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Detection the virulence-associated genes in *Shigella* Species Isolated from Diarrheal Samples in Babylon province

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Abstract
The genus *Shigella* comprises the most infectious and diarrhea genic bacteria causing severe diseases, mostly in children under five years of age. This study aimed to detect seven virulence genes (*ipaBCD, Vir*F, *sen, set1A, set1B, ial, ipaH,* and) in *Shigella* species (spp.) using polymerase chain reaction (PCR) and to determine the relation of *Shigella* spp. From patients diarrheal samples with hospitalization and bloody diarrhea in Babylon province .

INTRODUCTION

Shigellosis, or bacillary dysentery, continues to be a public health concern worldwide, mainly in the underdeveloped and developing regions with poor hygiene and limited access to clean drinking water (1,2). The genus *Shigella* is divided into four serogroups—*S. dysenteriae* (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D) (3). Shigellosis is an invasive illness of the human colon that leads to varied clinical symptoms ranging from mild watery diarrhea to severe colitis (4). The pathogenesis of shigellosis is related to various virulence factors located on the chromosome or large virulent *inv* plasmids (5).In which epithelial cell penetration and modification of the host response towards infection for dissemination from cell to cell occurs—is mediated by an invasion-associated locus (*ial*) and the invasion plasmid antigen H (*ipaH*) genes, respectively (6,7). Chromosomal genes, *set1A* and *set1B*, encode the *Shigella* enterotoxin 1 (*ShET-1*), and are among the factors associated with the watery phase of diarrhea . *Shigella* enterotoxin 2 (*ShET-2*) is involved in invasion and is located in large virulent plasmids (8).
ShET-1 and *ShET-2*, in addition to their enterotoxic activity, play an important role in the transport of electrolytes and water in the intestine (9). *Vir*F are located on large virulent plasmids and act as virulence determinants in intercellular spreading and invasion (10). Two distinct shiga toxins, *stx-1* and *stx-2*, are encoded by chromosomal genes and expressed only by *S. dysenteriae* serotype 1 and are similar to the shiga-like toxins of enterohemorrhagic *Escherichia coli* (EHEC) (11). These toxins lead to the expansion of vascular lesions in the kidney, central nervous system, and colon in a large number of cell types . Because of the high toxicity of the shiga toxin, infections with *S. dysenteriae* serotype 1 commonly have life-threatening complications (12).
The aim of the present study was to detect nine virulence factors genes (*ipaBCD*, *Vir*F, *sen*, *set1A*, *set1B*, *ial*, and *ipaH*) in *Shigella* species (spp.) using the polymerase chain reaction (PCR) and to determine the relation of *Shigella* spp. from diarrheal samples with hospitalization and bloody diarrhea .

MATERIALS AND METHODS

1. Clinical samples and laboratory identification
Twenty one or thirty ?? *Shigella* strains, including *S. sonnei* (n = 2), *S. flexneri* ((n=12) *S. dysenteriae* (n = 7), and *S. boydii* (n = 0), (2+21+7=30 no 21)???were used in this cross-sectional study. These strains were isolated from 426 stool samples from patients with diarrhea in Babylon province during an. January ,2017 to August,2017. The presence or absence of bloody diarrhea and any history of hospitalization were reported by the individual responsible for the clinical evaluation.
Cary-Blair transport medium (Oxoid, Basingstoke, HampshireHampshire, UK) was used for sample transportation to the laboratory, where each sample was subjected to immediate testing. In the laboratory, all specimens were cultured in different differential media, including MaCconkey agar,DCA,SS agar Xylose lysine desoxycholate (XLD) agar and Hektoen enteric (HEA) (Merck, Darmstadt, Germany), and then incubated at 37°C for 24 hours. All grown colonies were identified using a conventional biochemical culture base and a microbiological API 20E kit (bioMerieux, Marcy l'Etoile, France). Serological tests were performed on the *Shigella* strains using the slide agglutination method (14). All strains were stored in Luria-Bertani broth containing 15% glycerol at −80°C until use. Each sample was subjected to PCR amplification using 14 pairs (seven virulence genes) and(three species-specific genes) of different primers (15–18) PCR was performed using a polymerase chain reaction (PCR) instrument with master cycler gradient for the detection of various virulence- and species-specific genes (*set1Aset1B*, *ial virF*, *sen ipaBCD*, and *ipaH*). The overnight-grown colonies on the XLD agar plates were picked for template genomic DNA extraction by the boiling method. The total volume of the PCR mixture was 20 µl, containing 0.5 µl extracted template DNA, 2.0 µl 10× PCR buffer, 0.5 µl MgCl2 (50 mM), 0.5 µl deoxynucleotides (10 mM), 0.5 µl each virulence gene primer, 0.5 µl Taq DNA polymerase (5 U/mL) (Amplicon Co., Copenhagen, Denmark), and 13 µl ddH2O (In *set1A set1B*, 2 µl H2O was added).
2.The PCR conditions for the amplification of virulence genes included an initial denaturation at 94°C for 60 seconds, 35 cycles of denaturation at 94°C for 60 seconds, annealing at 58°C (variable) for 90 seconds, and extension at 72°C for 60 seconds, as well as a final extension at 72°C for 7 minutes. The reaction mixture was completed in a thermal gradient cycler for the detection of species-specific genes using the following PCR procedure: predenaturation at 95°C for 1 minutes, 35 cycles with denaturation at 94°C for 35 seconds, annealing at 55°C for 90 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes. The PCR products were subjected to electrophoresis using 1.0% agarose gel, stained with ethidium bromide, and observed under ultraviolet light.

RESULTS

1. *Shigella* species
Of the 426 diarrheal samples, 21 isolates of *Shigella* spp. were obtained using conventional biochemical and microbiological tests. All isolates were confirmed by the *Shigella* genus-specific PCR. The species-specific amplification test showed that 12, 2 and 7 strains of *S. flexneri*, , *S. sonnei* and *S. dysenteriae*, respectively, with no *Shigella boydii* isolate were isolated from all the tested samples. The study was performed on patients aged 1–60 years; as anticipated, children over one year of age were more affected by *Shigella* than the other age.
2.Molecular detection of virulence genes in *Shigella spp.:*
Virulence Genes Related to *Shigella spp* were investigated through specific primer for 21 isolates that identified by biochemical test and specific antisera, The conventional gene confirmed 12 isolates of *S. flexneri* 2 *S. sonnei*, and 7 *S. dysenteriae*, isolates). The *ipaBCD* , *paH* and *virF* genes was present in all isolates. Concerning others virulence genes, a vast

genetic diversity was shown among isolates; *set-1*B genes wer figuer e predominant in 23.8% of the isolates (3/21), followed by *set*-A and *ial* in 14.2% (5/21) of the isolates .The *sen/ospD3* (ShET-2) 28.5% of the isolates(6/21) , and *invE* genes were present at a frequency of 28.5% of the isolates(6/21), Some isolates carried *set-1*A but not *set-1B*, or vice versa.figuer (1) figure(2) figure (3)figure(4) figure(5) figure(6) figure(7) figure(8). In table, there is a significant difference P<0.05 among virulence genes of *Shigella spp* where *p* value is 0.028.

Table (1): Distribution of virulence factor genes according to *shigella* serogroup

Shigella spp	*Ial*	*ipaH*	*ipaBCD*	*setA*	*setB*	*virF*	*Sen/ospD3*	*invE*
Shigella flexeneri	8	12	12	3	5	12	1	0
Shigella dysenteriae	2	7	7	0	0	7	4	6
Shigella Sonnei	0	2	2	0	0	2	1	0

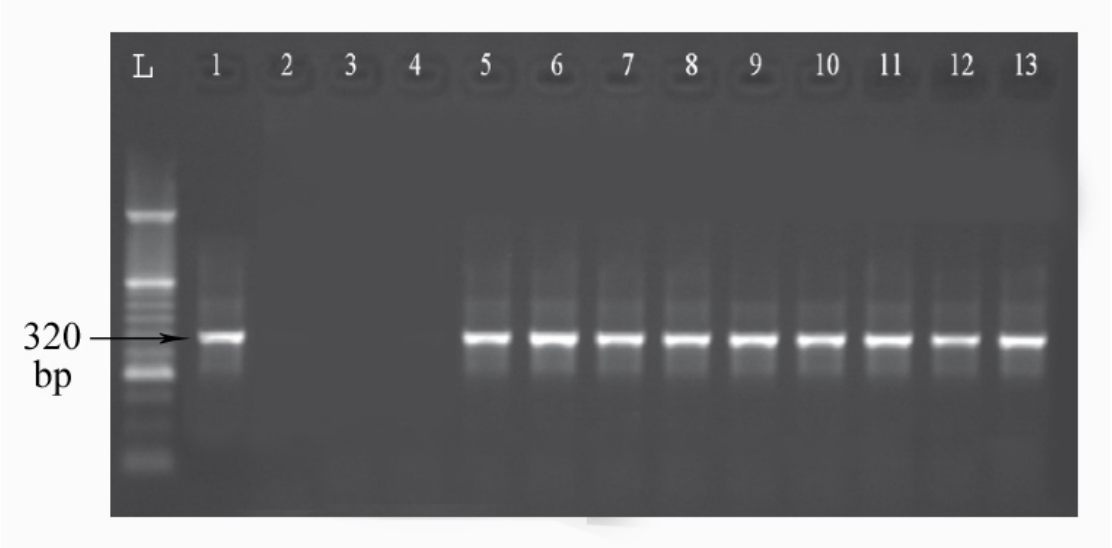
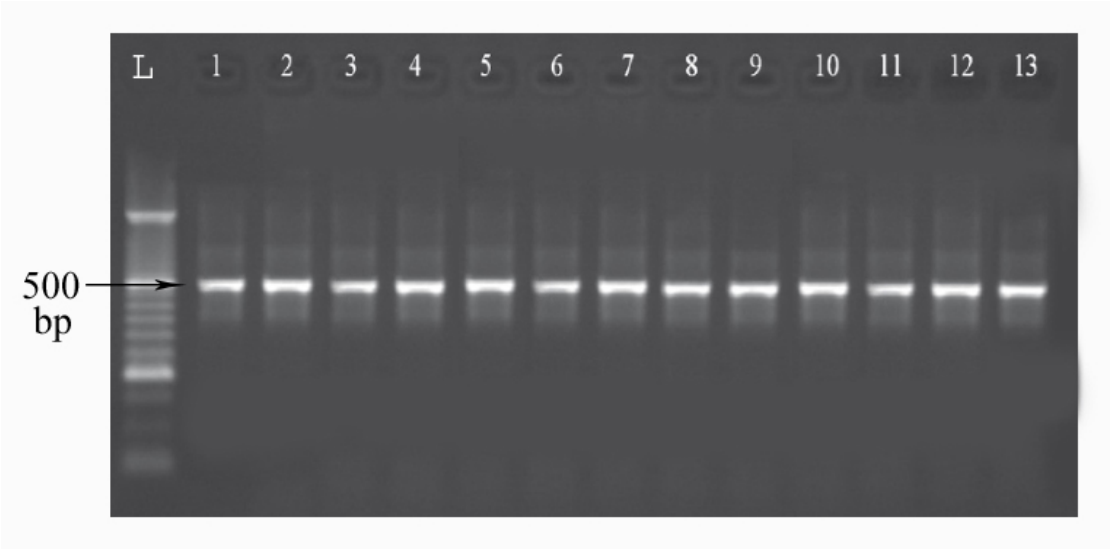


Figure (1)Agarose gel electrophoresis of PCR products for detection of (*ial*) gene amplicon product in. Lane 1-13 refer to *shigella spp* isolates' number. L Allelic ladder



Figure(2) agarose gel electrophoresis of PCR products for detection of *(ipaBCD*) gene amplicon product in. Lane 1-13 refer to *shigella spp* isolates' number .L Allelic ladder

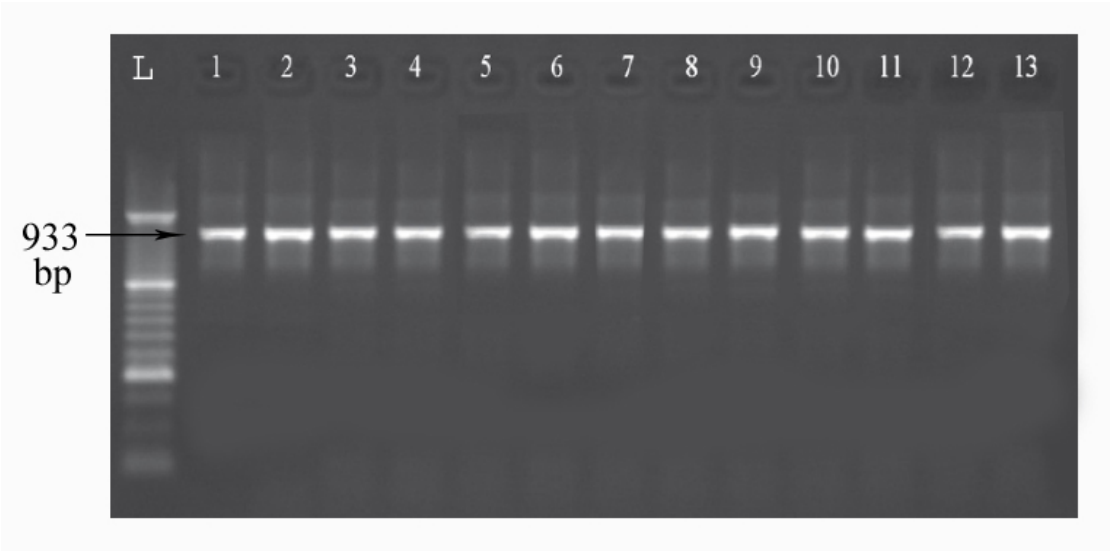


Figure (3)Agarose gel electrophoresis of PCR products for detection of (*ipaH*) gene amplicon product in. Lane 1-13 refer to *shigella spp* isolates' number. L Allelic ladder

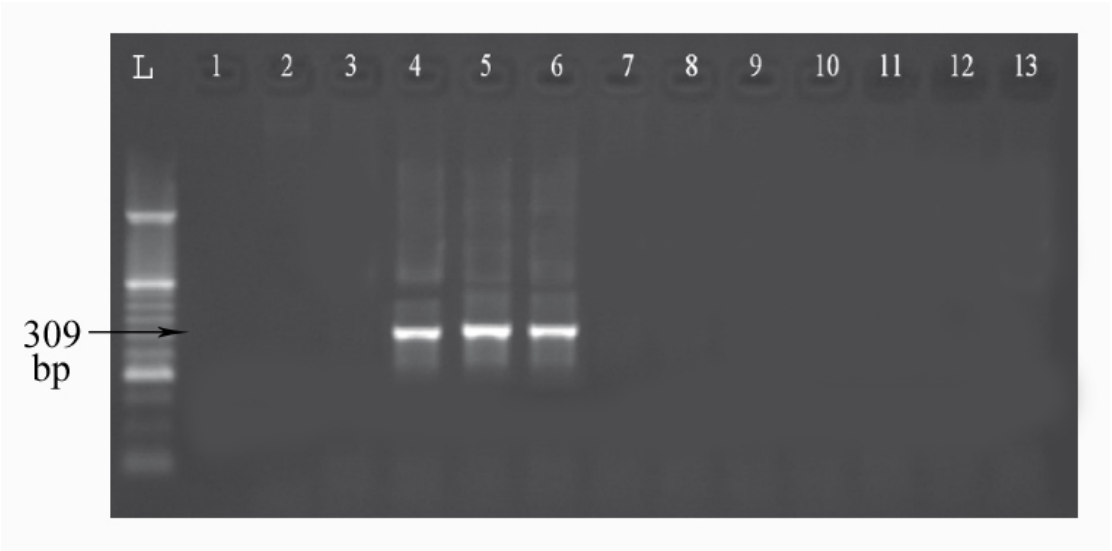


Figure (4)Agarose gel electrophoresis of PCR products for detection of (*setlA*) gene amplicon product in. Lane 1-13 refer to *shigella spp* isolates' number .L Allelic ladder

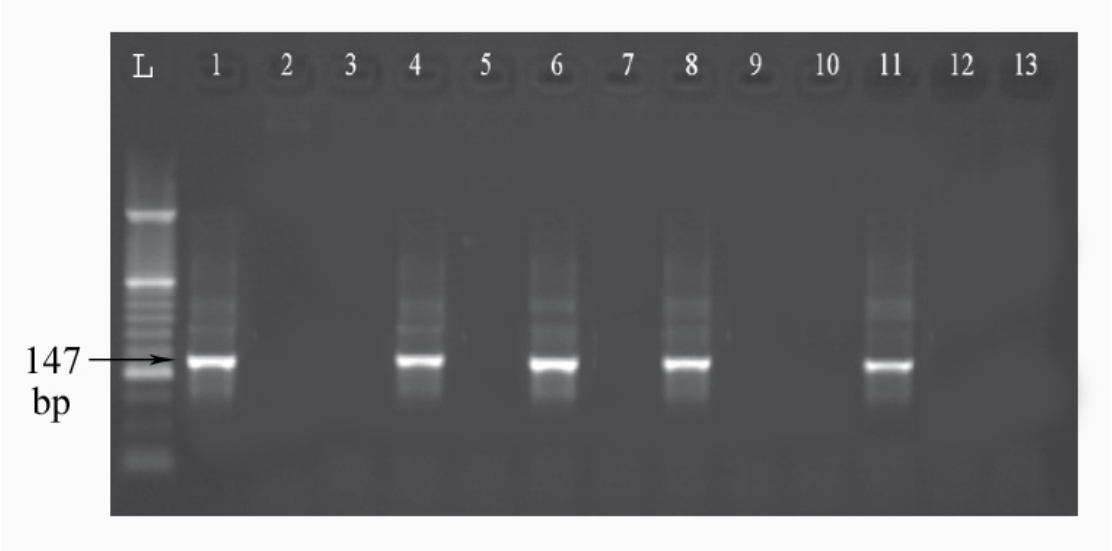


Figure (5)Agarose gel electrophoresis of PCR products for detection of (*setl B)* gene amplicon product in. Lane 1-13 refer to *shigella spp* isolates' number .L Allelic ladde

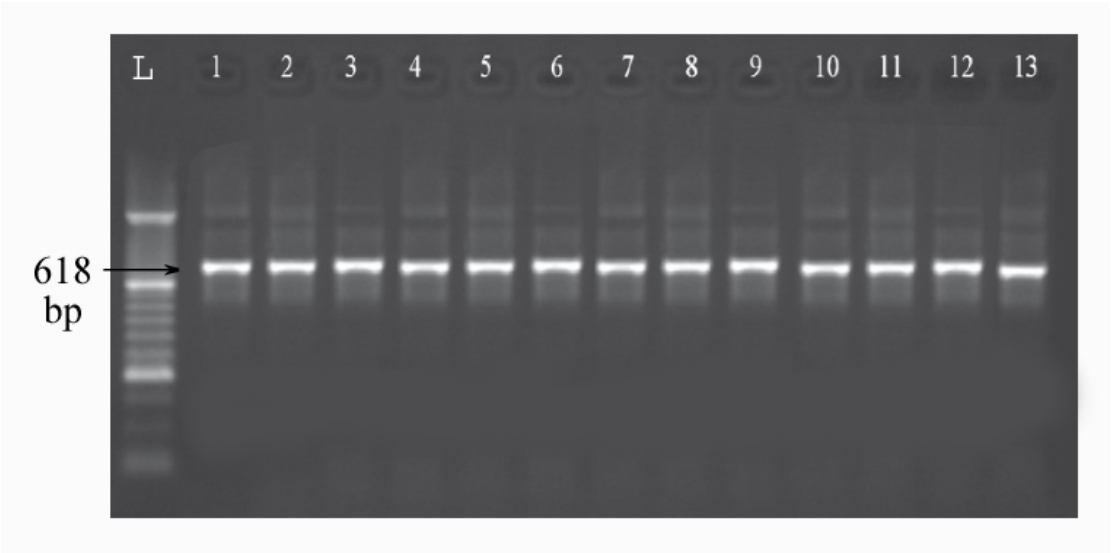


Figure (6)Agarose gel electrophoresis of PCR products for detection of (*vir F)* gene amplicon product in. Lane 1-13 refer to *shigella spp* isolates' number .L Allelic ladder

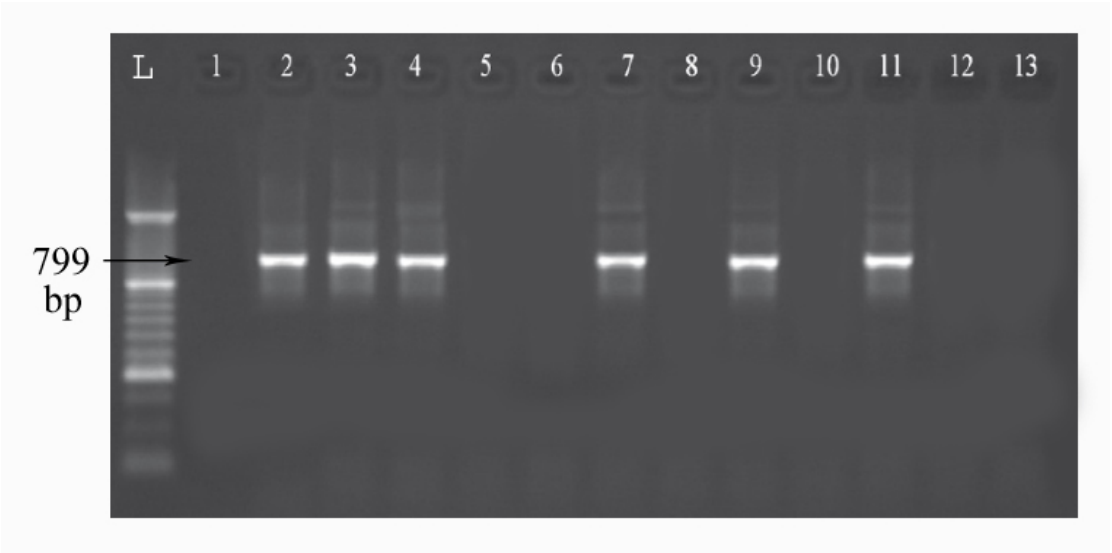


Figure (7)Agarose gel electrophoresis of PCR products for detection of (*sen/ospD3)* gene amplicon product in. Lane 1-13 refer to *shigella spp* isolates' number .L Allelic ladder

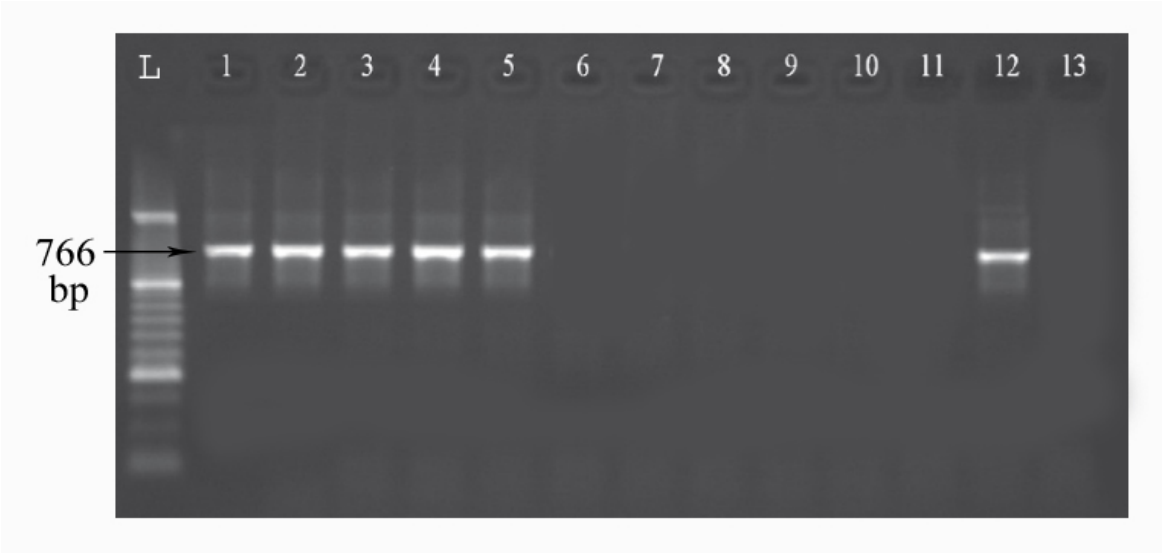


Figure (8)Agarose gel electrophoresis of PCR products for detection of (*invE*) gene amplicon product in. Lane 1-13 refer to *shigella spp* isolates' number .L Allelic ladder

DISCUSSION

In the current study, 21 *Shigella* isolates were obtained from all the tested stool samples. Conventionally identified isolates of *Shigella* were confirmed using *ipaH*-specific PCR assay. In our study, similar to (19), *ipaH* was detected in all *Shigella* culture-positive specimens (20). In accordance with these results (21) showed that *ipaH* is carried by all four *Shigella* species as well as by enteroinvasive *E. coli* (EIEC). In agreement with(22,23)results, the results of our study revealed that *vir*F and *ipaBCD* were found to be positive in all the strains. *Shigella* attaches to the target region through the two receptors.

Some of the virulence factors mentioned above are also situated in large virulent plasmids. 10/21 (47.6%) *Shigella* strains were found to carry *ial*, in our study; these results are approximately consistent with (22) . This contrast may be because *ial* is only located on the virulent plasmid and can cause deletion mutations (26). *inv*F was described first in uropathogenic *E. coli* (UPEC), but has now also been found in *Shigella* spp. The prevalence of *inv*F in *S. dysenteriae* has been found to be 6/21 (28.5%) (8). This data conflicts with that of studies conducted in India (27) . A large invasion plasmid gene (*sen*), which encodes *ShET2*, has also been reported in numerous *Shigella* spp. Similarly, *sen* has been detected in 7/21 (33.3%) *Shigella* isolates (26). Casabonne *et al* (22) showed that of the numerous *Shigella* isolates, carried the gene encoding *ShET-2*. The conflict is likely because of the loss of the large plasmid that contains the gene in different *Shigella* serogroups and the number of samples. *Shigella* enterotoxin 1 (*ShET-1*) is encoded by *set* located on the chromosomes of several clinical strains of *S. flexneri* (28). *ShET-1* has been found to stimulate fluid secretion into the intestine, thus, contributing to the watery phase of diarrhea (28,30). In our study, 3/21,5/21(14.3%,23.8%) isolates were found to carry both *set1A* and *set1B* respectively . (22), Vargas *et al*. [15], and Cruz et al. [23] showed that the prevalence of set1A and set1B was 7.0% (7/100), 3.92 (2/51), and 36.6 (11/30), respectively. In agreement with previous studies, the present study showed that *set1A* and *set1B* were detected only in *S. flexneri* strains (26).

The *inv*F is another virulence determinant related to *S. dysenteriae*; it is not excreted by the bacteria, but is released only during cell lysis (31). 6 (28.5%) *S. dysenteriae* isolate carries *inv*F. Bekal *et al*.'s (32) study detected *S. flexneri* isolates harboring the Shiga toxin 1- Among *Shigella* enterotoxin genes, both *sen* and *set* enterotoxins are significantly associated with bloody diarrhea. In Cruz *et al*.'s study (23), *ShET-2* was found to contribute to intestinal injury and bloody diarrhea.

The *ShET-2* coding *sen* is responsible for epithelial inflammation; in this research found a combination of the *ipaBCD*, and *ipaH*, (100%) S. *sonnei* isolates. In addition, Zhang *et al*. (16) found that 21/21 (100%) of *S. flexneri* isolates were positive for *ipaBCD*, *ipaH*, and *vir*F simultaneously; however, only *set1A*, and *set1B* were detected in *S. flexneri* strains.Of the 21 *Shigella* isolates, *S. flexneri* were found to carry *set* and *sen* in Casabonne et al.'s study (22).To the best of our knowledge, this is the first study on the distribution of virulence gene combinations, and these genes are related with hospitalization and bloody diarrhea among *Shigella* species .

In conclusion, this work has demonstrated the high prevalence of two enterotoxins, *ShET-1* and *ShET-2*, in *S. flexneri*, especially, among the hospitalized patients who were included in the study population. Among *Shigella* spp. *S. flexneri* was found to have a high number of virulence determinants. Bloody diarrhea and hospitalization were also found to be associated with the number of virulence determinants. Future studies should investigate the relations between shigellosis symptoms and virulence determinants

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