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PLASMID SCREENING OF MULTIRESISTANT SHIGELLA ISOLATES

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ABSTRACT

Twelve isolates (3.67%) out of 327 diarrheal cases were identified biochemically and serologically as shigellae. *Shigella flexneri* was the most frequently isolated subgroup of shigella (2.14%) followed by *Shigella boydii* (1.22%), and *Shigella dysenteriae* (0.3%), respectively. The susceptibility of the isolates to 18 different antimicrobial chemotherapeutic agents was performed by the disc diffusion method. All the isolates were resistant to 1-6 of the antimicrobial agents under test. The majority of shigella isolates (58.3%) were resistant to at least 4 of the antimicrobial agents. The resistance patterns of shigella isolates were heterogenous, since as many as 8 different patterns were recognized among the tested isolates. The plasmids of shigella isolates were detected by agarose gel electrophoresis. The results revealed very heterogenous profiles. Eleven different profiles were recognized for the 12 shigella isolates. All the shigella isolates were harboring at least 3 plasmids. The approximate molecular weight of shigella plasmids were ranged from 1. 2-5.2 Mega Dalton (MDa). The data revealed that there is no consistent relationship between the plasmid profiles and the resistance patterns of the isolates. Moreover, one of the plasmid profile was shared by 2 of the shigella isolates (isolate no. 80 & 256) although they have different susceptibility patterns. However, in some instances, isolates sharing some resistance markers, had one or more plasmid in common.

INTRODUCTION

Shigellae are commonly isolated from intestinal tract of human[1]. They are classical pathogens assumed to be the etiologic agents of the disease when they are encountered in the clinical specimens[2].

Emergence of multiresistant *Shigella* species is a serious problem in treatment failure of shigellosis in developing countries. Several useful antimicrobial agents including tetracycline, chloramphenicol, and ampicillin are no longer effective against *Shigella* species in many countries[3-5].

Resistance to antimicrobial agents may be mediated by genes that are encoded on the host cell chromosome, plasmids or transposons, which are capable of integrating into the plasmid and/ or into the chromosomes[6,7]. Multiple drug resistance among shigella are probably plasmid mediated[8,9]. Evidently, R plasmids have evolved by the acquisition and accumulation of new genes that are selected for by the use of antibiotics in human and veterinary medicine[10].

Plasmid profile analysis has been used as an epidemiologic tool in investigating outbreaks of enteric diseases. When used as a fingerprint for a strain, the plasmid profile may aid in differentiation of strains, identifying a source of infection, or evaluating the efficacy of control measures[11].

This study has been conducted to examine the plasmid profile of the shigella isolates and try to make a correlation; if any; between the plasmid profiles and the resistance patterns of the isolates.

EXPERIMENTAL

Materials:

All antibiotic discs were the products of Oxoid, England. Bromophenol blue, ethylene diamine tetra-acetic acid (EDTA), Tris-base, agarose (ultra pure, DNA- grade) and lysozyme were purchased from Sigma Co, USA. Chloroform and glacial acetic acid were the products of Prolabo, France. Ethidium

bromide, and iso- amyl alcohol were purchased from Merck, Germany. Shigella antisera were purchased from Difco Lab., Detroit, Michigan, USA.

Methods:

Clinical Cases:

Stool samples from 327 subjects suffering from acute diarrhea were collected from patient attending the out- patient clinics at the Hospitals of El- Minia Locality. Furthermore, fifty samples matched the healthy control subjects were collected. Stool specimens were obtained in sterile wide- opened containers tightly covered and processed within 2 hours.

Isolation and identification:

Isolation of shigella may be achieved by using deoxycholate citrate agar (DCA), salmonella-shigella agar (S-S agar) and MacConkey's agar[12]. Colonies that do not ferment lactose were picked up and subcultured on nutrient agar slants for further biochemical and serological identification according to Rowe and Gross [13] & Collins and Lyne[14].

Susceptibility tests:

Susceptibility tests to different antimicrobial agents have been carried out according to Kirby-Bauer single disc diffusion method[15]. The following antimicrobial discs were tested: sulfamethiazole (TH, 25 µg), sulfisoxazole-trimethoprim (SXT, 25 µg), oxytetracycline (OT, 30 µg), ampicillin (PN, 10 µg), piperacillin (PRL, 100 µg), chloramphenicol (C, 30 µg), nalidixic acid (NA, 30 µg), norfloxacin (NOR, 30 µg), ofloxacin (OFX, 10 µg), amikacin (AN, 30 µg), gentamicin (GM, 10 µg), kanamycin (K, 30 µg), neomycin (N, 30 µg), streptomycin (S, 10 µg), cephalexin (CL, 30 µg), cephradine (CE, 30 µg), cephalothin (KF, 30 µg), and Cefotaxim (CTX, 30 µg). The diameters of inhibition zones were recorded and interpreted according to Acar and Goldstein [16] and Bauer *et al.* [15].

Plasmid isolation on agarose gel electrophoresis:

The standard procedure for isolation and characterization of plasmid DNA depends upon lysing host bacterial cells; for example with lysozyme; and subsequently treating the lysate so that the smaller circular plasmid DNA molecules are separated from the relatively huge mass of chromosomal DNA[17]. Agarose gel electrophoresis technique was employed for the detection and preliminary characterization of plasmid DNA present in the clinical isolates. The DNA plasmids carried on *E.coli* V517 were used as molecular weight markers[18]. The approximate molecular weights of the unknown plasmids were determined in comparison to the known molecular weight markers.

RESULTS

Identification of shigella isolates:

The clinical isolates were subjected to three stages of identification[13]. First, staining reactions and culture characteristics of the isolates on simple, enriched, and selective media were determined. Shigellae are Gram-negative ,non-motile, lactose non- fermenting bacilli. In the second stage, twelve of the isolates were identified biochemically as shigella. Finally, the biochemically identified shigella isolates were confirmed serologically. As table (1) shows, seven isolates were serotyped as *Shigella flexneri* (58.3%), four were typed as *Shigella boydii* (33.3%) and one isolate was identified as *Shigella dysenteriae* (8.3%). Non of the isolates was typed as *Shigella sonnei*. In addition, shigella was not detected in the specimens of the fifty healthy control subjects.

Table (1): Serotyping patterns of Shiglla isolates:

Serotype	Positive isolates
S. flexneri	16, 27, 28, 44, 97, 102, 130
S. boydii	71, 80, 155, 256
S. dysenteriae	104

Susceptibility tests:

All the shigella isolates (100%) were susceptible to eleven antimicrobial agents namely, nalidixic acid (NA), ofloxacin (OFX), norfloxacin (NOR), amikacin (AN), gentamicin (GM), kanamycin (K), neomycin (N), piperacillin (PRL), cephalothin (KF), cephradine (CE) and cefotaxim (CTX).

The resistance markers of shigella isolates; summarized in table (2); showed that, each isolate

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Table (2): Resistance patterns and plasmid profiles of shigella isolates.

Isolate number	Resistance Patterns	Plasmid profiles (≈ Mol. Wt.)
104	OT, S, PN	4.3, 3, 2.7, 1.7, 1.5, 1.2
17	TH, SXT, OT, C, PN	4.3, 3.6, 2.8, 2.7, 1.5
27	TH, SXT, OT, C,S, PN	5.2, 4.3, 3.6, 3.5, 2.8, 2.7, 1.5
28	TH, SXT, OT, C,S, PN	3.6, 3, 2.7, 2.3
44	TH, SXT, OT, S, PN	4.3, 3.7, 2.7, 2.3, 1.2
97	TH SXT, OT, C,S, PN	4.3, 3.6, 3.55, 3.4, 2.7, 2.3, 1.5, 1.3
102	TH, SXT, OT, C,S, PN	3.7, 2.7, 2.3, 1.2
130	OT, S, PN	4.3, 3.55, 2.7, 2.3
71	TH, S	4.3, 3.6, 3.4, 2.7, 2.3, 1.5, 1.3
80	PN	4.3, 3.4, 1.5
155	TH, SXT, S, CL	5.2, 4.3, 3.5, 2.7, 1.5, 1.3
256	CL	4.3, 3.4, 1.5

was resistant to at least one antimicrobial agent. Seven of the isolates (58.3%) were carrying resistance determinants for 4 or more antimicrobial agents. Among them, 4 isolates were resistant to 6 antimicrobial agents. The resistance patterns of shigella isolates were heterogenous, since as many as 8 different patterns were recognized among the tested isolates (table 2).

Plasmid screening:

The results of screening were summarized in table (2) and figure (1). All the isolates harbored at least three different plasmids. In addition, 41.7% of the isolates were harboring at least 6 plasmids. A single isolate contained as many as 8 plasmids.

The approximate sizes of plasmids of shigella ranged from 1.2 to 5.2 MDa. The photo of agarose gel electrophoresis (figure 1) shows eleven different profiles indicating that, the isolates were very heterogenous epidemiologically.

DISCUSSION

In this investigation, the prevalence of *Shigella* genus in El-Minia Locality was studied. The absolute diagnosis of shigellosis is achieved by isolation of the organism from the stool[19]. In this study, twelve (3.67%) out of 327 diarrheal cases were harboring shigella. The patients subjected to this study do not probably represent the full spectrum of shigellosis in El-Minia Locality, but rather a subset of cases with severe manifestation.

The isolates were subjected to identification by the traditional methods of identification including biochemical reactions[13, 14].

The biochemically identified isolates were substantiated serologically. *Shigella flexneri* was the most common (58. 3% of the total shigella isolates), followed by *Shigella boydii* (33.3%), and *Shigella dysenteriae* (8.3%) No *Shigella sonnei* was detected. Different *shigella* species have been reported in different countries. Generally, as the level of environmental and personal hygiene rises, for example in USA and UK, *Shigella sonnei* was the most predominant *shigella* species[8,20,21]. On the other hand, isolation of *Shigella flexneri* in developing countries was more frequent than the isolation of *Shigella sonnei* [1,5,9,22 - 27].

Antimicrobial therapy decreases the duration of clinical symptoms and excretion of the pathogen in the stool[28], and is recommended in young children, and elderly with severe infections[9]. In this study, the highest antimicrobial activities have been shown by gentamicin (GM), amikacin (AN), kanamycin (K), neomycin (N), the 4-quinolones namely: ofloxacin (OFX), norfloxacin (NOR), and nalidixic acid (NA), as well as the cephalosporins. These data are highly consistent with that reported in other investigation. Where the shigella isolates were susceptible to AN and GM[29], and to the 4-quinolones including NA and OFX[23, 30] as well as to the cephalosporins[31].

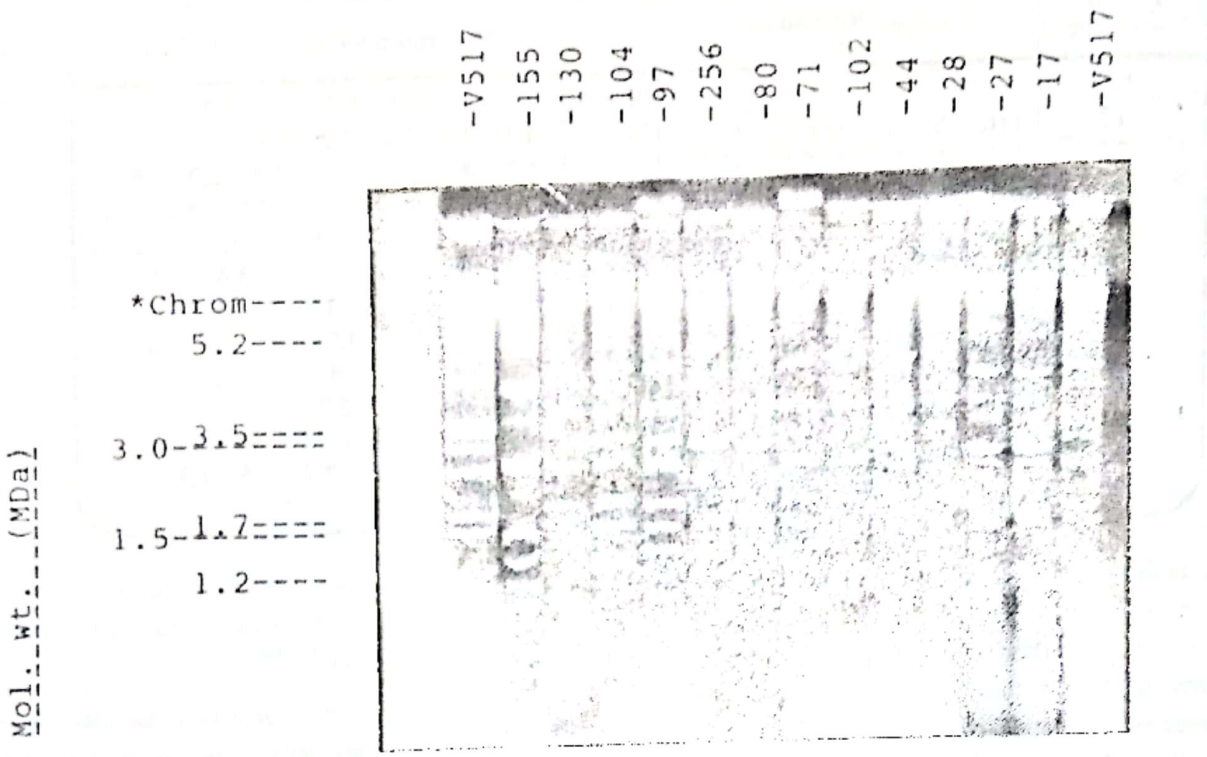

Fig (1): Agarose gel electrophoresis of Shigella isolates.

On the other hand, all the isolates were resistant to at least one antimicrobial drug. Moreover, about 83% of the isolates were resistant to 2 or more antimicrobial agents (table 2). The isolation of multiresistant isolates in this study is consistent with that reported in other developing countries such as Hong Kong[23] where 96% of shigella isolates were resistant to 2 or more antimicrobial agents. In Korea[1], 97% of shigella isolates were resistant to 3 or more drugs. In India, high prevalence of multi-drug resistant shigella was reported[32]. The emergence of multiresistant shigella may be attributed in part to the wide indiscriminate use of antimicrobial agents[9].

As many as 8 different resistance patterns were described for the 12 shigella isolates which indicates heterogenicity of the isolates (table 2). The conjugal transfer of resistance determinants from the microbial intestinal flora, which differ from one person to another, to shigella[33] as well as, the presence of resistance determinants for antimicrobial agents on transposons[10,34] might explain the heterogenicity of the patterns detected in the present study.

Several antimicrobial agents including chloramphenicol (C), ampicillin (PN), streptomycin (S) and cotrimoxazole (SXT) are no longer effective against *Shigella* species[4,5,23,24,35]. In the present study, 41.7% to 75% of the isolates were resistant to sulfamethiazole (TH), cotrimoxazole (SXT), oxytetracycline (OT), chloramphenicol (C), streptomycin (S), and ampiciallin (PN). These findings might limit the use of these drugs in the therapy of shigellosis in EL- Minia Locality. So, the need for a public awareness against the misuse of antimicrobial agents is important. In addition, the treatment of shigellosis in El- Minia Locality should be guided by the sensitivity pattern of the causative agent as has been recommended by the WHO[9].

Plasmid profile analysis provided further

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discrimination over that produced by serotyping and biochemical identification. It is reliable in differentiating the outbreak isolates from epidemiologically unrelated controls[36]. All the shigella isolates were harboring at least 3 different plasmids. 41.7% of the isolates were containing 6 plasmids or more. Moreover, isolate number 97 harbored as many as 8 plasmids. The approximate mol. wt. of shigella plasmids were ranged from 1.2 - 5.2 MDa. The harboring of a heterogenous population of plasmids by the shigella isolates ranged in number from 2 to as many as 10 was reported, and the size of most of these plasmids was< 6 MDa [37,38]. The plasmid profiles were very heterogenous, where eleven different profiles were recognized for the 12 shigella isolates (table 2 and figure 1). The heterogenicity of the plasmid profiles of shigella isolates might be explained in different ways:

a) the resistance determinants for an antimicrobial agent could be carried on various genetic elements such as R plasmids, transposons and/or chromosomes.

b) The resistance determinants for the same antimicrobial agent may be carried by more than one plasmid [39], or the plasmid may code for any other biological function such as toxin production, virulence, .. etc.

c) The misuse of antimicrobial agents in developing countries including Egypt, may select for intestinal resistant strains of *E.coli*; harboring various R plasmids which are different from one person to another[10]; and may serve as a source of the inter- or intra- generic transformation of resistance codes *in vivo* [33].

The results of resistance patterns and plasmid profiles, summarized in table (2) and figure (1), revealed that, there is no consistent relationship between the plasmid profiles and the resistance patterns of the isolates. However, plasmid profiles distinguished more strains than did the antimicrobial susceptibility patterns[40], where 11 different plasmid profiles were recognized. Meanwhile, only 8 different resistance patterns were described for the 12 shigella isolates. Moreover, one of the plasmid profiles was shared by two of the shigella isolates (number 80 and 256) although they have two different susceptibility patterns. This could be interpreted on the basis that, plasmid may code for any biological function such as toxin production, virulence,.. etc., other than resistance determinants. Similarly, shigella isolates with the same resistance patterns showing different plasmid profiles and *vice versa*, had been reported [41,42]. However, in most cases, isolates with the same resistance pattern or sharing resistance to certain antimicrobial agents shared at least one plasmid (table 2). Noticeably, plasmids of Mol. Wt. ≈4.3 MDa and 2.7 MDa were in common among ten of the isolates. In addition, 1.5 MDa plasmid was found in six isolates excluding isolates no. 80 and 256 (which have different resistance patterns), 2.3 MDa plasmid was also found in six isolates, and 3.6 MDa plasmid was found in 5 isolates. Evidently, as resistance patterns show, the isolates harboring these plasmids were sharing some resistance markers. Further genetic studies including conjugation, restriction, transformation, curing, and hybridization are required for finding a sound correlation of the antimicrobial resistance with plasmid profiles.

Generally, the heterogenicity of shigella isolates, collected from El-Minia Locality indicates that many clones are associated with shigellosis in this area.

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مسح توضيحى للبلازميدات النووية لعزلات الشيجيلا متعددة المقاومة

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فى هذا البحث تمت محاولة عزل ميكروبات الشيجيلا من ٣٢٧ مريضا بالاسهال الحاد من مرضى العيادات الخارجية بالمستشفى الجامعى ومستشفى الأمراض المتوطنة بالمنيا. وكذلك أخذت عينات من ٥٠ فردا لا تظهر عليهم أعراض مرضية فى غضون الشهر الأخير على الأقل.

تم استنبات عينات البراز لجميع الحالات على المنابت البكتيرية المختلفة بهدف فصل عينات الشيجيلا وتم التعرف عليها من خلال الفحص المجهرى والتفاعلات الكيميائية الحيوية وتم تصنيفهم طبقا للأمصال المختلفة الخاصة بكل ميكروب على حده. وقد تم عزل ١٢ عزلة من ميكروب الشيجيلا بنسبة ٣,٦٧٪ ولم يتم عزل ميكروب الشيجيلا من الأشخاص الأصحاء.

اتضح من النتائج أن ميكروب الشيجيلا فليكسنيرى هو الأكثر شيوعا بين عزلات الشيجيلا. تم عمل اختبار حساسية للميكروبات المعزولة للمضادات البكتيرية المختلفة (١٨ نوعا) بطريقة انتشار الأقراص (Disc diffusion) وقد أظهرت النتائج أن جميع عزلات الشيجيلات مقاومتها شديدة لواحد على الأقل من المضادات البكتيرية المختبرة وقد كان على سبيل المثال ٥٨,٣٪ من عزلات الشيجيلا مقاوما لأربعة مضادات بكتيرية على الأقل وكذلك وجد ٨ أنماط للمقاومة (Resistance patterns) بين الأثنى عشر عزلة.

وأظهرت الدراسة حساسية جميع عزلات الشيجيلا لكثير من المضادات البكتيرية مثل حامض النديديكسك والنورفلوكساسين والأوفلوكساسين والكلورامفنيكول والجنتاميسين والنيوميسين والبيراسيلين والسيفوتاكسيم والكاناميسين والسيفالوسين والسيفاردين.

كما تم فصل البلازميدات النووية من عزلات الشيجيلا عن طريق الفصل الأيونى للجيل الأجاروزى (Agarose gel electrophoresis).

فقد وجد احدى عشر نظاما لتوزيع البلازيمدات فى الاثنى عشر عزلة. وجميع العزلات تحتوى على ثلاثة بلازميدات مختلفة على الأقل كما أظهرت الدراسة أنه لا يوجد توافق بين أنماط المقاومة للمضادات البكتيرية المختلفة ومجاميع توزيع البلازيمدات مومما هو جدير بالذكر أن بعض العزلات التى أظهرت نفس نمط المقاومة أو اشتركت فى مقاومة بعض المضادات البكتيرية كانت مشتركة فى أحد البلازميدات النووية على الأقل.