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Resistance and Genetic Discrimination of *Shigella flexneri* Strains, using ERIC and ITS PCR

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This study was carried out to differentiate, genetically between *Shigella flexneri* strains isolated from river Nile water at Sohag governorate. The antibiotic resistance was targeted in this study as well. The antibiotic susceptibility of 11 *Shigella flexneri* strains was done using 10 different antibiotics. The genetic correlation of these strains was performed by using ERIC and ITS based PCR techniques. Eleven *Shigella flexneri*, strains were isolated from river Nile water in 7 areas along Sohag governorate. The ratio of *S. flexneri* in water samples comparing with total bacterial count was also recorded. The antibiotics susceptibility test showed that 100% of strains are resistant to Rifamycin, Cefalexin, and Erythromycin, whereas 91%, 82%, 54.5%, 45.5%, 36.5%, and 27.3% were resistant to Ceftazidim, Cefepim, Cefoxitin, Ertapenem, E-Moxiclav, and Norfluxacin respectively. The lowest strains percentages were resistant to Ciprofluxacin (18.2% of the strains). By using ERIC-PCR, nine different patterns were observed among the 11 isolates. The pattern was distinct for each isolate, except 3 isolates were fully identical in their profile. ERIC-PCR typing supported the existence of specific clones responsible for the shigellosis cases in the different cities and there was evidence of transmission between cities. ITS-PCR did not differentiate between the strains, which separated in only two groups, since it produced few bands profile, identical in most of tested strains. The strains isolated from Sohag city are joint factor with 3 strains isolated from 3 different regions in Sohag governorate representing 50% of the studied areas, because Sohag city is the center of the governorate, and includes the central hospitals receipt shigellosis cases from the other regions. This should alarm the officials to enhance the hygiene process and sewage control, to prevent contamination of drinking water with sewage.

Key words: *Shigella flexneri* ERIC, ITS, Resistance.

Shigella flexneri, is a facultative anaerobic bacteria belonging to the family Enterobacteriaceae. It is a Gram-negative rod that causes diarrhea and dysentery in humans. Potentially life-threatening, *S. flexneri*'s effects include bacteremia, hemolytic uremic syndrome (HUS) and toxic megacolon (Lew *et al.,* 1991). Shigellosis is the principle disease of diarrhea and dysentery caused by *Shigella flexneri*. Ten to 100 organisms are sufficient to cause this disease in human, and it transmitted from person-to-person by way of fecal-oral (Beletshachew *et al*., 2004). Shigellosis can be characterized as a disease with over 60% incidences in children ages 1-5 (Kotloff*et al.,* 1999).This pathogen typically infects men approximately twice as frequently as it infects women: 2.3 cases/100,000 people for males, 1.2 cases/100,000 people for females. Once contracted, 20% of *S. flexneri* infections require hospitalization (Beletshachew *et al*., 2004).

The incidence of shigellosis worldwide is determinedas 164.7 million cases per year,163.2 million were estimated in developing countries, where 1.1 million deaths occurred (WHO, 2005). More than 50% of these deaths included children

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younger than 5 years. The disease incidence in developing countries reaches 20 times greater than those in developed countries.This incidence varies within the same region (ECDOC, 2010).

More than 80% of microbial human diseases are waterborne.Water quality deterioration may occur due to the sources of fecal pollution including human, grazing cattle, natural animals' populations, septic tanks, failed sewage systems and summer storm activity. Consequently, many developing countries suffer from chronic shortages of freshwater because the accessible resources are heavily polluted.

As a result of antimicrobialuse over time, *Shigella* have developedresistance to many commonly used antimicrobial drugs. Mobile genetic elements including R plasmids, transposons,can help in resistance dissemination through isolates (Toro *et al.,* 2005; Navia*et al.,* 1999; Sur *et al.,* 2004). Drug resistancepatterns are influenced by many factors including geographic location, year in which the isolate wasobtained, class of antimicrobial agent, pressure exerted byantimicrobial use, and isolate source (Niyogi; Sur *et al.,* 2004). Resistance to traditional first-line drugs like ampicillin and trimethoprim-sulfamethoxazole is common, and resistance to some other antibiotics is increasing.

Persons with mild infections usually recover quickly without antibiotic treatment. However, appropriate antibiotic treatment may shorten the duration of illness and decrease the spread of infection. Antibiotic treatment is recommended for patients with severe disease, bloody diarrhea, or compromised immune systems.

AP-PCR, arbitrary primed PCR (Welsh *et al.,* 1990); RAPDs, randomly amplified polymorphic DNA (Williams *et al.*, 1990); rep-PCR, repetitive sequence-based PCR (Versalovic*et al*., 1994) and AFLP, amplified fragment length polymorphism (Vos*et al*., 1995). ERIC (Enterobacterial Repetitive Intergenic Consensus sequences) is one of Rep PCR is based on the specific conservative repeated sequences in different bacteria.

Sohag(the studying area)is a part of the Nile Valley, where extends from the northern border of Qena Governorate at latitude 26º72' N to the southern border of Assiut Governorate at latitude 26°57' N. It is located between longitudes 31°20' and 32°14' E as shown in Fig 1. The length of the River Nile in the studying area is 125 km.

The objective of this study is to use molecular characterization techniques for characterizing endemicand outbreak patterns of shigellosis inriver Nile in Sohag governorate, Egypt as a case study example. The resultsdescribed herein include bacterial distributions; antimicrobial resistance testing, ERIC, ITS PCR, and plasmid profiling, to evaluate the type of drug resistance, risk factorsassociated with high levels of drug resistance, and genetic diversityof strains present in Nile water in Sohag governorate region during a two seasons period.

MATERIALS AND METHODS

Sampling

Water samples were collected from 7 different locations in Sohag governorate as the following from North to South: Tima, Tahta, Sakolta, Sohag city and Akhmim, Girga, and Dar El-Salam (Fig. 1) in 250 ml clean sterile glass bottles, from 40 cm depth. All samples were transported in ice box, and the microbiological determinations were carried out within 6 hours.

Bacterial identification

The isolates have been identified on the bases of morphological, physiological, and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology. Serological testing was performed byusing a slide agglutination test using somatic (O) or flagella (H) antisera (bioMérieux kit), to identify *Shigella* sub species.

Antibiotic Susceptibility Testing (Bauer*et al.,* 1966)

All *Shigella* isolates were tested for susceptibility by the Kirby-Bauer's disk diffusion method against the following antibiotics: E-Moxiclav (30 µg), Rifamycin (30 mg), Cefalexin (30 mg), Erythromycin (15 mg), Ceftazidim (30 mg), Norfluxacin (10 µg), Ciprofluxacin (5 µg), Cefepim (30 mg), Ertapenem(10 µg) and Cefoxitin (30 mg).

This panel of antibiotics was selected based on the antibiotic prescription patterns in local hospitals and veterinary dispensaries, and the CLSIguidelines, (2006). Overnight grown cultures, on trypticase soy broth (optical density adjusted to MacFarland 0.5), were spread evenly on Mueller-Hinton agar plates. The plates were incubated at 37 °C for 24 h. The zones of inhibition

were measured and interpreted as resistant or sensitive according to CLSI (2006).

DNA Extraction

High molecular-weight, bacterial genomic DNA was purified form call lysates according to Koopmann (1999). The DNA was redissolved in a buffer containing 10 mMTris and 1 mM EDTA, and its concentration was determined spectrophotometrically.

ERIC PCR

ERIC (Enterobacterial Repetitive Intergenic Consensus) amplification was performed according to Louws *et al*., 1994.

ERICIR primers ^{5}(ATGTAAGCTCC TGGGGATTCAC)^{3}, ERIC2 ^{5}(AAGTAAGTGA CTGGGGTGAGGG)^{3} were used according to mentioned protocol.

The amplification program (Louws*et al*., 1994) performed for 30 cycles as following: Denaturizing at 94°C for 1 min; Annealing at 52°C for 1 min; Elongation at 65°C for 8 min; and final elongation at 65°C for 15 min.

ITS PCR (Jensen *et al*., 1993)

DNA samples were diluted to a concentration of 20 ng/µl prior to amplification. A 1.25-µl aliquot of bacterial genomic DNA was combined with 2.5 µl of reaction buffer (500 mMKCl, 100 mMTris-HCl [pH 8.8 at 25°C], 15 mM $MgCl_2$, 1% Triton X-100), 1 µl of a deoxynucleoside triphosphate (dNTP) mixture (concentration of each dNTP, 5 mM), 1.25 µl each of two 15-base oligonucleotide primers (primer G1 and L1[concentration, 50 ng/µl]), and 42 µl of deionized water. G1: 5'-GAAGTCGTAACAAGG-3', L1: 5'-CAAGGCATCCACCGT-3'

This mixture was heated to 94°C for 5 min, and 1.3 U of a thermostable DNA polymerase were added. Twenty five amplification cycles were performed with an automated thermocycler according to the following format: 1 min at 94°C; 2-min ramp to 55°C; 7 min at 55°C; 2 min ramp to 72°C; and 2 min at 72°C. The final cycle was followed by an additional 7 min at 72°C to complete partial polymerization. At the end of the cycling program 1 µl of 0.5 M EDTA was added as a stop solution, and the products were stored at 4°C.

The amplified DNA products were visualized by electrophoresis through an agarose gel. Horizontal slab agarose gel with a concentration of 1.5% in 1 x TAE buffer.

Cluster analysis

MVSP program, version 3.22 was usedfor cluster analysis of data obtained by ERIC, and ITS PCR.

RESULTS

Identification of *Shigella flexneri*

The isolated bacteria were subjected to the biochemical tests used in distinguishing and Identification of *Shigella*at the genus level. It identidied at the species level as *Shigella flexneri* by using ofa slide agglutination test using somatic (O) or flagella (H) antisera(bioMérieux kit).

Eleven *Shigella flexneri* strains were isolated from the studied areas. Table 1, show the numbers of isolated *Shigella flexneri* strains from different locations, compared with the total bacterial counts (TBC), total coliform, and fecal coliform.

Antibiotic Susceptibility

Table 2, Fig.2, and Fig. 3 show in detail, the sensitivity of *Shigella flexneri* toward 10 used antibiotics, whereasall strains are resistant to

Table 1. Microbiological characteristics of River Nile water in different regions in Sohag governorate

Site (strain code)	TBC (cfu/ml)	*T.coliform* (cfu/ ml)	*F.coliform* (cfu/ ml)	*Shigella* bacterium (cfu/ml)	Shigellaflexneri strains
Tima (S1)	3×10^3	15	5	1	1
Tahta (S2)	7×10^3	80	35	3	1
Sakolta (S3)	18×10^3	6	4	3	1
Sohag (S 4, 5, 6)	3×10^3	50	42	10	3
Akhmim (S7, 8)	11×10^3	30	19	6	2
Girgra (S9, 10)	6×10^3	63	25	7	2
Dar-Elsalam (S11)	7×10^3	40	26	1	1

TBC: Total bacterial count T. coliform: Total coliformF. coliform :Faecal coliform

Table 2. Antibiotics susceptibility of *Shigellaflexneri* strains.

	S	I	R
S1 (Tima)	E, CL, FEP, RF, CAZ,	Amc, CIP, NOR, ETP, FOX	-
S2 (Tahta)	E, CL, FEP, RF, CAZ	Amc, CIP, NOR, ETP, FOX	-
S3 (Sakolta)	E, CL, FEP, RF, CAZ, NOR, ETP, FOX	CIP	Amc
S4 (Sohag)	E, CL, RF, CAZ, FOX	Amc, Cip, NOR, ETP	FEP
S5 (Sohag)	E, CL, Amc, FEP, RF, CAZ, FOX	Cip, NOR, ETP	-
S6 (Sohag)	E, CL, Amc, FEP, RF, CAZ,	Cip, NOR, ETP, FOX	-
S7 (Akhmim)	E, CL, FEP, RF, ETP, CAZ, FOX	NOR, CiP, Amc	-
S8 (Akhmim)	E, CL, Amc, FEP, RF	CIP, NOR, ETP, CAZ, FOX	-
S9 (Girga)	E, CL, RF, ETP, CAZ, FOX	Amc, FEP, NOR	CiP
S10 (Girga)	E, CL, FEP, RF, Cip, NOR, ETP, CAZ	Amc	FOX
S11 (Dar-Elsalam)	E, CL, FEP, RF, CAZ, Amc, CIP, NOR, ETP, FOX	-	-

Amc: E-Moxiclav, RF:Rifamycin, CL:Cefalexin, E: Erythromycin, CAZ:Ceftazidim, NOR: Norfluxacin, CIP:Ciprofluxacin, FEP:Cefepim, ETP:Ertapenem, FOX:Cefoxitin, S:Suscebtibl

Moxiclav, Cefalexin, Cefepim. As shown in Fig. 3, 100% of strains are resistant toRifamycin, Cefalexin, and Erythromycin, whereas 91%, 82%, 54.5%, 45.5%, 36.5%, and 27.3% were resistant to Ceftazidim, Cefepim, Cefoxitin, Ertapenem, E-Moxiclav, and Norfluxacin respectively. The lowest strains percentages were resistant to Ciprofluxacin (18.2% of the strains).

ERIC-PCR

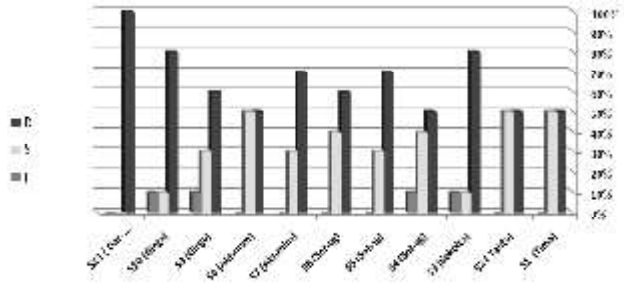
The ERIC-PCR technique successfullytyped all isolates examined. Nine different patterns wereobserved among the 11 isolates (Fig. 4). The pattern was distinct for each isolate, except 3 isolates were fully identical in their profile as shown in fig. 4 and in the dendrogram (Fig 5).The strains show different and distinct profile, except S3, S5, and S9, which were isolated from Sakolta, Sohag city, and Girga. These 3 strains were fully identical in their profile.

ITS-PCR

This technique did not differentiate the strains. The strains separated in only two groups, one of them included the strains S1, S5, and S10 which isolated from Tima, Sohag city, and Girga, respectively (Fig. 6 and Fig. 7).

As shown in Fig. 4, the strain (S11) isolated from Dar-Elsalam was resistant to all used antibiotics. Strains (S10, S3) isolated from Girga and Sakolta respectively, were resistant to 80 % of used antibiotics, whereas sensitive and have intermediate resistant to 10% equally. S7 and S5 *Shigella flexneri* strains isolated from Akhmim and Sohagrespectively, were resistant to 70% of antibiotics, whereas 30% were sensitive. Strains, S8, S2, S1 isolated from Akhmim, Tahta, and Tima respectively were resistant and sensitive to 50% of antibiotics equally. Strain S4 isolated from Sohag, were resistant, sensitive intermediate to 50%, 40%, and 10% of antibiotics respectively, and the strain S9 isolated from Girgawas resistant, sensitive intermediate to 60%, 30%, and 10% of used antibiotics respectively. The *Shigella flexneri* (S6) strain isolated from Sohag was resistant to 60% and sensitive to 40% of the tested antibiotics.

Fig. 1. Sampling sites showing 7 areas in River Nile along Sohag governorate



R= Resistant S= sensitive I= Intermediate R

Fig. 2. Antibiotics resistance percentages according to isolation areas

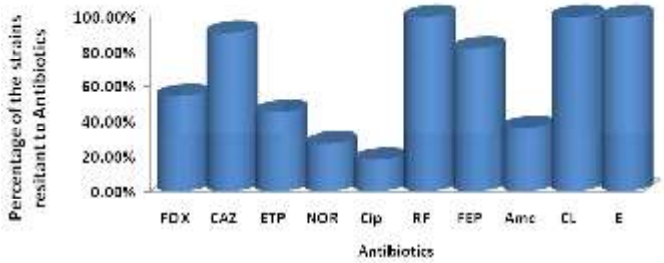


Fig. 3. Percentage of *Shigella flexneri* strains resistant to antibiotics

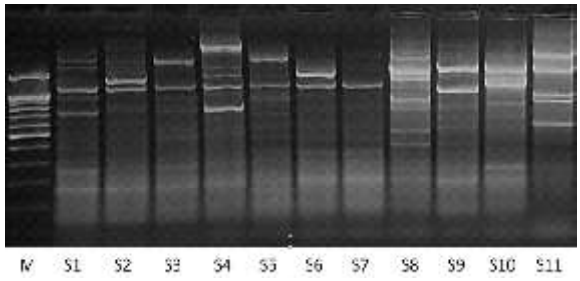


Fig. 4. ERIC-PCR profile patterns. M: 100 bp DNA ladder, S1: *Shigella flexneri* strains isolated from Tima, S2: from Tahta, S3: from Sakolta, S4: from Sohag city, S5: from Sohag city, S6: from Sohag city, S7: from Akhmim, S8: from Akhmim, S9: from Girga, S10: from Girga, and S11 isolated from Dar-Elsalam

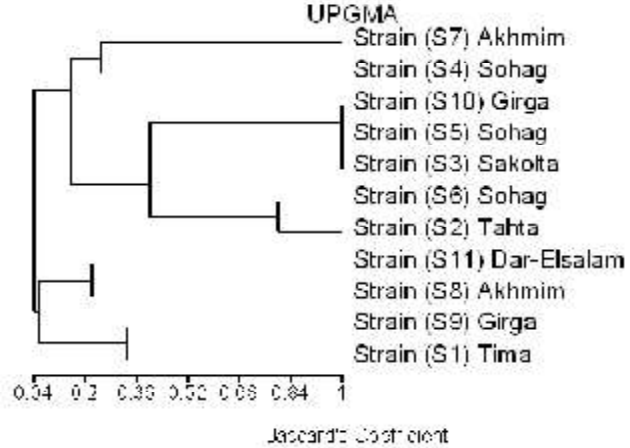


Fig. 5. Dendrogram showing the genetic correlation between *Shigella flexneri* strains using ERIC-PCR fingerprinting technique

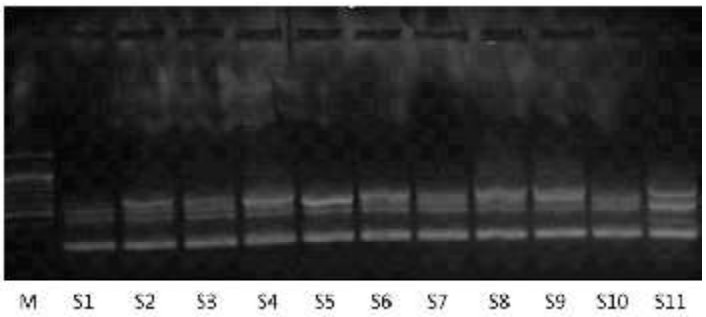


Fig. 6. ITS-PCR profile patterns. M: 100 bp DNA ladder, S1: *Shigella flexneri* strains isolated from Tima, S2: from Tahta, S3: from Sakolta, S4: from Sohag city, S5: from Sohag city, S6: from Sohag city, S7: from Akhmim, S8: from Akhmim, S9: from Girga, S10: from Girga, and S11 isolated from Dar-Elsalam

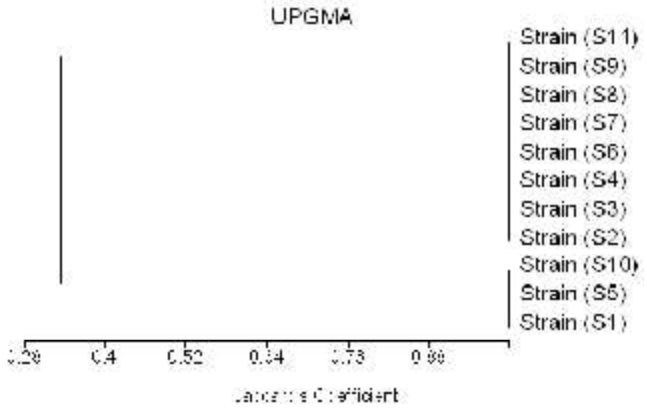


Fig. 7. Dendrogram showing the similarity coefficient between *S. flexneri* strains using ITS amplification technique. S1: *Shigella flexneri* strains isolated from Tima, S2: from Tahta, S3: from Sakolta, S4: from Sohag city, S5: from Sohag city, S6: from Sohag city, S7: from Akhmim, S8: from Akhmim, S9: from Girga, S10: from Girga, and S11 isolated from Dar-Elsalam

DISCUSSION

Shigella spp., is usually present in drinking water as a result of water contaminated with human feces, which is contains human enteric pathogens causing human diarrhea (Scott *et al*., 2008). Shigellosis outbreaks resulted usually from consumption of feces contaminated water, especially in developing countries with inadequate sanitation facilities.

Total number infectedcases of *Shigella* that occur yearly throughout the World is estimated to be 164.7 million, including 163.2 million cases in developing countries, 1.1 million of which result in death. The Children under 5 represent 61% of all deaths attributable to shigellosis (Emch *et al*., 2008; Germani and Sansonetti, 2003)

In the last decades, Important epidemics were reported: (1) in 1970 in Central America where 112,000 people were affected and 13,000 died; (2) in 1985, in Texas (USA), 5,000 people became infected after ingestion of contaminated lettuce; (3) in May-June 1994, domestic cases were detected in several European countries, including Norway, Sweden, and the United Kingdom. Epidemiological evidence incriminated imported iceberg lettuce as the vehicle of transmission; (4) in 1996, in Paris, with 153 reported patients (Kapperud *et al*., 1995).

There is worldwide concern about the emergence of drugresistant strains of enteric pathogens. 1n 2012, Debdutta *et al.,* reported that 50 *Shigella* isolates were resistant to ampicillin, 96 per cent to nalidixic acid, 94 per cent to tetracycline, 82 per cent to ciprofloxacin, 80 per cent to ofloxacin and 70 per cent to norfloxacin.

Shai Ashkenazi *et al.,* 2003, have demonstrated that, resistance of *Shigella* to tetracycline,trimethoprim–sulfamethoxazole, and

ampicillin increased in 1998/2000 than in 1991/1992 in Israel. They identified a significantly increased resistance to tetracycline (from 23% to 87%), high resistance to trimethoprim–sulfamethoxazole (94%) and ampicillin (85%) and emerging resistance to quinolones (0.5-2%).

In our study, the lowest resistance percentage towards 10 antibiotics used, was 50% in each of strains S1, S2, S4, and S8 isolated from Tima, Tahta, Sohag, Akhmim respectively. Hundred percent of *Shigella flexneri* isolates in this study were resistant to 5 or more antibiotics. These results are higher than those obtained by Sawsan *et al.,* 2009,whom reported that, 38% of *Shigella* spp., isolated from Monterey region of California,were resistantto five or more antibiotics. All of isolated *Shigella flexneri* were fully resistant to Rifamycin, Cefalexin, and Erythromycin, which consider an alarm for the usefulness of these antibiotics in shigellosis treatment. The same is toward Ceftazidim, Cefepim, that 91% and 82% of isolated *Shigella flexneri* were resistant to them respectively. The lowest resistant degree was toward each of Norfluxacin, and Ciprofluxacin. Twenty seven (27%), 18% of isolated *Shigella flexneri* strains were resistant to NOR and CIP respectively. According to these results, the last two antibiotics can be used as drug of choice in shigellosis treatment. This level of drug resistance obtained in this study is also higher than national NARMS (National Antimicrobial Resistance Monitoring System for enteric bacteria: human isolates final report, 2004 United States Department of Health and Human Services; 2007).Therefore, a special and continuous effort should be taken to manage the antibiotics treatment in the ideal way to prevent the development of raised antibiotics resistance.

ERIC is a DNA fingerprinting technique which is used in genotyping of many organisms. Kosek *et al.,* 2012, have been evaluated the performance of enterobacterial repetitive intergenic sequence-based polymerase chain reaction (ERIC-PCR) typing versus the current standard for the typing of *Shigella* pulsed gel electrophoresis (PFGE). They typed 116 *Shigella* isolates from a village in an endemic setting over a 20-month period using both methods. PFGE identified 37 pulse types and had a discrimination index of 0.925 (95% confidence interval = 0.830-1.00), whereas ERIC-PCR identified 42 types and had a discrimination index of 0.961 (95% confidence interval = 0.886-1.00). Each of PFGE and ERIC-PCR showed a 90.4% correlation in the designation of isolates as clonal or non-clonal in pairwise comparisons. They also reported that both systems were highly reproducible and provided highly similar and supplementary data compared with serotyping regarding the transmission dynamics of shigellosis in this community. ERIC-PCR is considerably more rapid and inexpensive than PFGE and may have a complementary role to PFGE for initial investigations of hypothesized outbreaks in resource-limited settings.

In 1995, Peter *et al.,* reported that ERIC-PCR technique represents a rapid and simple means for typing *S. sonnei* witha level of discrimination equivalent to that of PFGE but greater than those of plasmid profile analysis,restriction endonuclease analysis of plasmids, and ribotyping. In Iran 2010, Reza *et al*., have used ERIC-PCR in discrimination between 13 *S. flexneri* strains isolated from a prison in Iran as a result of shigellosis outbreak. They found that these strains produced only a single pattern with 13 DNA band fragments in all selected *S. flexneri* strains associated with the outbreak.

In this study, ERIC is used for discrimination between 11 *Shigella flexneri* strains isolated from different regions in river Nile along Sohag governorate.Nine patterns were obtained from the eleven strains used in this study as shown in fig. 4. As seen in dendrogram (Fig. 5), only 3 strains, S3, S5, and S10 isolated from Sakolta, Sohag city, and Girgarespectively were 100% identical in their profile. The next most similar in their profiles, are strains S2 and S6 which isolated from Tahta and Sohag city respectively. The two strains have about 76% similarity coefficient. From these results, it is clear that, the strains isolated from Sohag city are joint factor with 3 strains isolated from 3 different regions in Sohag governorate representing 50% of the studied areas.This may be explained that Sohag city is the center of the governorate, which includes the central hospitals receipt shigellosis cases from the other regions. This should alarm the officials to enhance the hygiene process and sewage control, to prevent contamination of drinking water with sewage.

According to the results of ITS-PCR, this

technique cannot be used for strain differentiation, since it produced few bands profile, identical in most of tested strains.

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