz-e-Tebbi Koodakan, Ali Asghar, and Mofid hospitals) between Jan. 2001 to Dec. 2003. Bacterial strains were identified by standard microbiological and biochemical methods. *Shigella flexneri* strains were stored at -70°C in peptone and glycerol. These bacteria were routinely grown at 37°C on MacConkey agar. Reference strains of *Shigella flexneri* 2a (ATCC 2067) was obtained from Pasteur Institute of Iran and PTCC from Iran Reference Laboratory.

DNA isolation

DNA isolation was performed using sodium perchlorate method (11). In brief, a single colony was picked up and grown in 5 ml Lauria Bertony broth overnight and centrifuged. Then, $0.1 \times \text{SSC}$ (15 mM NaCl, 1.5 mM sodium citrate) was added. The pellets were resuspended in 0.6 ml lysozyme solution, incubated at 37°C for 1 hr, and followed by addition of TE buffer containing sterile distilled water, sodium perchlorate (4M), and SDS 10%; it was incubated at 37°C water bath for 1 hr. Then, NaCl (5N) and cold ethanol 90% was added. Isolated DNA was dissolved in sterile distilled water.

PCR Protocol

Hot start PCR method was done in 2 steps. Since *icsA* gene consists of 3309 bp and results in a large PCR product, the gene was divided into two fragments; a 1709 bp fragment and a 1600 bp one. Two pairs of primers, F_3R_1 and F_1R_3 , were used in this study, as follows:

F_1 : 5'-TTT CAG GGG TTT ATC AAC C-3'
 R_1 : 5'-CAT CAT GTG CAC AAA AAC C-3'
 F_3 : 5'-TCA GGG GAG GGA GAT CTC ATT TTG G-3'
 R_3 : 5'-CCA AAA TGA GAT CTC CCT CCC CTG A-3'

The template DNA, the primers and the Taq polymerase were added to a buffer containing Mg^{2+} ions. The reactions were carried out in a gradient thermal cycle (Corbet). The cycling profile was as follows: 95°C for 10", 95°C for 40", 58°C for 1', 72°C for 1', and 72°C for 1'. Finally, the PCR products were visualized by agarose gel electrophoresis (0.8%) in TAE buffer. DNA fragments were visualized by ethidium bromide staining and photographed under UV light illumination (11).

These 2 bands were compared with DNA ladder (Gene Ruler™ # SMO 331).

Protein preparation and SDS-PAGE

Whole membrane preparations were used to examine the protein profiles of the inner and outer membrane of both isolated and reference organisms. In this method, cell pellets 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, 3mM EDTA (pH 7.3) was added. Terminal pellets were suspended in 1xLUG buffer [Tris-HCl (pH 6.8) 50ml, 0.25 M SDS 25ml, Glycerol 2 gr, β -Mercaptoethanol 5ml, 1% bromophenol blue 2 ml, distilled water 100 ml] (12).

Finally, the protein mixture was electrophoresed through a polyacrylamide gel. The gel was stained with Commassie brilliant blue R 250 and de-stained with a mixture of ethanol and acetic acid. The 120-kDa band, which seems to be related to *IcsA* protein band, was compared with protein ladder (Ruler™ # SMO 661, Fermentas).

Results

From 550 isolates of *Shigella* species (from Nov. 2001 to Sep. 2004), 275 (50%) of isolates were *Shigella flexneri*, 234 (42.5%) *S. sonnei*, 28 (5%) *S. boydii*, and 13 (2.36%) *S. dysenteriae*. One hundred of *S. flexneri* strains were randomly selected.

During this study, 41% of patients were female and 59% were male and, the age distribution was between 6 months to 7 years old.

PCR method

Our results showed that among 100 isolated strains, both bands of 1600 bp and 1709 bp were detected in 46 isolates (46%) (Fig 1).

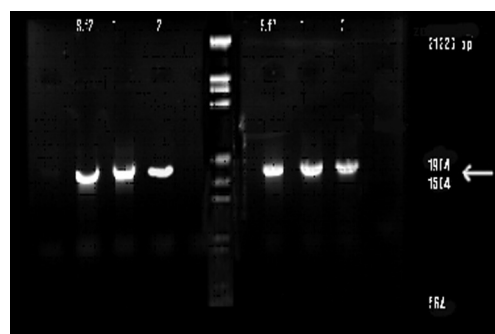


Fig 1: PCR product gel electrophoresis
 Lane1:S.F2, *Shigella flexneri*2a, PCR product 1600bp
 Lane2:sample1, PCR product 1600bp
 Lane3:sample2, PCR product 1600bp
 Lane4: Ladder SMO#331
 Lane5: *Shigella flexneri* 2a, PCR product 1709bp
 Lane6:sample1, PCR product 1709bp
 Lane7:sample2, PCR product 1709bp

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

Group	Protein bands (kDa)								Bacteria %	
	30	40	50	60	70	85	100	120		150
	+	+	+	+	+	+	+	+	-	20.6
	+	+	+	-	-	-	-	-	-	12.1
	+	+	+	+	+	+	+	-	-	7.3
	+	+	+	+	+	+	-	-	-	6.0
	+	+	+	+	-	-	-	-	-	2.3
	+	+	+	+	+	+	+	+	-	17.0
	+	+	+	+	+	-	-	-	-	1.2
	+	+	-	-	-	-	-	-	-	2.4
	-	-	-	-	-	-	-	+	-	12.3
	+	+	+	+	-	-	-	+	-	1.2
	+	+	+	+	+	+	-	+	-	3.0
	+	+	+	+	+	+	+	+	-	14.6

-:no exist
+:exist

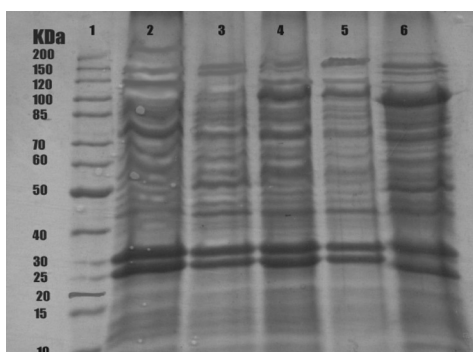


FIG 2: SDS-PAGE of some strains of *S. flexneri*

- 1- ladder
- 2- *S. flexneri* 2a standard
- 3- negative sample
- 4, 5, 6- positive samples

There were 12 distinct groups based on protein bands. Both 95 kDa (secreted form) and 120 kDa (mature form) were coexisted in group I (20.6%), group II (17.0%), and group XII (14.6%) (Table 1, Fig. 2). The 35 kDa band was seen in all groups, except for group IX.

Discussion

One hundred *Shigella flexneri* strains obtained from children stool specimens were chosen for determination of the *icsA* gene by PCR and determination of a 120 kDa protein band probably related to IcsA protein by SDS-PAGE. IcsA is both necessary and sufficient for actin assembly in *Shigella flexneri* (2). Moreover, the ability to intracellularly spread is dependent upon the activity of IcsA too (6, 7, 13, 14, 15). A similar intracellular actin-based movement was reported for *Listeria monocytogenes* by Tilney and Portnoy, indicating that this pathogen is capable of lysing its phagocytic vacuole, moving intracellularly and spreading from cell to cell (13).

Maurelli *et al.* amplified *icsA* gene from the virulence plasmid and examined expression and secretion of IcsA in BS 103 (a virulence plasmid-cured derivative of 2457 T, to generate

BS 489), BS 489, and BS 543; BS 583 was used as a control. A 120 kDa band was visible in both BS 583 and BS 489, indicating that the cellular levels of IcsA were similar and no IcsA protein was detected in BS 543, which is an *icsA*-deleted strain. The expected 95 kDa secreted form of IcsA was detected only in strains containing the virulence plasmids 2457 T and BS 583. Finally, Maurelli showed that about 50% of wild type parent (2457 T) *S. flexneri* strains were labeled with the specific monoclonal IcsA antiserum (14). Although we did not confirm our results with monoclonal antibody and Western blot, a 120 kDa (probably related to IcsA protein) band was detected in 46% of the bacteria (46 cases).

In the study of Goldberg *et al.*, bacterial proteins from wild type and *icsA* gene in *S. flexneri* were prepared, fractionated, and examined by SDS-PAGE, Coomassie staining, and Western blot (4, 16). In outer membrane fractions, two bands present in wild type *S. flexneri* were absent in *icsA* mutant strains; a large band at approximately 120 kDa (corresponding to mature IcsA) and a small band migrating at approximately 35 kDa. The latter was assumed to be cleaved β -domain of IcsA (4). Only $31 \pm 3\%$ of stationary phase wild type strain 2457 bacilli in PCR method had detectable IcsA in their surface (16).

Our results showed that 46% of *Shigella flexneri* strains had *icsA* gene detected by PCR. Our results are different from Goldberg *et al.* ones. These different results could be related to geographical and environmental conditions.

Finally, since the *icsA* gene and IcsA protein were not found in all *Shigella* strains, it seems that not all strains have a similar pathogenesis. On the other hand, as the demonstration of *icsA* gene by PCR in all *Shigella* strains (46%) corresponded to the presence of a 120 kDa protein band by SDS-PAGE (46%), it seems that both tests may confirm each other. However, the PCR may be

more accurate than SDS-PAGE.
It is recommended to check the presence of

icsA gene in other *Shigella* strains and also
Enterobacteriaceae.



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میزان پراکندگی ژن ICSA و باندهای پروتئینی آن در بین شیگلا فلکسنری های جدا شده از سه بیمارستان تهران

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چکیده

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*** هدف:** تعیین پراکندگی ژن ICSA و پروتئین آن در بین سویه های شیگلا فلکسنری جدا شده از نمونه های بالینی شهر تهران
*** مواد و روش ها:** ۱۰۰ سویه از باکتری های شیگلا فلکسنری توسط روش های استاندارد باکتری شناسی و بیوشیمیایی شناسایی شد. جداسازی DNA بر اساس روش سدیم پرکلرات Hot start PCR و به کمک دو جفت پرایمر انجام شده و محصولات هر مرحله به کمک الکتروفورز در ژل ۰/۸ درصد و بافر TAE جدا و بررسی شدند. استخراج پروتئین کامل باکتری های شیگلا فلکسنری به منظور تعیین الگوی پروتئینی و مشاهده نوار احتمالی پروتئین ICSA (نوار ۱۲۰ کیلو دالتونی) به کمک روش SDS-PAGE انجام شد. تعیین فنوتیپ تهاجمی جهت شناسایی سویه های تهاجمی باکتری های شیگلا فلکسنری به کمک رنگ کنگورد صورت پذیرفت.

*** یافته ها:** شیگلا فلکسنری از ۱۰۰ سویه جدا شده و در ۴۶ مورد (۴۶ درصد) هر دو محصول ۱۶۰۰ و ۱۷۰۹ کیلو بازی با روش PCR مشخص شدند. همچنین نوار ۱۲۰ کیلو دالتونی که احتمالاً مربوط به پروتئین ICSA می باشد، در ۴۶ نمونه مشاهده شد. دامنه نوارهای پروتئینی از ۳۰ الی ۱۵۰ کیلو دالتون بودند.

*** نتیجه گیری:** ICSA، پروتئینی لازم و ضروری در شیگلا فلکسنری برای تجمع اکتین است. از آنجایی که در این مطالعه ژن ICSA و پروتئین آن از همه سویه های شیگلا جدا نشدند، به نظر می رسد که بیماری زایی همه سویه های این باکتری یکسان نباشد. از طرف دیگر، همزمانی وجود ژن ICSA با روش PCR در ۴۶ مورد و مشاهده نوار ۱۲۰ کیلو دالتونی که احتمالاً مربوط به پروتئین ICSA بوده در ۴۶ مورد، گویای این نکته است که ظاهراً نتایج حاصل از این دو روش تایید کننده یکدیگر می باشند. همچنین، چون صحت آزمون PCR بر اساس توالی اولیگومرهای پرایمرها بودند، ممکن است صحت آزمون PCR بیشتر از روش SDS-PAGE قابل بررسی باشد.

کلیدواژگان: ژن ICSA، پروتئین ICSA، PCR، SDS-PAGE