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Bacteriological and Molecular Study on *Shigella* Isolates From Human, Food and Animals

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

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سورة الشعراء

" اهـــــــداء "

الى من علمني ان الدنيا كفاح وسلاحها العلم والمعرفة الذي احمل اسمه بكل افتخار

الذي سعى وشقى لأنعم بالراحة والهناء الذي لم يبخل بأي شيء من اجل دفعي لطريق النجاح

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الى قدوتي الاولى التي رائتني قلبها قبل عينها وحضنتني احشائها قبل يديها

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ودعائها سر نجاحي سهرت الليالي تنير دربي الى شجرتي التي لا تذبل الى الظل الذي اوي اليه

في كل حين . " امي الحنونة "

الى رفيقة دربي منذ صغري في مشوار دراستي .... اختي الغالية

يا من ملء حياتي بهجة وسرور وسندي في الحياة .... اخي العطوف

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كل من شجعني وساندني بدعمهم وتلقيهم النصائح وغنيا لهم لي

بالخير بصدق واخلاص ....بدون استثناء

شكرا لوجودكم في حياتي

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Abstract

Shigella species were classified within the family *Entero-bacteriaceae* as it is facultatively anaerobic non-motile Gram's negative bacilli. It included four species *S.flexneri ,S.sonnei ,S.dysenterai and S.boydii.*

The aims of the current study are to explore the rate of drug resistance among the isolates as multi or extensively drug when exposed to different types of Antibiotics. *Shigella* species was isolated from human and animal source by standard bacteriological methods and affirmed by Polymerase Chain Reaction detection. This study also aimed to figures out the impact of certain demographic and strain diversity on the rate of *Shigella* antibiotic resistant genes detected through PCR technique.

A total of (360) including (175) stool human samples were collected from various age groups of diarrheal patients who attended different hospitals in Baghdad City, plus (85) food samples and (100) animal stool samples during a period from October (2019) to October (2020). Conventional bacteriological methods are used for cultivation and identification of *Shigella*, included many differential and selective media like Xylose Lysine Deoxycholate agar, Hektoen Enteric agar, *Salmonella Shigella* agar, MacConkey agar and *Shigella* broth. Further verification was achieved by biochemical tests through (Api20 E) system and (Vitek 2) system, and eventually confirmed by PCR assay. Statistical Product and Service Solutions (SPSS) (version 25) was used for statistical analysis of the results obtained, significant differences were followed when (P) value is equal or less than (0.05) ($P \leq 0.05$).

The results showed that the *Shigella* detection rate from human samples was (12) (6.9%), from beef meat was (2) (6.7%) and from sheep meat was (1) (5.3%). No isolates were recovered from animal samples. *Shigella genus* was identified by the use of specific primers to (*invC*) gene in PCR assay. This gene was detected in all isolates from human and food samples which gives positive results. Furthermore, specific genes were also detected by the use of same technique and specific primers are used for *rfc*, *wbgZ*, *rfpB* and conserved hypothetical protein gene of *Shigella flexneri Shigella sonnei , Shigella dysenteriae, and Shigella boydii* respectively.

The obtained results revealed from human samples were (4.0%, 1.7%, 0.6%, and 0.6%) respectively for *Shigella flexneri, sonnei, dysenteriae* and *boydii*. In regards to food samples , *Shigella flexneri* & *Shigella sonnei* were both identify (3.3%) in beef meat while *Shigella flexneri* was detected (5.3 %) in sheep meat.

High infection rate was found in male (98) (56.0%) versus (77) (44.0%) among females. The high isolation rate was (12.5 %) recovered from <10 years age group, while age groups of (30 to 60) years showed the lowest rate. High rate was reported in rural area (8.9 %), children (12.8 %) , Governmental employee (8.2 %) and Housewife (5.1 %) and high percentage was reported (60.0%) of type of diarrhea was bloody mucoid.

The antibiotic susceptibility test was done by Kirby-Bauer method and Vitek 2 system against 11 different antibacterial agents. The *Shigella* isolates from human showed high resistance to several antibiotics ; (11) (91.7%), equal rate (10) (83.3%) and (9) (75%) of isolates were resistance to ampicillin, tetracycline, Cefotaxime, and trimethoprim-sulfamethoxazole respectively.

While the high sensitive to several antibiotics; (12) (100%),(11) (91.7%), (10)(83.3%) of isolates were sensitive to imipenem, ciprofloxacin and pipracillin-tazobatam, respectively.

The rate of multi-Drug Resistance (MDR) showed that (11) of (91.6 %) isolates from human samples were resistance to three or more antimicrobial categories, while (1) isolate (8.3 %) was found to be extended in its resistance (EDR). Whereas, the results of food specimens high resistance to several antibiotics; (2) (100%) of *Shigella* recovered from beef meat specimens were resistant to Ampicillin, Tetracycline,while all (2) (100%) were sensitive to Ciprofloxacin, Imipenem, Pipracillin–Tazobatam, and Chloramphenicol . Regarding the (1) isolate from sheep meat, it was resistant to Ampicillin, Tetracycline, Trimethoprim-sulfamethoxazole, Cefotaxime and Ceftriaxone.

While it is sensitive to Ciprofloxacin, Imipenem, Pipracillin–Tazobatam, Chloramphenicol, Ceftazidime and Nalidxic acid.(2 / 3)(66%) of isolates showed MDR resistant to three or more antimicrobial categories while (1/3) (33.3%) of isolates were not MDR but showed resistant to (≤ 2) antimicrobial categories.

The Extended Spectrum β-Lactmases isolates from human samples were distributed as *bla-tem* (10 / 12) (83.3%) , *tet A* 9 /12 (75.0%) , *ctx-m* 8 /12 (66.7%) , *cat* gene 5 / 12 (41.7%), while from food samples including beef meat and sheep meat were found all positive isolated detected to all antibiotics resistance gene except *cat* gene was not detected. Virulence gene were found *ipaH* positive results in all isolated from human and food samples, *ial* gene 11/1 (91.7 %) , 3/3 (100), human and food samples respectively , while *setl A* and *setl B* 2 /12 (16.7 %) detected from human samples only but not detected from food samples .

The present results indicated that the amplified *tetB* fragment exhibited one nucleic acid variation, G184A, with a missense Ala144Thr effect on the *tetB*-encoded tetracycline efflux MFS transporter protein. Concerning the bla_{-TEM} amplicon, the results showed that the amplified bla_{-TEM} locus exhibited two nucleic acid variations (T269G and A452G) with two respective missense effects (Asn134Thr and Leu73Pro) on the bla_{-TEM} encoded class A broad-spectrum beta-lactamase TEM-1. As in the case of the bla_{-TEM} amplicon, the *set1B* amplicons were also exhibited two nucleic acid variations (G106A and C128G), and both variations showed two respective missense effects (Pro743Leu and Gly736Arg) on the *set1B* encoded serine protease autotransporter toxin Pic. Concerning *Ial* amplicons, only one nucleic acid variation (T179C) was identified, which showed a silent (Leu24 =) consequence on the Ial-encoded protein. Meanwhile, *ipah* amplicons did not exert any detectable variation as all its sequences exhibited complete homology with the reference genomic DNA sequences.

Based on the observed variations in the investigated bacterial amplicons, all generated comprehensive phylogenetic trees indicated that the investigated sequences were positioned in the *Shigella flexneri* sequences. The utilized *Ial*-based trees indicated a higher degree of clear and non-overlapping positioning and discrimination among the currently investigated samples over the other utilized amplicons. In contrast to the *Ial*-based tree, bla_{-TEM} based tree showed the least accurate phylogenetic positioning than the other utilized trees. In all cases, all the observed nucleic acid variations were only minor deviations within the same identified species.

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List of Abbreviations

Full name	Abbreviations
%	Percentage
&	And
±	Plus or Minus
µ	Micro
µg	Micro gram
µl	Microliter
g / ml	gram / milliliter
A	Acid
Amp	Ampicillin
AmpC	Ampler type C
API20 E	Analytical Profile Index 20 *Enterobacteriacea*
ASCO	Advanced Scientific Bureau
AST	Antibiotics Sensitive Test
BHI	Brain Heart Infusion
Bp	Base Pair
BSC	Bio Safety Cabinet
C	Choramphenicol
CAZ	Ceftazidime
CDC	Center for Disease Control

CIP	Ciprofloxacin
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CRO	Ceftriaxone
CTX	Cefotaxime
CTX-M	Cefotaxime Beta-Lactamase hydrolyze Cefotaxime
D.W	Distilled Water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxy Nucleoside Tri-Phosphate
EDR	Extensively Drug Resistant
ESBL	Extended Spectrum β-Lactamase
F	Forward Primer
HEK	Hektone Enteric Agar
HIV	Human Immunodeficiency Viruses
hrs	Hours
IL	Interleukin
IMP	Imipenem
IND	Indole
Ipa	Invasion Plasmid Antigen
ISO	International Organization for Standardization
JAMES	Advanced Kovacs Reagent
K	Alkaline
kb	Kilo Base
M Cell	microfold Cell
MAC	MacConkey Agar
MDR	Multi Drug Resistant
min	Minute
ml	Milliliter
mmol/L	mmol/Liter

MSM	Men Who Have Sex With Men
NA	Nalidixic Acid
NCBI	National center for biotechnology Information
ºC	Degree Celsius
OXA	Oxacillinase b-Lactamase Active on Oxacillin
PCR	Polymerase Chain Reaction
PMN	Polymorphnuclear
PTZ	Pipracillin- Tazobactam
R	Reverse Primer
RBCs	Red blood cells
rRNA	Ribosomal Ribonucleic Acid
S & Sh	*Shigella*
Sec	Second
Setl A & B	*Shigella* Enterotoxin 1
SD	Standard Deviation
SF	Selenite F broth
SHV	β-lactamase Sulfahydral Variant
SOP	Standard Operating Procedure
Spp.	Species
S-S-agar	*Salmonella Shigella* Agar
STX	Shiga toxin
SXT	Trimethoprim- Sulfamethoxazole
T3SS	Type III Secretion System
Taq	*Thermus Aquaticus*
TDA	Tryptophan Deaminase
TE	Tetracyclin
TEM	Beta-Lactamase named after First Patient Isolated from Temarian named Temoneira
TSI	Triple Sugar Iron

USA	United States of America
UV	Ultra-Violet
VP	Voges-Proskauer
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate Agar
TE buffer	Tris –Acetate –EDTA Buffer

Chapter One Introduction

1.1. Background

Diarrheal diseases was ranked Worldwide as the third disability-adjusted life - years among children younger than 10 years in 2019 (GBD, 2020). Annually, there are about (1.8) million death attributed to diarrhea due to different pathogens. Bacteria, viruses and protozoal pathogens, were the main causes of gastroenteritis in developing countries, was associated to increases in morbidity and mortality and considered as public health of first priority (Shahin *et al*., 2019; Ugboko *et al*., 2020).

Shigella species was reported among the eight enteric pathogens reported by CDC as it causes of the majority of bacillary dysentery in developing countries (Tack *et al*., 2020).

Shigella species were classified within the family *Enterobacteriaceae* as it is facultatively anaerobic non-motile Gram's negative bacilli (Kotloff *et al*., 2018). It included four species *S.boydii, S.dysenterai, S.flexneri and S.sonnei*. Furthermore, different serotypes were detected in each species, the identification of these serotypes depend mainly on differences in somatic antigen of LPS, and in the severity of the disease they caused watery diarrhea, fever, and cramps of abdomen were the main characteristics of Shigellosis (Anderson *et al*., 2016).

The manifestation of *Shigella* infections ranged from watery diarrhea to bloody stool with fever, prostration and abdominal crump, extra intestinal or intestinal complications due to the infection were also reported (Miron *et al*., 2000; Vubil *et al.,* 2018).

Many virulence factors were associated with *Shigella* infections, and these factors were carried or encoded pathogenicity islands on bacterial chromosome and the plasmid of virulence. They initiated the infection and reduced the acquired immune response making the host with possibility of reinfection (Mattock and Blocker, 2017).

The only natural hosts for *Shigella* are humans, as a number (180) of *S.sonnei* or *S.flexneri* units or as low as ten colony forming units of *S. dysenteriae* can produce infection with clear clinical symptoms (Niyogi, 2005).

Standard conventional culture methods and biochemical tests for isolation and identification of *Shigella* species are time consuming and sometimes not accurate when very few microbes are present and masked by other dominant normal flora. Accordingly most sensitive and accurate molecular biological techniques are used like polymerase chain reaction (PCR) and multiplex PCR to detect and identify *Shigella* species (Ojha *et al*., 2013 ; Ranjbar *et al*., 2014).

Antibiotics were used long time ago for treatment of shigellosis these antibiotics included ciprofloxacin, nalidixic acid, ampicillin, and trimethoprim sulfamethoxazole as these agents assist in killing of bacteria and helps in recovery from illness (Sati *et al.*, 2019).

Shigella species are resistant to some of these antibiotics, making the treatment is unable to terminate the infection particularly during outbreaks or in severe cases (Paula *et al*., 2010; Bhattacharya *et al*., 2012).

During the past decade, many *Shigella* species were resistant to wide range of antimicrobials were emerged and the rate of resistant has increased notably (Kosek *et al*., 2010; Qiu *et al*., 2012; Kahsay and Muthupandian, 2016).

Shigella flexneri and *Shigella dysenteriae* showed flouroquinolones resistance that increases yearly and was attributed to mutation in topoisomerase IV and gyrase DNA sequences, in addition to resistance to quinolone that mediated by plasmid genes reported in isolates from India, USA, China, and Japan (Taneja *et al*., 2016; Muthuirulandi Sethuvel *et al*., 2017).

Furthermore, resistance to cephalosporin mediated by AmpC and ESBL in species of *Shigella* were reported by Taneja et al., (2012). *Shigella flexneri* isolates from clinical human cases and tested for different antibiotics showed MDR that was attributed to *acrA* gene overexpression and efflux pump role (Yang et al., 2008).

Additionally, some other *Shigella* species that were isolated from stool samples of patients with dysentery showed MDR that was attributed to mutation in *tolC* and *acrA* genes DNA sequences (Mehata *et al*., 2010).

Accordingly it has been suggested to reduce the burden of *Shigella* disease and restrict mobility with antimicrobial resistance required development of alternative modes of treatment and vaccines that may reduce the threat of shigellosis (Baker and The, 2018).

1.2. Aims of The Study

1- Molecular diagnosis of *Shigella* species using PCR technique from human and animal sources.

2- Exploring the antibiotic resistant patterns of *Shigella* species against (11) antibiotics or antibacterials and to detect the rate of resistance to different drugs to point out the (MDR) and (EDR) of these isolates.

3- Molecular detection and sequencing of certain antibiotic resistance gene and virulence gene by using conventional PCR technique.

4- Figure out the impact of certain demographic and strain diversity on the rate of antibiotic resistance.