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ORIGINAL ARTICLE

Identifying IpaB Gene in Shigella Bacteria and Determining the Antibiotic Sensitivity of Shigella strain

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ABSTRACT
The current study is conducted in view to the fact that Shigellosis is known as a cause of death of children with diarrhea in developed countries. Investigation of antibiotic resistance of these bacteria is highly important. IpaB gene is known as virulence genes that can be adopted as identifier in Shigella diagnosis. In this investigation, among 100 samples of Shigella collected from Tehran, one adopts biochemical and serological tests for 4 genus of Dysenteriae, Sonnei, Boydii and Flexneri. The test of examining the antibiotic sensitivity of these strains has been carried out by propagation in Agar method for 14 different antibiotics. PCR of ipaB gene has been performed using devoted PCR for all strains. The results of conducted study show that 35 strains (35%) are dedicated to Sonnei Shigella with the greatest percentage. Also the results show that nearly 50% of strains have been resistant to Tetracycline and Cotrimoxazole .It has been specified by performing PCR test that the ipaB gene exists in all strains.
Keywords: *Shigella, IpaB gene, antibiotic sensitivity*

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INTRODUCTION

Shigellosis is one of the five infectious diseases that now is besetting human. Contamination with Shigella species (negative-Gram bacteria) results in bacilli diarrhea disease that is an acute inflammatory colon disease. Many efforts have been taken place for developing vaccine against this disease and one hope plasmid invasive antigens IpaB due to immune system adequate induction [1].

Shigella genus is one of main factors of diarrhea in human and *Bacillus anthracis* the etiologic agent of anthrax. One of the most important pathogenic factors of Shigella is IpaD protein. Assimilation of N terminal of ipaD gene together with pa20 gene that has an immunogenic role can be set forth as a candid for recombinant vaccine. Shigella is a negative-Gram bacillus of colon that infections arising from it have generated serious problems in developed and developing countries. Genus of Shigella is divided into four groups including Dysenteriae, Flexneri, Sonnei and Boydii. Prevalence of Shigella-related infections is highly easy due to low dose of pathogenic agent of this bacteria as well as easy transfer from one person to another and indirect contamination through food substance and contaminated water [1].

The pathogenic is due to bacteria invasion or intrusion to colon mucus together with destruction of coverage tissue and developing acute inflammatory colitis in Lamina Propria and finally wounding of colon mucus by losing blood and releasing inflammatory elements and mucus into colon lumen. Under this circumstance bacteria prevents room water absorption in the colon and the volume of excrement changes and as a results the patient alternatively suffers from bloody and mucous diarrhea [2].

Among pathogenic agent in these bacteria one can point out to toxins, proteases, bacteria adhesive agents. Pathogenic genes in charge of Shigella disease are resided in Chromosomes and plasmid (inv) [3]. As for preventing and treating this bacteria, washing hands before eating and cooking food decreases the hazard of catching the disease. Bloody diarrhea can be treated by antibiotics such as Ampicillin, Trimethoprim/sulfamethoxazole and fluoroquinolones. It is necessary to supply water for patient too.

Antidiarrheal agents can intensify the diarrhea. Currently, there is no vaccine for treating such disease [4].

Over studies it has been revealed that the Shigella is the third bacterial agent separated from children suffering diarrhea. This infection is native all around world. The epidemic usually takes place in regions with high population and poor sanitary state. Today with expansion of resistance against such bacteria toward usual antibiotics, the treatment of this infection has encountered some problems. In addition taking ineffective antibiotics considering the failure of complete treatment of the disease results in prevalence and expansion of this contamination. However, one should not forget that one the etiological cause of prevalence and anomy of diseases in a region is rise of resistance of bacteria against the common and used antibiotics [5].

Considering the importance of this bacteria in Iran and shortage of conducted studies in this regard, within this study, one examines the ipaB gene and its importance in fast diagnosis of Shigella isolated from patients without clinical symptoms by using molecular method of PCR together with phenotypic usual method with studying clinical strains antibiotics resistance.

Shigella can be considered as an intracellular invasive bacteria and presence of 10 bacteria in water or food can give rise to disease symptoms. Every year more than 164 million cases of contamination and 1.1 million cases of death emanating from infection to these bacteria are reported around the world and 69% of its victims are consisted of children under 5 years old. The main etiological cause of this epidemic in developed country and recently in Iran has been *S. sonnei* and resistance to various antibiotics has been the reason of bacterial expansion. Currently, no usual vaccine is available against Shigella and it's due to some problems in developing vaccine against these bacteria that among them one can point out following cases.

Complicated pathology of bloody diarrhea syndrome is a representation of various genetic compounds controlling the pathogenesis of this organism. Chromosomal and extra-chromosomal genome for developing this phenotype is the invasion to colon epithelial cells that its genetic compound is placed on a great plasmid of 120-140 megadaltons. This plasmid is available in all types of Shigella. These genes code various proteins such as invasive plasmid antigens (IpaA, IpaB, IpaC and IpaD) that are necessary pathogenic agents. Studies show that main part of genes involved in invasion in Shigella is conserved Dysenteriae and Flexneri. The prominent role of these proteins varies from apoptosis induction by IpaB to connection of host cells and active polymerization by Ipac. It has been revealed that IpaB-IpaC complex enters the host cell membrane and forms a canal for admission of other Shigella proteins into host cell [6].

To date, no report is published based on existence of ipaB Gene in other bacteria, only [7] in their article have stated that Salmonella invasive proteins B (SipB) and Yersinia B external protein (YobP) have homology with Shigella IpaB protein. Considering existence of IpaB gene in all species of Shigella and bacteria EIEC and their nonexistence in other bacteria and insignificant difference of this gene among species of Shigella, one can use this gene as a way for identification of shigellosis disease in clinical cases.

Shigella bacteria strains have been gradually become resistant to most of common and cheap antibiotics. And this results in failure of antibiotic treatment. Therefore development of a vaccine for controlling bloody diarrhea diseases seems to be necessary. To date many efforts has been done for finding an effective and safe vaccine. However, none of them were not so effective and safe so that can be considered and used as a vaccine. Various studies have shown that immune system develops a quite good response against pathogenic agents such as IpaC_LPS-IpaB and as a result there are many hopes to these agents for developing vaccine.

Lack of suitable animal model, indirect evidences from mechanism involved in human immune system and probably high abundance of bacterial transformation that is coupled with losing the ability of admission of eukaryotic cells. From other side the ideal vaccine is one whose development and consumption is easy, therefore the Shigella alleviated live vaccines are matter of special attention [8].

Over maintenance and re-cultivation, Shigella loses its great plasmid with high frequency and this can occur for mitigated bacteria too, in this case the obtained bacteria lacks the ability to admission in epithelial cells. Lack of ability of bacteria in entering the target cell causes that antigens of these bacteria lose the potential in inducing the immune system through dendritic cells as progenitor cells offering antigens.

In a study examined the pattern of antibiotic sensitivity and determining ipaH gene in Shigella strains isolated from selected provinces of country. In this research 100 samples of Shigella have been collected from all parts of Iran. These strains have been identified by biochemical and serological tests for 4 species of Dysentery, Sonnei, Boydii and Flexneri. The antibiotic sensitivity test of these strains with the propagation in agar methods has been performed for 14 different antibiotics. According to the results of this study 36 Sonnei Shigella strain (73%), 9 Flexneri Shigella strain (18%) , 3 Boydii Shigella strain (5%)

and 2 dysentery Shigella strain (4%) have been identified. The results indicate that nearly 50% of strains are resistant to tetracycline and cotrimoxazole. The degree of resistance to the studies antibiotics in Sonnei Shigella strains was greater than others. The presence of ipaH gene has been confirmed in all strains. Examining the ipaH virulence gene shows that one can use this gene as an indicator for rapid identification of Shigella species.

Prevalence of Shigella-related infections is highly easy due to low dose of pathogenic agent of this bacteria as well as easy transfer from one person to another and indirect contamination through food substance and contaminated water [1]. Considering the importance of this bacteria in Iran and shortage of conducted studies in this regard, this study examines the ipaB gene and its importance in fast diagnosis of Shigella isolated from patients for more rapid identifying of Shigella species and preventing from developing resistance because of irregular taking of antibiotics.

MATERIAL AND METHODS

In this study, one examined 100 Shigella isolates that have been collected from samples of patients participated in national plan of examining the intestinal pathogenic bacterial agents within Iran provinces and known as healthy patients or vectors of Shigellosis from Tehran province over 2006 through 2011 and transmitted to Iran Pasteur institute.

Developing pure cultivation from samples

At first the samples have been cultivated in Agar SS Salmonella Mueller agarand after 24 hours incubation at the temperature of 37 degree centigrade, in case of impurity of cultivation, colonies that were suspicious of Shigella in terms of morphology and pigment were transmitted again to the Mueller Agar milieu for purification. Then they obtained colonies have been examined for recognizing positive samples.

Identifying Shigella samples

All Biochemical tests have performed from 24-houred cultivation of bacteria in the Mueller-Hinton Agar milieu. The isolates have been cultivated in terms of reaction on TSI milieu as well as lysine, citrate and urea milieus.

Examining isolates antimicrobial resistance

Antibiotic sensitivity test has been carried out using the Kirby-Bauer method and by Mueller-Hinton cultivation milieu and McFarland microbial suspension on the 100 isolates of different Shigella species. Examining the isolates antibiotic sensitivities have been carried out for 15 antibiotics in various groups including:

Amoxicillin Clavulanic acid, Ampicillin, Cefotaxime, Cefalotin, Gentamicin, Amikasin, Ceftazidime, Ceftizoxime, Netilmicin, imipenem, tetracycline, ciprofloxacin, nalidixic acid, chloramphenicol, Cotrimoxazole.

Extraction of Chromosomal DNA

For extracting bacterial DNA at first the lysis phase is carried out by boiling. Then by using phenol-chloroform from bacterial lysis solution, the required DNA has been isolated through a phase including separating the matters excluding DNA, DNA purification and thickening it.

PCR test

All matters used in this PCR tests in this study are provided from (MBI –Fermentas, GmbH, Leon-Rot, Fermentas Germany) and one has adopted the Thermocycler device for performing the PCR tests. For proliferation of ipaB gene one uses

- Primer couples
- Forward IpaB F
- 5'-TGGAAAAACTCAGTGCCTCT-3
- and Reverse IpaB R
- 5'-CCGTCCGTAAATTCATTCT-3'

Also for proliferation of ipaB gene the following PCR mixture is produced and used:

The double-distillated water 11.9 µL, buffer of PCR 2.5 µL, MgCl2 25 millimolar 3 mL, Dntp (10millimolar) 1 µL, F & R primer each one (10 picomole per µL) 0.7 µL, polymerase Taq (5 units per µL) 0.2µL, DNA 5 µL, the final volume for each reaction 25 µL.

The used thermal cycles are as follows:

Initial denaturation: 95^{o}C, 5 minute, one time, denaturation: 95^{o}C, 50 second, connection: 56^{o}C, I minute, proliferation: 72^{o}C, 1 minute (30 times). Final proliferation, 72^{o}C, 1 minute, 1 time.

-Electrophoresis of PCR product

Required amount of the 0.5 X TBE buffer is poured in the electrophoresis tank and the tray containing gel 1 % is placed in the tank. By the sampler 2.8 mL of marker is poured into one of wells of the gel. In this study one used voltage of 90 volts for conducting the electrophoresis.

After completing electrophoresis, the gel is placed in Ethidium Bromide with density of 50mg per mL and then is placed in distilled water for 5 minutes. Then the gel is brought out of distilled water and the gel is imaged in imagery set.

Serology results

In terms of frequency, the isolated Shigella serogroups 40% of case i.e. 40 isolates belong to Sonnei Shigella, 29 percent to Flexneri species, 18 percent to Boydii genus and 13 percent to Dysenteriae genus. As it was expected, over summer the greatest extent of contamination cases are observed and the dominant serogroup has been the Sonnei Shigella that has devoted the greatest frequency to itself.

The results of Antibiotic sensitivity test

Considering the figure 1 the greatest resistance of strains of *Shigella dysenteriae, Boydii and Sonnei* is Cotrimoxazole antibiotic. As for *Shigella flexneri* strain the degree of resistance to cefalotin is greater than other antibiotics.

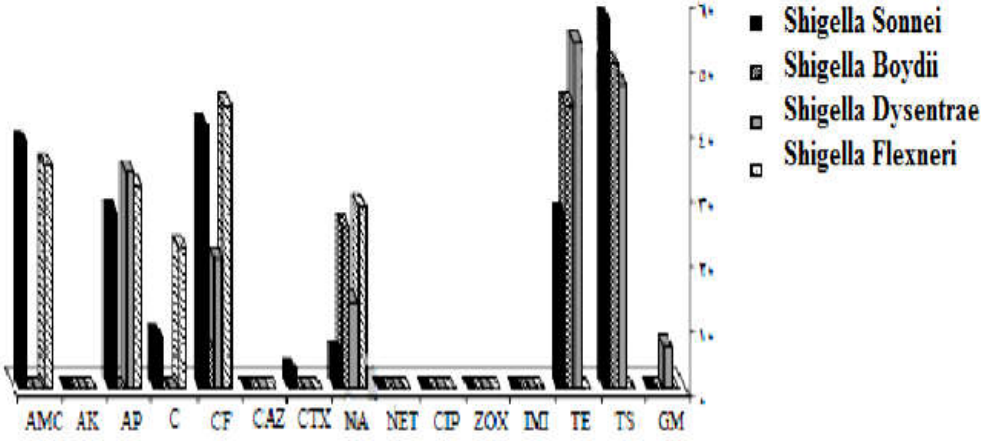

Figure 1: Comparing the percentage of resistance of Shigella isolates by separating serogroups (AMC amoxicillin clavulanic acid, Ak Amikasin, AP Ampicillin, C Chloramphenicol, CF cefalotin, CAZ Ceftazidime, CTX Cefotaxime, NA nalidixic acid, NET Netilmicin, CIP Cipromoloxamine, ZOX Ceftizoxime, IMI imipsinom, TE tetracycline, TS co-trimoxazole, GM gentamycin).

PCR test for identifying ipaB gene

The expected PCR product for proliferation of ipaB gene is 423 base pairs of ipaB that as for all strains (100 %) some pieces exactly in the same size can be observed.

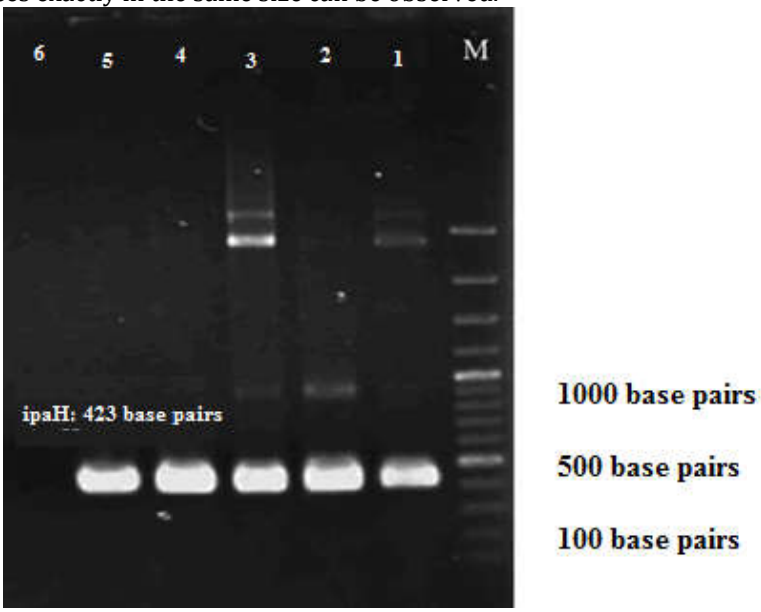

Figure1: PCR1 related to ipaB gene number 1, 2, 3 and 4 of a sample of studied strains. The number 5 is positive control (standard strain) and the number 6 is negative control.

DISCUSSION AND CONCLUSION

Shigellosis is an acute gastroenteritis and is one the important etiologic cause of death and affliction of children to diarrhea in developing countries. This disease takes place by bacteria belonging to Shigella genus.

The degree of occurrence of shigellosis in the world is estimated nearly 164.7 million that includes 69% of death of children under age of 5 years old. Epidemics occur usually in regions with large population and poor sanitary conditions. Recent studies in Iran and examination of these bacteria in diarrhea samples suggests that Shigella is among important etiologic cause of bacilli Dysenteriae in our country, Iran that is resistant to wide range of antibiotics [11].

In this study, the identified isolates serotyping has shown that the greatest frequency is related to Shigella sonnei (37%) and Flexneri, Boydii and Dysenteriae are 34% and 16% respectively. It is worthy to note that Shigella Sonnei comparing with other species brings about more alleviated disease. In a study carried out by [12]on 82 strains of Shigella, similar results have been obtained: 74.3 % Sonnei Shigella, 19.5 % *Shigella Flexneri*, 3.6 % *Shigella boydii* and 2.4 % *Shigella dysenteriae*. [11]Examined 32 strains and reported 58.9% *Shigella Sonnei*, 36.4% Shigella Flexneri, 3.3 % *Shigella boydii* and 1.3 % Shigella Dysenteriae that was similar to results of this paper [11].

These studies have shown that in recent years no changes have been occurred in degree of occurrence of Shigella serogroups in Iran. Though Iran is a developing country, but prevalence of Sonnei Shigella as a dominant serogroup is similar to cases in the developed countries such as Canada and U.S.A. and like these countries the infection from Shigella in children has been reported in greater extent. While in Taiwan and Bangladesh the infections of these bacteria have been reported from Flexner serogroup Shigella [13].

In the past, the premise was that the ratio of *Shigella flexneri* and Shigella sonnei in a region is representation of the sanitary standard situation in that region, as the overall level of individual and environmental sanitary raises, the amount of Flexneri Shigella (serogroup B) decreases, while the ratio of occurrence of Sonnei Shigella (Serogroup D) increases. Also one presumes that one of the important causes of prevalence of Shigella serogroups in different counties is the effective factors of natural selection that among them the environmental and individual factors can be mentioned. Over comparing among various serogroups in different seasons, it can be observed that occurrence of Shigellosis in summer season is more than other seasons and that is perhaps due to the problem of living up to sanitary practices for restriction of water resources and from other side, the population of vector insects propagating bacteria across society increases in this season [14].

Adequate antibiotic treatment of Shigellosis decreases symptoms period, and bacteria repulse. Most of Shigella enteritis respond to treatment by antimicrobial drugs, but from other side the rise of resistant strains and from other side the self-restricting nature of the disease have resulted in that only in some serious and lethal cases of the disease the dedicated treatment takes place. Rise of stains with various medicinal resistances has been reported from around the world. In this study the result obtained from antribiogram is such: all strains have been 100% sensitive to Amikasin, Ceftazidime, Cefotaxime, Netilmicin, Ciprofloxacin, Ceftizoxime, imipenem and Gentamycin, also they showed relatively high sensitivity (90 %)to antibiotics such as Amoxicillin clavulanic acid, ampicillin, Chloramphenicol, cefalotin, nalidixic acid. But they showed 50% resistance to Tetracycline and cotrimoxazole. In the study of [15] the greatest obtained resistance was 90.24 % related to Antibiotic Cotrimoxazole. The conducted study by [11] has exhibited the high resistance of Shigella to Cotrimoxazole (>94%) and relative resistance to ampicillin, Chloramphenicol, Nalidixic acid, cefixim and kanamycin (>6%). Comparing with conducted research in Iran fortunately the degree of resistance to cephalosporin of third generation such as Ceftazidime and Cefotaxime as well as antibiotics such as ciprofloxacin are still low. So that in this study 100% of strains have exhibited sensitivity to this antibiotic and this suggests that Shigella strains in Iran have been less exposed to antibiotics such as cephalosporin and quinolone and aminoglycoside. Therefore for preventing from developing resistance in these strains one should apply sufficient supervision in prescribing drugs without physician prescription for treating such infections [11].

IpaB gene is among pathogenic genes that place on the chromosome as well as bacteria plasmid. Due to the importance of this gene many studies has carried out on it and it has been asserted that this gene can be a significant marker in early diagnosis of bacteria with respect to other intestinal bacteria. Considering the significance of these bacteria in Iran, this study examines the IpaB gene as the pathogenic prevalent gene in Shigellastrains isolated from patients' people without clinical symptoms using PRC [12]. In this study the presence of this gene has been confirmed within 100 percent of Shigella bacteria samples. Within studies of [15] on 82 strains of Shigella the similar results have been attained too. In the study of [8] all of studied Shigella strains have contained the IpaB gene too. Considering the restriction of

experimented samples in this study, it seems that as the number of isolates rises, the results become more accurate.

According to findings there is no significant relationship between the presence of this gene in vector people and the patient. Once can conclude from these examinations that since the ipaB gene is chromosomal as well as plasmid-related, then it has more than one copy on the genome and it exists on all of Shigella strains and also due to the fact that the vectors lack clinical symptoms, therefore one can use dedicated primers of IpaB gene to adopt the PCR method as a highly rapid method with high sensitivity for identifying Shigella strain. However, due to limitations in this method such as adjusting the PCR program for identifying the desired gene, the adoption of cultivation seems to be required beside this technic.

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