Antimicrobial Susceptibility, Heavy Metals Tolerance and Plasmid Curing of *Shigella* Species Isolated from El- Dakahlia, Egypt

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Abstract The aim of this study was carried out determine the antibiotic susceptibility, heavy metals tolerance and plasmid curing of *Shigella* species isolating from diarrheal stool samples were collected from different hospitals in El- Dakahlia and Nile River waters, Egypt, from January 2012 to August 2014. After characterization and identification the results obtained show that 172 isolates isolated from stool samples belong to four *Shigella* species (*Shigella sonnei* 48.25 %, *Shigella flexneri* 29.65 %, *Shigella dysenteriae* 13.95 % and *Shigella boydii* 8.13 %) while 5 isolates isolated from Nile River water were found belong to (*Shigella sonnei* 40%, *Shigella dysenteriae* 40 % and *Shigella flexneri* 20 %). The antibiotics susceptibility of *Shigella* sp. to 11 antibiotics revealed that the most potent antibiotics were found co-amoxycylav, ciprofloxacin and ceftriaxone respectively while penicillin, ampicillin, co-trimoxazole and chloramphenicol respectively give low activity. The tolerance of *Shigella* sp. to heavy metals, (cadmium, nickel cobalt and zinc) revealed that all isolates sensitive to 1 and 0.1M concentration. Plasmid profile analysis of ten isolates *Shigella sonnei* shown that this isolates having numerous plasmid ranged from 8.5 to 4.3 kb. Treat isolates with SDS 2% for 24 hours to plasmid curing after recovery subject to antibiotic sensitivity and heavy metals tolerance. In conclusion, *Shigella*-associated diarrhea remains relatively common in Egypt and can be used ciprofloxacin and ceftriaxone for treat *Shigella* sp. infection. The heavy metal tolerance of *Shigella* sp. associated with resistance to antibiotics ampicillin, tetracycline and chloramphenicol. Present *Shigella* sp. in Nile River waters indicates polluted with sewage waters and becomes sources of some epidemic diseases.

Keywords: *Shigella* species, antibiotics susceptibility, heavy metals tolerance and plasmid curing

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1. Introduction

Shigella spp. is a virulent bacterium of *Enterobacteriaceae* family. Shigellosis is an acute gastroenteritis caused by shigellae, including *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*. Shigellosis remains a public-health problem in most developing countries where communities are ravaged by poverty, war, poor sanitation, personal hygiene, and water supplies [1]. Epidemiologic reports show that about 140 million people suffer from shigellosis with estimated 600,000 deaths per year worldwide [2,3]. A major food-borne threat to public health in many developed countries where the issues of sanitation are concerned [4,5,6]. *S. sonnei* is spread mainly by means of fecal-oral transmission. Other possible modes of transmission can be from ingestion of contaminated food or water and subcutaneous contact with inanimate objects. *S. sonnei* infectivity dose is very low, as few as 100-200 bacteria are needed to cause a clinical infection, Shigellosis [7,8]. The emergence of

antimicrobial resistance within members of the *Enterobacteriaceae* family is posing serious problems in the treatment of outbreaks of infections. Since its first report in studies conducted in the 1950s, multiple-drug resistance transmitted by plasmids among *Shigella* species has been reported from many countries [9,10]. Moreover, an increase in resistance against many different drugs has been observed in the last two decades. In India, over 70% of *Shigella* isolates were resistant to two or more drugs including ampicillin and co-trimoxazole during 2002 to 2007 [11]. Reports from Indonesia [12], Bangladesh [13], Malaysia [14] and Nepal [15] show increasing prevalence of *Shigella* isolates with multiple resistance to ampicillin, trimethoprim-sulphamethoxazole, tetracycline, and nalidixic acid. Similar resistance profiles have been also reported from Africa [16] Central America [17], Europe [18] and South America [19]. The genetic determinants that confer resistance to antibiotics are mostly located on plasmids (known as R-plasmids). These extra-chromosomal DNA sequences are often transferable to other bacteria in the environment and can be responsible for the emergence of resistance to multiple antibiotics [20]. The use of plasmid-

curing agents in combination with antibiotics may serve as a possible way to contain the development and spread of antibiotic resistance encoded by antibiotic resistant R-plasmids. This work was aimed to study the evaluation incidence of *Shigella* sp. resistant to antibiotics and heavy metals having plasmids in hospital and Nile River waters and curing plasmid.

2. Materials and Methods

Three hundred forty five stool samples used in this study were collected from patients having vomiting and diarrhea symptoms in hospitals El- Dakahlia, Egypt, and twenty water samples collected from Nile River waters at different sites from period between January 2012 to August 2014. The samples collected processed by standard microbiological methods and cultivated on MacConkey agar, Salmonella-Shigella agar and deoxycholate citrate agar. All media were readily prepared (Oxoid, England).

2.1. Assessment and Purification of Bacterial Isolates

The plates containing of MacConkey agar, Salmonella-Shigella agar and deoxycholate citrate agar media were inoculation with samples collected and incubated at 37°C for 24 and 48 hrs. respectively. The grown colonies were selected, picked up and transferred to agar slants containing the same medium. The purified isolated were subjected to a scheme of experimental identification.

2.2. Bacterial Identification

The pure culture was identify based on morphology, physiology and biochemical tests Microbiological Methods 6th [21], Bergey's Manual of Determinative Bacteriology [22] and confirmed by the slide agglutination test using polyvalent and monovalent antisera (Denka Seiken, Japan).

2.3. Antibiotic Susceptibility Testing

Susceptibility to antibiotics was assessed using the Kirby-Bauer disc diffusion technique while results were read following the Clinical Laboratory Standards Institute's guidelines National Committee for Clinical Laboratory Standards 2010 [23]. The panel of antibiotics used included: ampicillin (10µg), penicillin (10µg), chloramphenicol (30µg), co-amoxyclov (20/10µg), aztreonam (30µg), trimethoprim /sulphamethoxazole (co-trimoxazole, 1.25/23.75µg), ceftriaxone (30µg) nalidixic acid (30µg), tetracycline (30µg), streptomycin (30µg) and ciprofloxacin (5µg) The suspension of the test organism in nutrient broth was matched with 0.5 McFarland turbidity standards to give a concentration of 1.5×10^8 CFU/ml, while the inoculated plates were incubated at 35°C for 18 – 24 hours. The degree of susceptibility of the test isolates to each antibiotic was determined on basis inhibition zone diameter. Multidrug resistance was defined in this study as resistance to three or more antibiotics tested. All antibiotic discs were purchased from Oxoid. UK.

2.4. Tolerance of Heavy Metals by Using Agar Dilution Method

All isolates were also tested to determine tolerance to four heavy metals including (cobalt, cadmium, nickel and zinc) was carried out by using agar dilution method modified by Narasimhulu *et al.*, 2010 [24]. Prepare Mueller-Hinton agar media and approximate concentration molar (M) 1M, 0.1M, 0.01M and 0.001 for each heavy metals, added to flask contained 50 ml Mueller-Hinton, mixed well, and autoclaved at 121°C for 15 minutes. After autoclaving, inoculating by refreshed growth *Shigella* isolates and incubated at 37°C for 24 hrs. growth, meaning resistance and if there is no growth meaning sensitive.

2.5. Plasmid Isolation

Overnight *Shigella* (10 ml) culture was used for the plasmid isolation. After isolation and purification the bands were visualized in 0.7% agarose gels with 0.5 mg/ml of ethidium bromide in 10 mM Tris-acetate buffer. The procedure was conducted according to the method described by (Anderson D.G. and McKay 1983) and Frere 1994 [25,26].

2.6. Plasmid Curing

Shigella sp. isolates high resistant antibiotics and heavy metals subjected to plasmid curing. The plasmid curing was done by exposing the overnight grown culture at (37°C) and 2% Sodium Dodecyl Sulphate (SDS) [25,27]. After plasmid curing the *Shigella* isolates subjected to antibiotic sensitivity and heavy metals tolerance.

3. Results

One hundred seventy two *Shigella* isolates isolated from 345 stool samples and five *Shigella* isolates isolated from 20 water samples. This isolates subject to scheme for characterization and identification according to morphological physiology and biochemical tests and confirmed by the slide agglutination test using polyvalent and monovalent antisera (Denka Seiken, Japan). From identification results, the isolates isolating from stool samples were found belong to *Shigella* species as the following (83 isolates *Shigella sonnei* 48.25 %, 51 isolates *Shigella flexneri* 29.65 %, 24 isolates *Shigella dysenteriae* 13.95% and 14 isolates *Shigella boydii* 8.13%) while 4 isolates isolated from water samples belong to 2 isolates *Shigella sonnei*, 2 isolates *Shigella dysenteriae* and 1 isolates *Shigella flexneri* Table 1.

Table 1. Ratio of *Shigella* isolated from stool and water samples

Bacterial species	Prevalence	
	stool	water
<i>Shigella sonnei</i>	83 (48.25 %)	2 (40 %)
<i>Shigella flexneri</i>	51 (29.65 %)	2 (40%)
<i>Shigella dysenteriae</i>	24 (13.95 %)	1 (20 %)
<i>Shigella boydii</i>	14 (8.13 %)	0.0

The susceptibility of *Shigella* sp. isolates to 11 antibiotics, the results obtained showed that the most potent antibiotic against those isolates co-amoxyclov, ciprofloxacin and ceftriaxone while penicillin, ampicillin,

co-trimoxazole, chloramphenicol and tetracycline respectively give low activity and high resistant ratio against isolates. In generally *Shigella* sp. isolated from stool samples more resistant to antibiotics tested from than *Shigella* sp. isolated from Nile River waters Table 2 and Table 3. In present study, the effects of four heavy metals on *Shigella* sp. isolates were investigated with different molar concentration, from heavy metals (cadmium, nickel, cobalt and zinc). Results obtained revealed that all isolates

were sensitive to four heavy metals at concentration 1 and 0.1M but resistant at concentration 0.01M Table 4 and Table 5. The plasmid profile analysis shown that *Shigella sonnei*. have numerous plasmid ranged from 8.5-4.3kb Figure 1. This isolates subject to plasmid curing with SDS 2% for 24 hours after recovery subject to antibiotic sensitivity and tolerance heavy metals comparing between results before and after plasmid curing, showed change on antibiotics sensitivity and tolerance of heavy metals.

Figure 1. Visualization of plasmids in an agarose gel (0.7 %). Lane M represents marker from lane 1 to lane 10 plasmids isolated from *Shigella sonnei* resistance to antibiotic and heavy metals tolerances

Table 2. Percentage resistance of *Shigella* sp. isolated from stool samples antibiotics before and after plasmid curing

Antibiotics	<i>S. sonnei</i>		<i>S. flexneri</i>		<i>S. dysenteriae</i>		<i>S. boydii</i>	
	before (%)	after (%)	before (%)	after (%)	before (%)	after (%)	before (%)	after (%)
Ampicillin	67.2	50.4	75.52	58.8	74.99	20.83	72.42	28.52
Penicillin	74.4	51.6	80.36	56.84	79.16	50.0	78.56	35.71
Streptomycin	28.8	20.4	29.4	23.52	29.16	8.33	28.57	14.28
Tetracycline	49.2	38.4	60.76	43.12	54.16	25.0	50.0	28.57
Chloramphenicol	56.4	45.6	66.64	49.0	58.33	25.0	42.85	21.42
Co-trimoxazole	68.4	39.6	76.44	54.88	58.33	41.66	57.13	28.52
Nalidixic Acid	19.2	14.4	17.64	0.0	16.66	4.166	35.71	21.42
Ceftriaxone	3.6	0.0	9.8	3.92	4.16	0.0	14.28	0.0
Co-amoxyclov	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Aztreonam	24.0	15.5	27.44	15.68	20.83	8.33	14.28	0.0
Ciprofloxacin	0.0	0.0	0.0	0.0	4.16	0.0	0.0	0.0

Table 3. Percentage resistance of *Shigella* sp. isolated from Nile river to antibiotics before and after plasmid curing

Antibiotics	<i>S. sonnei</i>		<i>S. flexneri</i>		<i>S. dysenteriae</i>	
	before (%)	after (%)	before (%)	after (%)	before (%)	after (%)
Ampicillin	100	50	100	0.0	100	0.0
Penicillin	100	50	100	0.0	100	0.0
Streptomycin	50	0.0	50	0.0	0.0	0.0
Tetracycline	100	0.0	50	0.0	100	0.0
Chloramphenicol	50.	0.0	100	0.0	0.0	0.0
Co-trimoxazole	50	0.0	50	0.0	0.0	0.0
Nalidixic Acid	50	0.0	50	0.0	0.0	0.0
Ceftriaxone	50	0.0	50	0.0	0.0	0.0
Co-amoxyclov	0.0	0.0	0.0	0.0	0.0	0.0
Aztreonam	50	0.0	50	0.0	100	0.0
Ciprofloxacin	0.0	0.0	00	0.0	0.0	0.0

Table 4. Tolerance of *Shigella* sp. isolated from stool samples to heavy metals before plasmid curing

Metals conc.(M) Isolates	Cobalt			Zinc			Nickel			Cadmium		
	1	0.1	0.01	1	0.1	0.01	1	0.1	0.01	1	0.1	0.01
<i>S. sonnei</i>	0	0	R	0	0	R	0	0	R	0	0	R
<i>S. flexneri</i>	0	0	R	0	0	R	0	0	R	0	0	R
<i>S. dysenteriae</i>	0	0	R	0	0	R	0	0	R	0	0	R
<i>S. boydii</i>	0	0	R	0	0	R	0	0	R	0	0	R

R = resistant, 0 = no growth or sensitive

Table 5. Tolerance of *Shigella* sp. isolated from Nile river to heavy metals before plasmid curing

Metals conc.(M) Isolates	Cobalt			Zinc			Nickel			Cadmium		
	1	0.1	0.01	1	0.1	0.01	1	0.1	0.01	1	0.1	0.01
<i>S. sonnei</i>	0	0	R	0	0	R	0	0	R	0	0	R
<i>S. flexneri</i>	0	0	R	0	0	R	0	0	R	0	0	R
<i>S. dysenteriae</i>	0	0	R	0	0	R	0	0	R	0	0	R

R = resistant, 0 = no growth or sensitive.

Table 6. Tolerance of *Shigella* sp. isolated from stool samples to heavy metals after plasmid curing

Metals conc.(M) Isolates	Cobalt				Zinc				Nickel				Cadmium			
	1	0.1	0.01	0.001	1	0.1	0.01	0.001	1	0.1	0.01	0.001	1	0.1	0.01	0.001
<i>S. sonnei</i>	0	0	0	R	0	0	0	R	0	0	0	R	0	0	0	R
<i>S. flexneri</i>	0	0	0	R	0	0	0	R	0	0	0	R	0	0	0	R
<i>S. dysenteriae</i>	0	0	0	R	0	0	0	R	0	0	0	R	0	0	0	R
<i>S. boydii</i>	0	0	0	R	0	0	0	R	0	0	0	R	0	0	0	R

R = resistant, 0 = no growth or sensitive.

Table 7. Tolerance of *Shigella* sp. isolated from Nile River to heavy metals after plasmid curing

Metals conc.(M) Isolates	Cobalt				Zinc				Nickel				Cadmium			
	1	0.1	0.01	0.001	1	0.1	0.01	0.001	1	0.1	0.01	0.001	1	0.1	0.01	0.001
<i>S. sonnei</i>	0	0	0	R	0	0	0	R	0	0	0	R	0	0	0	R
<i>S. flexneri</i>	0	0	0	R	0	0	0	R	0	0	0	R	0	0	0	R
<i>S. dysenteriae</i>	0	0	0	R	0	0	0	R	0	0	0	R	0	0	0	R

R = resistant, 0 = no growth or sensitive

4. Discussion

Shigellosis accounts for a significant proportion of morbidity and mortality, especially in developing countries [28,29]. Four species of the genus *Shigella*, *S. sonnei*, *S. flexneri*, *S. dysenteriae*, *S. boydii*, cause a wide spectrum of illnesses ranging from watery diarrhea to fulminant dysentery [30,31,32]. The frequency of occurrence of *Shigella* sp. differs by country and in different populations within a country [30,33]. The samples used in this study were collected from patients having vomiting and diarrhea symptoms in hospitals El-Dakahlia, Egypt, and water samples collected from Nile River waters at different sites from period between January 2012 to August 2014. According to result obtained from isolation and identification were found the predominant isolate *S. sonnei* with a mean prevalence of 48.25 %, followed by *S. flexneri* 29.65 %, *S. dysenteriae* 13.95 % and *S. boydii* 8.13 % from total isolates, while isolates isolated from Nile River waters samples was found as the following, *S. sonnei* represented 40%, *S. dysenteriae* 40 % and *S. flexneri* 20 % from total isolates. The results obtained of this study shows change in the pattern of species isolated in Egypt by Remon *et al.*, 2004 [34]. *S. flexneri* is dominant to isolate in developing countries such as Bangladesh, Pakistan, Indonesia, India, some countries in Africa, and Iran [35,36] while *S. sonnei*

is the major *Shigella* isolate in developed countries [35]. Shawky and Saleh 2007 isolating *Shigella* sp. from Nile River Damietta branch and Ahmed *et al.*, 2013 isolated 46 isolates *S. flexneri* from Egypt [37,38]. In this prospective, community-based study, we were able to demonstrate that *Shigella* was an important cause of diarrhea in patients the Nile River Delta of Egypt. The antibiotics susceptibility revealed that most potent activity against all *Shigella* isolates co-amoxyclov, ciprofloxacin and ceftriaxone. These findings were in agreement with reports of other studies indicating *Shigella* isolates are sensitive to these antibiotics [28,39,40,41,42]. While penicillin, ampicillin, co-trimoxazole, chloramphenicol and tetracycline respectively give low activity and high resistant ratio against isolates. In this study was demonstrated a high level of antimicrobial resistance in *Shigella* species isolated from this samples. The multidrug resistant isolates represented incidence especially the emergence of resistance to streptomycin indicates that designing a surveillance system for antimicrobial resistance in Egypt and the introduction of integrated guidelines for the appropriate use of antibiotics are urgently needed. In present study, the effects of four heavy metals on *Shigella* sp. isolates were investigated with different molar concentration, the heavy metals were used cadmium, nickel, cobalt and zinc. Results obtained revealed that all isolates were sensitive to four heavy metals at concentration 1M and 0.1. Finally all isolates resistance to

four heavy metals at concentration 0.01M. The interpretation of these results may be due to the fact that *Shigella* sp. have many mechanisms for heavy metals resistance. Detoxifying mechanisms developed by resistant microorganisms such as complexation by exopolysaccharides, binding with bacterial cell envelopes, metal reduction, metal efflux etc. These mechanisms are sometime encoded in plasmid genes facilitating the transfer of toxic metal resistance from one cell to another [43]. Prasad *et al.*, 2009, found that all isolates were sensitive to heavy metals (Cd²⁺, Ag⁺, Ar²⁺, Co²⁺, Ni²⁺, Hg²⁺, and Pb²⁺) at concentration 0.1M, and most of them were resistant to heavy metals at concentration (0.0001M) [44]. The interaction between heavy metals and antibiotic resistance are of three types: heavy metals interaction with antibiotic compounds, heavy metals interaction with antibiotic resistance genes or even their products and heavy metal interaction with bacterial properties like conjugation. Nishino *et al.*, 2007 of cations heavy metals complex with antibiotics [45]. The heavy metal tolerance associated with resistance to antibiotics such as ampicillin, tetracycline, chloramphenicol, erythromycin, kanamycin and streptomycin [46]. The plasmid profile analysis demonstrated the virulence plasmid ranged from (8.5 to 4.3 kb). This plasmid was not used for pattern comparison of isolates because of its documented instability on subculture [47,48]. Most the *Shigella sonnei* isolates were found to harbour a more one and similar plasmid. In the study, numerous plasmid patterns were found in each of the *Shigella* species. Tacket *et al.* 1984 also found multiple plasmid profiles in all *Shigella* species [49]. Litwin *et al.* 1991 studied 74 *Shigella* sp. they found plasmid patterns for each species were distinct [50]. It was found this study there were relation between plasmid bands and multiple antibiotic resistant pattern and heavy metals tolerance. Curing of plasmids was carried out with SDS, sub culture plasmid curing was achieved by growing the strains, treatment with SDS 2%. The plasmid elimination was accompanied by drastic changes in antibiotic resistance and morphology of the colonies [51]. Some isolates lose resistant to penicillin, ampicillin, cotrimoxazole, tetracycline and chloramphenicol when treated SDS and increase inhibition zone diameter. With comparison between results before and after plasmid curing, showed change on antibiotics resistant and tolerance of heavy metals. The loss of antibiotic and heavy metals resistance was concomitant with the loss of plasmid content so that the results showed that some cured isolates had lost their antibiotic and heavy metals resistance. This indicates that the resistance determinants of tested antibiotics were located on plasmids.

5. Conclusion

In conclusion, *Shigella sonnei* is predominating isolates on diarrhea stool samples isolated from El-Dakahlia, Hospital Egypt. Although some of *Shigella* isolates multidrug resistance but can be used ciprofloxacin for treatment *Shigella* sp. Infection. The heavy metal tolerance associated with resistance to antibiotics such as ampicillin, tetracycline and chloramphenicol. The Present *Shigella* sp. in Nile River waters this indicated that is polluted with sewage water and represented incidence on

healthy people and distribution of epidemic diseases. Therefore need to additional control to prevent spread, include antibiotics development with some plasmid curing agent and integrated guidelines for the appropriate use of antibiotics are urgently needed.

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