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Molecular Identification and Antibiotic Susceptibility of *Shigella* Species Isolated from Diarrheal Patients

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بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

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Dedication

To…

The Kindness icons my **parents**

The sincere my **brothers** and my **sisters**

My soul mate my husband **Ali**

With lots of love

Shaima

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Summary

Shigella species are among the common bacterial causes of diarrheal diseases. *Shigella* species caused Shigellosis with low infectious dose, which is an intense enteric infection that is described by a liquid and mucoid diarrhea, otherwise called bacillary dysentery.

The aim of current study was to investigate the isolation rate and antibiotics resistance profiles of *Shigella* species isolated from acute diarrheal patients , determine the occurrence of Beta-lactamases genes and determine the molecular correlation of these isolates.

A total of 282 stool samples were collected from various age groups of diarrheal patients who visited and admitted to Al-Hussain Children Hospital , Maternity and Children Teaching Hospital and Al-Diwaniyah Teaching Hospital during a period from November 2017 to May 2018.

Four selective and differential media were used for identification of *Shigella* species to increase the chance of isolation, which are Xylose Lysine Deoxycholate agar, MacConkey agar, Hektoen Enteric agar and Salmonella Shigella agar. The suspected colonies identified biochemically by Api20 E system, 23 isolates gave positive results. Further confirmatory test was done by Vitek2 system, 21 isolates gave positive results.

Polymerase chain reaction was done to identify *Shigella* genus by using *invC* gene as a specific primer, 19 isolates were positive. Specific target genes were used to differentiate *Shigella* species, whereas *rfc* gene, *wbgZ* gene, *rfpB* gene and conserved hypothetical protein gene for *Shigella flexneri*, *Shigella sonnei*, *Shigella dysenteriae* and *Shigella boydii*, respectively. The obtained results revealed that *Shigella* isolation rate was (6.73%, 19/282). The positive cases involved 6 (31.5%)

males and 13 (68.4%) females. The higher isolation rate (78.9%, 15/19) was appeared in ≤ 10 years age group, while ages of 11 to 20 years showed low percentage (15.7%, 3/19) and ages of 41 to 50 years showed (5.2%, 1/19). The most common isolated species was *Shigella flexneri* (52.63%, 10/19) followed by *Shigella sonnei* (42.10%, 8/19) and *Shigella boydii* (5.26%, 1/19).

The antibiotic susceptibility test was done by Kirby-Bauer method and Vitek2 system against 20 different antibacterial agents. The *Shigella* isolates showed high resistance rates to cefotaxime (94.7%), ampicillin (89.4%), tetracycline (84.2%) and trimethoprim/sulfamethoxazole(78%). Moderate resistance rates showed to ticarcillin (68.4%), ceftriaxone (57.8%) and pipracillin (57.8%). The isolates were fully sensitive (100%) to imipenem, meropenem and colistin and highly sensitive to ciprofloxacin (94.7%), cefoxitin (94.7%), pipracillin-tazobactam (89.4%), cefepime (84.2%) and pefloxacin (84.4%). Moderate sensitivity was showed to ceftazidime and minocycline (78.94%), levofloxacin (63.15%) and aztreonam (57.89%) while nalidixic acid had equal percentage of resistance and sensitivity. The majority of *Shigella* isolates were multidrug resistant (94.7% , 18/19) because demonstrated resistance to different antibiotic classes.

Additionally, the *Shigella* isolates investigated molecularly for occurrence of β-lactamases genes including ($bla_{CTX\text{-}M}$, bla_{TEM} , bla_{SHV} *and* bla_{AmpC}). The distribution of β-lactamases genes was as follow: *S. flexneri* isolates harbored $bla_{CTX\text{-}M}$ (30%, 3/10), bla_{AmpC} (60%, 6/10) and bla_{TEM} (20%, 2/10), *S. sonnei* isolates harbored $bla_{CTX\text{-}M}$ (75%, 6/8), bla_{AmpC} (62.5%, 5/8) and *S. boydii* isolate harbored only bla_{TEM} (100%, 1/1), while bla_{SHV} was not detected in this study.

Finally, all *Shigella* isolates were genotypable by enterobacterial repetitive intergenic consensus_ polymerase chain reaction (ERIC-PCR) . The number of

enterobacterial repetitive intergenic consensus bands generated ranged from 1 to 6 with different molecular sizes. ERIC-PCR analysis of the isolates resulted in eleven different patterns (E1-E11) with 1 to 8 DNA bands resulting in considerable diversity of genetic types of 19 *Shigella* spp. isolates.

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List of Abbreviations

abbreviations	Key
%	Percentage
±	Plus or Minus
µl	Microliter
^{0}C	Degree Celsius
Amp	Ampicillin
AmpC	Ampler type C
API20 E	Analytical Profile Index 20 Enterobacteriacea
AUG	Augmentin
Bp	Base Pair
CAZ	Ceftazidime
CDC	Center for Disease Control
CIP	Ciprofloxacin
Cpe	Cefepime
CRO	Ceftriaxone
CS	Colistin
CTX	Cefotaxime
CTX-M	Cefotaximase, Beta-Lactamase hydrolyze Cefotaxime
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphate
DW	Distilled Water
ERIC	Enterobacterial repetitive intergenic consesus
ESBL	Extended spectrum Beta-Lactamase
FOX	Cefoxitin
gms	Gram
IL	Interleukin
IND	Indol
Ipa	Invasion Plasmid antigen
Ipm	Imipenem
JAMES	Advanced Kovacs Reagent
LEV	Levofloxacin
MDR	Multi-drug resistance
MEM	Meropenem
Min	Minocycline

min	Minute
ml	Milliliter
MOH	Ministry of Health
mRNA	Messenger ribonucleic acid
NA	Nalidixic acid
NCBI	National center for biotechnology information
NSAIDs	Non Steriodal Anti-inflammatory drugs
ONPG	O-nitrophenyl-beta-D-galactopyranoside
PBP	Pencillin Binding Protin
PCR	Polymerase Chain Reaction
Pef	Pefloxacin
Pi	Pipracillin
PTZ	Pipracillin- Tazobactam
rRNA	Ribosomal Ribonucleic Acid
S	*Shigella*
sec	Second
SHV	Sulfahydral variant
spp	Species
SS	Salmonella Shigella
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
Ti	Ticarcillin
UNICEF	United Nations Children,n Emergency Fund
UPGMA	Un-weighted Pair Group Method with Arithmetic Mean
VP	Voges-Proskauer
WHO	World Health Organization
x g	Times Gravity
XLD	Xylose Lysine Deoxycholate

Chapter one

Introduction
and
Literatures Review

1. Introduction and literatures review

1.1. Introduction

Diarrhea stays a vital public health problem and it is the second principle reason for mortality and morbidity in children globally (Bryce *et al*., 2005). The global mean mortality from diarrhea at 760 000 deaths yearly (WHO, 2013). unluckily, diarrhea is more dominant in the developing world (Bryce *et al*., 2005). About two million children under 5 years of age die yearly as result of diarrhea in developing world (Kosek *et al*., 2003).This high prevalence in the developing world may be an outcome of the lack of safe drinking water, insufficient sanitation, deficient hygiene and malnutrition, which markedly rises the danger of contracting diarrhea (Bonkoungou *et al*., 2013). Amelioration in sanitation, nutrition, education and early treatment with oral rehydrating salts (ORS) are considered to have contributed to the contraction in mortality numbers from an approximated 1.5-2.5 million deaths in 2010 to the current approximated 760 000 child deaths (Durley *et al*., 2004, Black *et al*., 2010).

Shigella genus are strict human pathogens that target the gastrointestinal tract and induce acute bacillary dysentery that known as shigellosis. *Shigella* are Gram-negative, facultative anaerobic, non-motile and facultative intracellular pathogens that are firmly related to *Escherichia coli* but have improved particular features of physiology, pathogenicity and serology (Ud-Din and Wahid, 2014). There are four species of *Shigella: Shigella dysenteriae, S. flexneri, S. boydii* and *S. sonnei*, each possess their own global burdens and epidemiological form and all of which are capable of inducing disease in human beings. *Shigella* infection is highly contagious can transmits by the fecal-oral

way, individual to individual contact or ingestion of polluted food or water (Livio *et al*., 2014). Diarrhea is an first symptom of shigellosis and might be begun as the pathogen attack the small bowel leading to inflammatory colitis (Ashida *et al*., 2015).

Antimicrobial-drug resistant bacteria present an increasingly fatal threat to human health. The Center for disease control approximates that 14,000 deaths are the consequence of antimicrobial-drug resistant bacteria. Bacteria including *Shigella* spp. have progressed a plenty of various ways to resist the action of antibiotics, in addition to transfer these resistance traits once acquired. An significant number of these mechanisms are mediated by enzymatic activities (Bonomo and Tolmasky , 2007).

In spite of the fact that shigellosis is predominantly a self-limiting sickness, the World Health Organization rules suggest antimicrobial medication treatment as a means of decreasing deaths, symptoms of illness , and excretion time of organism ; the choice of present medication is ciprofloxacin (WHO, 2005). The crucial concern is multidrug re-sistance, and in especial the progressing average of ciprofloxacin resistance revealed for *Shigella* isolates from Asian and African territories (Gu *et al*., 2012). Additionally, resistance to recommended second-line antimicrobial medications, for example, the third generation cephalosporin ceftriaxone and the macrolide azithromycin, is rising (WHO, 2005).

According to our knowledge there is little information of diarrhea burden caused by *Shigella* spp. in Iraq and particularly in Al-Diwaniyah city. Therefore, there is an urgent need to investigate the distribution of *Shigella spp.* isolated from diarrheal patients and demonstrate their

antibiotics resistance patterns. The continuous monitoring of antimicrobial drug susceptibility is prescribed to choose proper anti-infection agents for the powerful treatment of shigellosis in this region. An Iraqi study in Baghdad was firstly distinguished resistance of fluoroquinolone due to *qnr* gene between *Shigella* spp. isolates in Iraq which is indicated that resistance of plasmid mediated quinolone has risen in Iraqi pediatric patients (Abdulrahman *et al*., 2015).

1.2. Aim of the study

The aim of current study is to investigate the isolation rate and resistance profiles of antibiotics among *Shigella* species isolates obtained from acute diarrheal patients in Al-Diwaniyah, Iraq hospitals.

The following objectives were followed to access the study aim :

1. Detection of *Shigella* species in study samples recovered from hospitalized patients by conventional and molecular methods.
2. Determining the antibiotics susceptibility patterns of *Shigella* isolates by Vitek2 system and disk diffusion method .
3. Determining the occurrence of Beta-lactamases genes including extended spectrum β-lactamases ($bla_{CTX\text{-}M}$, bla_{TEM} , bla_{SHV} *and* bla_{AmpC}) that responsible for antimicrobial drug resistance of *Shigella* species.
4. Using of Enterobacterial repetitive intergenic consensus (ERIC)-PCR technique to investigate the genetic diversity and molecular correlation among *Shigella* isolates.

1.3. Literatures review

1.3.1. Diarrhea

Gastroenteritis is an irritation of the gastrointestinal tract involving both the stomach and small intestine and described by diarrhea with vomiting due to infection of the small and large bowel (Mandell, 2011). World Health Organization (2005) determines diarrhea as the passing of at least three or more abnormally loose or watery stools per day, or more repeated than typical for a person. However, the most critical is the stools consistency rather than the number. usual passage of formed stools isn't characterized as diarrhea such as babies nourished on just breast milk habitually pass soft, pasty and pale feces. Bowel frequency of a healthy person ranges from three bowel motions daily to one bowel activity every three days with a normal feces constituency range from paste like to solid and pellet stool (Haslet *et al*. 1999).

Diarrheal disease is a highest reason of childhood sickness and death especially in developing nations and a major reason of malnutrition and malabsorption in children in these nations (Joosten and Hulst, 2008). It is the second cause of mortality of children under 5 years and is responsible for an appreciate 760 thousands deaths per year with about 80% of these deaths appearing in the first two years of life (WHO, 2013). In addition, diarrhea disturbs weight gain in children, affects their memory and analytical skills adversely and reduces their school attentions thereby their future is crippled (Watkins, 2006).

1.3.2. Causes of diarrhea

Progresses in science over the past three decades has led to the revelation and isolation of various microbial causes of diarrhea.

Pathogens isolated include viruses, bacteria and protozoa with rotavirus and diarrheagenic *Escherichia coli* (DEC) being the most frequently isolated pathogens from stool samples in developing countries. Underlying conditions such as malnutrition, lack of drinkable water, insufficient sanitation and poor hygiene increases the risk of spreading diarrhea in developing countries (Bonkoungou *et al*., 2013).

A study published in Baghdad concludes that the essential risk factors for death in severe diarrhea are intensive dehydration, male sex, and low body mass, dietary deficiency, and living in rural sections and artificial nutrition (Nasheit *et al*., 2006).

Another study published in Baghdad , shows that Baghdadian children stay in danger of recurrent diarrhea episodes and another complications which may influence their improvement status (Alaa *et al*., 2014).

1.3.3. Disease burden of diarrhea

Although a great reduction in mortality of children between 2000 and 2010, diarrhea and pneumonia remains the chief cause of avoidable deaths, interpreting about 30% of all child deaths globally. This toll is greater than deaths from malaria, AIDS and measles joined in the same period. In 2010, conservative estimates suggest that there were 1.731 billion of diarrheal episodes and about 36 million of these proceeded to severe episodes in children less than five years of age. in 2011, estimates reported about 700 000 episodes of diarrhea with a high proportion happening in developing countries (Liu *et al*., 2012). Universally, there is an estimated 1.7 billion cases of diarrheal disease yearly with the greater part of these cases happening in South Asia and Africa. A great rate of these deaths, 72%, happens in the early two years of life (WHO, 2009, Liu *et al*., 2012).

In Iraq , for age groups less than five years, diarrheal illnesses, infections of respiratory tract , other communicable sicknesses, and congenital malformations represent principle causes of death. The total number of cases of diarrheal disease in children less than 5 years old was accounted by the Ministry of Health to be 732,954 with a case mortality percentage 0.08%. Almost certainly, reporting is inadequate and the obtainable figures underestimate the bigness of the trouble. There were 3.6 cases of diarrheal disease per child yearly as indicated by the MOH/WHO/UNICEF survey directed in 1997. A comparison of the ratio of children under 5 with diarrhea in the two weeks before the survey demonstrates that Iraq has one of the most noteworthy rates (Alwan, 2004). An Iraqi study showed that the prevalence of diarrhea was 63.5%, whereas 8.5 months old are the median age of children (Alaa *et al*., 2014).

1.3.4. Etiology of diarrhea

Diarrhea can occur due to infectious and noninfectious causes. The gastrointestinal infections which characterized as diarrhea is originate by a great range of microbes involving viruses, protozoa and bacteria (Webb and Starr, 2005). A local study in Baghdad showed that the most common bacterial causes of diarrheal cases are *Escherichia coli* (27.2%) , *Campylobacter jejuni* (11.4%) and *Shigella* species (6.2%) (Saeed *et al*., 2010).

Bacteria including diarrheagenic *Ecsherichia coli*, *Shigella*, *Salmonella* and *Campylobacter* species are commonly isolated from about 15% of diarrheal diseases from children. Also, cholera is a common cause of diarrhea in sub-Saharan Africa and Asia (Charles and Ryan, 2011). Jointly, rotavirus and diarrheagenic *E. coli* are the most common

microbes isolated from diarrheal stool samples in developing countries (WHO, 2013).

Protozoans are associated with about 10% of childhood diarrhea. The most commonly protozoans isolated from diarrhael stool are *Giardia lamblia*, *Cryptosporidium* spp. and *Entamoeba histolytica* (Elliott, 2007).

Sometimes, diarrhea originate from non-infectious sources. Some medications such as NSAID and lactose containing foods consumed by lactose intolerant individuals do evoke diarrheal signs. Certain diseases cause diarrhea like Inflammatory Bowel Disease and Irritable Bowel Syndrome (O'Ryan *et al*., 2010). Other causes of non-infectious diarrhea are chronic ethanol ingestion, ischemic bowel disease, malabsorption of bile salts and microscopic colitis. Excess secretion of certain hormones such as serotonin by hormone-secretion tumors also cause diarrhea. Interestingly too, infants and toddlers may emerge chronic mild diarrhea without any visible infection. This is known as Toddler's diarrhea (Kasper and Harrison, 2005, Wedlake *et al*., 2009).

1.3.5. Treatment and management of diarrhea

Treatment of diarrhea is generally supportive. Orally rehydration treatment has been the crucial of diarrhea management programme to inhibit the life threat dehydration. Fluids replacement must start at home and given on the beginning of a diarrheal episode (WHO, 2009). The addition of zinc to oral rehydration solution decreases both the period and severity of diarrheal episodes in addition to decrease stool volume and the necessity for further medical interest. (Lazzerini and Ronfani, 2008). If the oral rehydration solution is not available, other fluids homely prepared by utilizing accessible low cost ingredients such as cereal drinks made from a thin mush of rice, corn , potato or other easily obtainable

low cost grains homely available. Breast milk is a superior drink for fluid replacement and must persist to be delivered to diarrheal infants concurrently with other oral rehydration fluids (WHO, 2013).

Patients who have inflammatory or bloody diarrhea should not be given anti-motility agents. Antimicrobial therapy is indicated for some non-viral diarrhea because most is self-limiting and does not need therapy. Patients with hemorrhagic colitis should have a careful check including complete blood cell picture with blood film, blood urea nitrogen concentration and creatinine levels to diagnose hemolytic uremic syndrome. Individual with travelers' diarrhea with frequent soft stools in an 8-hour period associated with vomiting, nausea, abdominal pain, fever or bloody stool may utilize from antimicrobial treatment. Antibiotics regiments are usually between 3-5 days. Patients suspected of having systemic infection should be given parenteral antimicrobial drugs (CDC, 2005).

1.4. *Shigella* species

Shigella belongs to the phylum Proteobacteria , class Gammaproteobacteria, order Enterobacteriales, family Enterobacteriaceae. *Shigella* is a genus of Gram-negative, facultative anaerobic, non-spore forming, non-motile, rod shaped bacteria closely related to *E. coli* and *Salmonella* and responsible for human shigellosis.

Shigella causes an infectious disease called shigellosis which presents as dysentery and affects only humans and apes and no other animals (Ryan and Ray, 2004). Symptoms of shigellosis generally start one to two days after exposure to bacteria and involve diarrhea (usually contain blood and mucus), fever, abdominal cramps, stomach pain, and pain with stool passing. Shigellosis often resolves in five to seven days. Some of infected people may have no manifestations at all, but may still pass

the *Shigella* pathogens to others. However, in immunocompromised individuals such as children and the elderly, the diarrhea may aggravate and patients might need hospitalization. Severe shigellosis combined with high fever often results in seizures in children under two years old.. Classically, bacillary dysentery or shigellosis is diagnosed by the laboratory methods that may detect *Shigella* spp. in stools samples of infected individuals (Amenyedor, 2013).

1.4.1. Serogroups of *Shigella* spp.

Shigella species are divided into four main serogroups, designated A, B, C and D. Serogroup A recognized as *S. dysenteriae*, serogroup B for *S. flexneri*, serogroup C for *S. boydii* and serogroups D for *S. sonnei*. They can be further classified into serotypes based on O antigen of the cell envelope lipopolysaccharide. *S. boydii* has 20 serotypes, *S. flexneri* has 6 serotypes and 14 subserotypes, *S. dysenteriae* has 15 serotypes with 2 provisional serotypes and *S. sonnei* has only one serotype. Physiologically, serogroups A to C are similar, but *S. sonnei* can be differentiated biochemically . About 60% of cases in the developing nations are produced by *S. flexneri* with 77% of cases in the developed nations produced by *S. sonnei*. *S. sonnei* is also responsible for 15% of cases in the developing world. *S. dysenteriae* is considered to be the common isolates of epidemics of dysentery, especially in crowded inhabitance like prison, slum and refugee encampments (Hale and Keusch, 2006; WHO, 2012).

1.4.2. Growth and culture characteristics

Shigella are slime Gram negative rods; coccobacillary shapes happen in young cultures, facultative anaerobes but grow best aerobically and

produce convex, round, translucent colonies with intact edges with a diameter of about 2 mm in 24 hours. All *Shigella* ferment glucose. Except for *Shigella sonnei,* they do not ferment lactose. The incapability to ferment lactose discriminates *Shigella* on differential culture media. *Shigella* produce acid from carbohydrates but seldom produce gas. They may also be classified into mannitol fermenters and non mannitol fermenters (Jawetz, 2013). (Table 1-1).

Table (1-1) Differentiation between *Shigella* spp. by biochemical tests

Shigella spp.	Mannitol	Ornithine Decarboxylase
Shigella dysenteriae	–	–
Shigella flexneri	+	–
Shigella boydii	+	–
Shigella sonnei	+	+

1.4.3. Transmission

Shigella spp. are highly contagious and transmit originally by the fecal oral route via the touching of contaminated fingers to the mouth. Infection consider as a sequel of inadequacies in basal hygiene and sanitation particularly in the developing countries. Infection usually occurs in toddlers who are commonly not toilet trained and more plenty at day care centers where children from different backgrounds come together. *Shigella* pathogens also can be delivered into polluted food or via drinking or swimming in polluted water. Little amount of organisms (≤100 bacterial cells) are needed to cause shigellosis. Food handlers are a principle origin of contamination as well as vegetables irrigated with

contaminated sewage. House flies, *Musca domestica*, also possible to transmit the *Shigella* from person to another (Wutoh *et al*., 2000).

Transmission is influenced by varieties in environmental factors like rainfall and temperature. Raise in infection frequency has been exhibited during summer months (Farshad *et al*., 2006, Yismaw *et al*., 2008).

1.4.4. Epidemiology

A survey in 1999 on *Shigella* infection between 1966 and 1997 showed that *Shigella* are responsible for an approximated 164.7 million episodes of diarrhea yearly; 163.2 episodes yearly happened in developing world with 1.1 million mortality (Kotloff *et al*., 1999).

Overpopulation, hygienic inadequacy, pollution of water supplies by sewage and HIV related immunodeficiency are the basic causes for 99% of Shigellosis episodes occurring in developing world. 1.5 million episodes yearly happened in industrialized world. 69% of total infections and 61% of total deaths were occurred in kids less than 5 years of age. *S. dysenteriae* (serotype1) is the causative promoter of epidemic bacillary dysentery within the Indian landmass, South East Asia and Central African Republic, it is also responsible for 30% of *S. dysenteriae* episodes in endemic areas; however recent researches have demonstrated that in some areas serotypes 2 - 12 are becoming prevalent (Talukder *et al*., 2003). Infection by *S. dysenteriae* (serotype1) is especially intensive due to it also generates Shiga toxin . *S. flexneri* was appeared to be the prevalent species in endemic areas (Table 1-2), with serotype 2a being the main strain.

In Iraq, a recent research was demonstrate that *S. flexneri* was the common cause of shigellosis with 54.2% while *S. sonnei* and *S.*

dysenteriae represented 37.3% and 8.5%, respectively (Abdulrahman *et al*., 2015).

Another Iraqi study also showed that 11.96% of diarrhoeal episodes among children were caused by *Shigella* spp. (Ibraheem, 2016).

Table (1-2) *Shigella* isolates median in developed and developing nations (Kotloff *et al*., 1999)

	S. flexneri	*S. sonnei*	*S. boydii*	*S. dysenteriae*
Developing countries	60%	15%	6%	6%
Industrialized countries	16%	77%	2%	1%

1.4.5. Pathology

The host response to initial infection is described by the stimulation of an acute inflammation, which is joined by infiltration of polymorphonuclear cells (PMN), causing massive devastation of the colonic mucosa. Apoptotic macrophages destruction in sub-epithelial tissues permits survival of the *Shigella* invasion, and inflammation helps more *Shigella* entry. General pathology includes erythema, mucosal dropsy, brittleness, superficial ulceration, and central mucosal hemorrhage including the rectosigmoid union mainly. Microscopic pathology includes necrosis of the epithelial cells, goblet cell exhaustion, polymorphonuclear cells and lamina propria infiltration via mononuclear cells , and crypt abscess development. In addition to these sequences of pathogenic events, only *S. dysenteriae* (type 1) capable of developing the

strong Shiga toxin that forbids synthesis of protein in eukaryotic cells and that might result in extra intestinal complications, involving hemolytic uremic syndrome and death. (Phalipon and Sansonetti, 2007).

1.4.6. *Shigella* Pathogenesis

Instructions about the *Shigella* pathogenesis were primarily gained from researches of *S. flexneri*. Upon intake, *S. flexneri* penetrates the human body. *Shigella* spp. have the ability to express acidity resistance system and come via stomach to reach the large bowel, where cellular invasion happens (Gorden and Small, 1993). Invasion of *S. flexneri* required genes that clustered on a 31 kb region of a bulky virulence plasmid. This region encodes a type III secretion system (T3SS) and several other effector proteins, for example IpaA, IpaB, IpaC and IpgD. Amongst these proteins, IpaB is an basic virulence factor for the domination of T3SS excretion, escape from phagosome, and apoptosis of macrophage (Blocker *et al*., 1999; Guichon *et al*., 2001; Suzuki *et al*. 2005; Schroeder *et al*., 2007; Schroeder and Hilbi, 2008; Roehrich *et al*., 2010)

The invasion events can be characterized by 6 steps: the passage within the Microfold cells (M cells); the escape from the cells of immune system; cells invasion; intracellular multiplication; intracellular and intercellular spreading ; and the damage of the host cells. The *Shigella* are transported through the colonic epithelium layer in M cells by transcytosis, then swallowed by the resident macrophages in the underlying lymphoid pouch. Within the macrophages, the *Shigella* destroy the phagosome and stimulate apoptosis. The apoptotic macrophages deliver the pro inflammatory cytokines interleukin (IL)-1 and IL-18 and originate inflammatory and immune responses including

neutrophils recruitment to mediate bacterial removal (Sansonetti *et al.*, 1982; Schroeder *et al.*, 2007). The surviving bacteria assemble T3SS which facilitate the basolaterally invasion of colonic epithelial cells. Effector proteins were excreted by T3SS into the epithelial cells to stimulate endocytosis of bacteria (Blocker *et al.*, 2003; Roehrich *et al.*, 2010).

The endocytosed *Shigella* then destroy the vacuoles, replicate inside cytoplasm, and propel unidirectionally by actin based motion. At the cell boundary, the invading bacteria assemble the T3SS once more, followed by the *Shigella* endocytosis by the neighboring cells. The *Shigella* then destroy the double membrane vacuole and replicate in cytoplasm, get going the spread of infection (Philpott *et al.*, 2000; Schroeder and Hilbi, 2008). As a consequence of infection , colonic cells are destroyed and their absorption of nutrients and water is impaired , resulting in watery diarrhea with blood and mucus.

1.4.7. *Shigella* invasion

The infectious dose essential for *Shigella* infection is as low as 100 microscopic organisms (DuPont *et al.*, 1989). The colonic epithelium invasion and cell to cell spreading is the major promoter of the intensive inflammatory reaction joined with *Shigella* infection. A type III secretion system (T3SS) facilitates the uptake of *Shigella* into epithelial cells. The proteins of the T3SS are encoded by a bulky 220kb virulence plasmid and produce a needle like macromolecular design that permits the passage of effector proteins through the membrane of the target eukaryotic cell. Proceeding to proteins passage, *Shigella* first attaches to the target cell, in spite of lack of classic adhesion proteins. New study has indicate that the *Shigella* surface protein IcsA acts as an adhesion that is stimulated by bile

salts, and aids connection with target host cells after earliest stimulation of theT3SS (Zumsteg *et al*., 2014).

As well as , bile salts stimulate the OspE1 and OspE2 secretion which stay on the outer membrane of bacteria and raise polarized cells adherence (Faherty *et al*., 2012). The eventual assembly of the T3SS also stimulates by bile-salts especially deoxycholate. Additionally, bacterial connection to filopodia via T3SS elements, IpaB and IpaD also stimulates adherence and invasion (Stensrud *et al*., 2008).

Interestingly, a study was proved that anaerobic conditions affect the *Shigella* secretion by the T3SS via fumarate and nitrate reductase (FNR)-mediated suppression of *spa32* and *spa33* transcription. Oxygen detection in the area directly close to the epithelial barrier mitigates this transcriptional inhibition, eliciting expression of both *spa32* and *spa33* resulting in the T3SS activation and effective invasion. Altogether, these determinations show that *Shigella* has developed to extremely sense when it is within the suitable intestine environment to elicit efficient adherence and T3SS activeness (Marteyn *et al*., 2010).

At the terminate of the T3SS is the apex complex consist of IpaB, IpaC, and IpaD (Veenendaal *et al*., 2007). Assemblage of IpaB and IpaC onto the needle simplifies by IpaD, and IpaB and IpaC are hydrophobic proteins that are capable of inserting into host cell membrane to produce a pore which permits for delivery of effectors (Blocker *et al*., 1999; Veenendaal *et al*., 2007). The insertion of cell membrane and T3SS activeness is stimulate via IpaB connection to cholesterol in the cell membrane of the host (Hayward *et al*., 2005; Epler *et al*., 2009). Previous to insertion, IpaB acts to inhibit secretion by the T3SS, behaving as a

molecular spigot that is eliminated with host cell membrane insertion (Roehrich *et al*., 2010).

T3SS-dependent delivery of effector proteins produces an area of actin remodeling and polymerization that eventually resulting in membrane ruffling and the uptake of the associated *Shigella.* Eventually, *Shigella* anchoring to the positioning of membrane ruffling supports by interaction of IpaA with host-cell vinculin. Once *Shigella* is internalized, it quickly runs away from the entrance vacuole. Insertion of IpaB and IpaC into the vacuole membrane is supposed to form pores stimulating vacuole destruction (Izard *et al*., 2006).

1.4.8. The toxins of *Shigella*

As well as the generation of virulence factors from the virulence plasmid, *Shigella* additionally generate further toxins encoded on the chromosome or virulence plasmid like *Shigella* enterotoxin1 (ShET-1), *Shigella* enterotoxin 2 (ShET-2), and Shiga toxin (Stx).The acute liquid diarrhoea is caused by ShET-1 and ShET-2 ,which are capable of inducing fluid secretion into the gut lumen (Niyogi *et al*., 2004). ShET-1 is composed of one A subunit and numerous B subunits, which are chromosomally encoded by the *set1A* and *set1B* gene, respectively. The B subunits are able to bind specifically to the target cell receptors. The single A subunit achieves the toxic enzymatic reactions inside the target cell. T3SS secrets a second enterotoxin ShET-2, which is encoded by the *sen* gene on the virulence plasmid. ShET-2 is capable of inducing an inflammatory event affect the epithelial cells through IL-8 secretion (Farfán *et al*., 2011).

Shiga toxin (Stx) is an exotoxin generated by *S.* dysenteriae serotype1 and particular serotypes of *E. coli* exclusively (Sperandio and Pacheco,

2012). Stx has cytotoxic effects on different host cells resulting in progression of vascular lesions in the colon, kidney, and central nervous system (Schroeder and Hilbi, 2008; Lee *et al*., 2010; Lee *et al*., 2013; Zaidi and Estrada-García, 2014). Stxs exhibits an AB5-toxin structure including an enzymatic A subunit non-covalently joined with five B subunits, which are encoded by the *stxA* and *stxB* gene, respectively, on the chromosome. The A subunit is a cytotoxic protein and has action on the 28S rRNA subunit of eukaryotic ribosomes and blocks protein synthesis and induces the cells death by apoptosis (Lee *et al*., 2010; Zaidi and Estrada-García, 2014). The pentamer B subunit controls the binding form of the holo toxin to the sensitive glycolipid Gb3 receptor on the host cell surface. After Gb3 attachment, Stxs are uptaked through endocytosis and carried from early endosomes to the trans Golgi network and then to the endoplasmic reticulum. Immediately, the A sub-unit is split into an enzymatically active 27 kDa A1 piece and a 4 kDa A2- piece via furin. Only the toxin A1 piece is retro translocated into the cytoplasm and prompts the cell apoptosis event (Sperandio and Pacheco, 2012).

1.4.9. The virulence factors encoded on virulence plasmid

The cellular pathogenesis and clinical presentation of *Shigella* infection are a sequel of complex activities of a variety of *Shigella* virulence factors. *Shigella* virulence plasmid is about 220kb. It encoded for 50 to 60 virulence related genes on a 31-kb entry region known as the *ipa-mxi-spa* region. This region encodes the assembling components of T3SS, which are Mxi and Spa proteins. Invasion plasmid antigens (Ipas) involve IpaA, IpaB, IpaC and IpaD proteins. The crucial virulence factors in *Shigella* are Ipa proteins. IpaA structure has three vinculin joining sites (VBSs) that can energize vinculin by the joining to the vinculin head domain (Park *et al*., 2011). Vinculin is the adapter protein shared in the stability

of centric adhesions. IpaA can adjust actin polymerization via regulating the addition or elimination of actin monomers. As a result, these proteins can trigger a cytoskeletal rearrangement of the host cells for increasing *Shigella* endocytosis into cells during epithelial cells invasion (Lee *et al*., 2014). The IpaB and IpaC proteins are situated on the apex of the T3SS canal to regulate the secretion system, aiding the *Shigella* to run away from the phagosome and stimulate the macrophage apoptosis. IpaC protein scan also stimulate cell actin polymerization ruffle development during *Shigella* infection, independent of IpaB (Kueltzo *et al*., 2003; Terry *et al*., 2008; Mounier *et al*., 2009). IpaD proteins are also situated on the apex of the T3SS canal and can regulate T3SS secretion (Roehrich *et al*., 2010; Dickenson *et al*., 2013; Roehrich *et al*., 2013; Schiavolin *et al*., 2013; Dohlich *et al*., 2014).

Various origins of virulence plasmid sequences have been recorded. Each form encoding is analogous, like the pSS of *S. sonnei* (Jiang *et al*., 2005), pC301 of *S. flexneri* 2a (Zhang *et al*., 2003), pWR501 of *S. flexneri* 5a (Venkatesan *et al*., 2001), and pWR100 of *S. flexneri* 5a (Buchrieser *et al*., 2000). In addition to the 31-kb entry area, there are no antibiotic resistant genes encoded on the virulence plasmid. Numerous insertion sequences (ISs) are encoded on the virulence plasmid. The virulence plasmid additionally includes conjugation related genes. By the aid of another plasmids that have conjugation capability, the virulence plasmid is able to acquire access from one bacterial cell into another via the pilus; therefore, the virulence plasmid is capable of mobilizing (Sansonetti *et al*., 1982; Venkatesan *et al*., 2001).

1.4.10. Shigellosis complications

Shigella infection is frequently limited to the gastrointestinal tract. However, there are incidents of extra intestinal spreading infections such as bloodstream infection with mortality rate of 46% (Muthuirulandi *et al*., 2017). About 3% of infected cases with *Shigella flexneri* may continue to develop eye irritations, joint pains, and painful urination which may persist from a month to several years. This condition is diagnosed as Reiter's syndrome. This can further progress into reactive arthritis (Gaston and Lillicrap, 2003). Individuals with the Human Leukocyte Antigen B27 (HLA-B27) have a genetic susceptibility of developing reactive arthritis as a late complication of *S. flexneri*. HLA-B27 has been highly associated with a group of autoimmune disorders known as sero negative spondyloarthropathies. *S. dysenteriae* infected individuals may rarely develop hemolytic uremic syndrome (HUS). HUS characterized by convulsions in children originating from a fast temperature increasing linked with metabolic alterations. It is also associated with Shiga toxin production. (Ram *et al*., 2008).

1.5. Bacterial antibiotics resistance

The emergence of bacterial antibiotics resistance, as a result of their development process has now been revealed as an issue of global emergency by the World Health Organization (WHO, 2014). It has led to increased decline rates of treatment of infectious diseases triggered by bacteria, for example, therapies to which they were previously sensitive no more act. Bacteria have intrinsically resistance to its inherent characteristics or gain this resistance ability during mutations and gene transfer. Different strategies of antibiotics resistance involve deficient drug penetration into the cell, outflow of antibiotics through efflux

pumps, target alteration by mutation and hydrolysis of antibiotics (Blair *et al*., 2015) (Table 1-3). Antibiotics resistance has been recorded in both Gram positive bacteria and Gram negative bacteria (Fernández and Hancock, 2012; Blair *et al*., 2015). One such truth is that emergence of multidrug resistance *Shigella* species (Tariq *et al*., 2012). *Shigella* species are the leading organism of Shigellosis, which is an acute gastroenteritis infection (Srinivasa *et al*., 2009).

Table (1-3): Appointed present problems related to antimicrobial drug resistance, according to drug class (Blair *et al*., 2015).

Antibiotic class	Resistance Mechanism
Cephalosporins	Extended spectrum β-lactamases, chromosomal cephalosporinases
β-Lactamase inhibitors	β-lactamases hyperproducers , new β-lactamases resistant to inhibitors, chromosomal cephalosporinases
Carbapenems	Zinc metallo enzymes and other β-lactamases
Vancomycin, teicoplanin	Modified cell wall precursors with decreased affinity for vancomycin
Quinolones	DNA topoisomerase alterations, efflux mechanisms, permeability alteration
Trimethoprim–sulfamethoxazole	Resistant enzymes in folic acid synthesis Pathway
Erythromycin, new Macrolides	Methylation of the bacterial ribosome producing resistance to macrolides, clindamycine, and streptogramine B antibiotics
Aminoglycosides	Modifying enzymes o Aminoglycoside

1.5.1. Development of *Shigella* antibiotic resistance

There are multiple mechanisms by which the antimicrobial resistance may occur. In *Shigella* species, antimicrobial resistance is frequently due to class 1 and class 2 integrons which include resistance gene cassettes. Integrons are movable and transferrable from one bacterial cell to another, supplying a flexible access for bacteria to harmonize to the environmental tension caused by antibiotics. This mechanism of action may explain the spreading of resistance genes and the emergence of MDR strains, and explain why *Shigella* resistance patterns vary worldwide (Zhang *et al*., 2014).

The first therapies chosen to treat shigellosis were sulphonamides, and followed by tetracycline and then by chloramphenicol. Resistance to all of these is developed by *Shigella* spp. and so consequently treatment altered by ampicillin and co-trimoxazole. Nevertheless, treatment of choice were again shifted to nalidixic acid as a result of resistance development to the former therapies by *Shigella*. Later, ability to resist nalidixic acid developed and directly after that fluoroquinolones were introduced. But recently fluoroquinolones resistant strains have been isolated from different origins. World Human Organization suggests ceftriaxone, pivmecillinam and azithromycin for treatment of infectious cases by fluoroquinolones resistant *Shigella* species (Taneja and Mewara, 2016). Nevertheless, ceftriaxone resistant and azithromycin resistant isolates have also been showed in some places (Mahbubur *et al*., 2007). As a consequence there is turning into a threat to humanity and a matter of great concern.

1.5.2. Extended spectrum β-Lactamases as a leading cause of antimicrobial drug resistance

Even if there is a several kinds of strategies of bacterial resistance to β-lactam antibiotics, the most considerable are the β-lactamases, which are enzymes able to hydrolyze the β-lactam ring of penicillins, cephalosporins, and related antimicrobial medications, making them inactive. There are numerous of β-lactamases, which differ in substrate specificity and host extent (Bush *et al*., 1995, Livermore, 1995). Much of the motivation to develop new β-lactam antibiotics has been failed because the emergence of bacteria that develop β-lactamases able to damage present antibiotics. The previous cephalosporins (such as, cephalothin) are liable to cleavage by a different of β-lactamases frequently present in Gram-negative rods, involving the chromosomal cephalosporinases of pseudomonas, enterobacter, and other genera, in addition to the main plasmid-produced enzymes of Enterobacteriaceae. The former enzymes additionally hydrolyze a variety of penicillins and in contrast to the chromosomal cephalosporinases, are generally in activated by β-lactamase inhibitors like clavulanic acid (Bush *et al*., 1995).

Alterations of the structure of cephalosporins generate the cephamycins, involving cefotetan and cefoxitin, that are resistant to several plasmid-mediated β-lactamases (Bois *et al*. 1995). More development generated the extended-spectrum cephalosporins ceftazidime, cefotaxime, and ceftriaxone, as well as aztreonam (a monobactam), which have good stability contra several β-lactamases.

The broad usage of extended-spectrum cephalosporins is due to their safety, efficiency, and advantageous pharmacokinetics. In the early 1980s, resistance to these medications emerged in Gram-negative rods

with chromosomally encoded β-lactamases, predominately as mutations consequence that led to the basic production of these naturally inducible enzymes (Sanders and Sanders, 1988).

Enteric Gram-negative rods with transportable resistance to extended-spectrum cephalosporins were first demonstrated in the Western Europe in the mid1980s (Knothe *et al*., 1983). The encoding genes of the extended spectrum β-lactamases were perfectly kept on self-transportable plasmids that usually loaded other determinants of antibiotic resistance (Sirot *et al*., 1988; Rice *et al*., 1990). Due to these genes may be carried on transposable components, they may transfer into different plasmids, allowing the spreading of extended-spectrum β-lactamases within Gram-negative rods (Sirot *et al*., 1991). The dominant β-lactamases in Gram-negative bacteria are TEM-, OXA-, SHV- and CTX-M (Muthuirulandi *et al*., 2017). In *S. flexneri*, ampicillin resistance is principally due to *bla*-$_{oxa}$ gene while in *S. sonnei* it is principally due to the existence of *bla*-$_{TEM}$ gene.

Mutations widen the ESBLs spectrum of like TEM-1,TEM-2 and SHV-1, which are responsible for resistance to third-generation cephalosporins (Taneja and Mewara, 2016).

The frequency of extended-spectrum β-lactamase generation between Gram negative rods differs from nation to nation and between institutes within a nation, at minimal in part because of styles of antibiotic use. Iraqi study showed that *Shigella* isolates had high rate of β-lactamase production reach to 75.6% (Al-Rahman, 2013). Another study in Iran was detected the frequency of ESBL producing *Shigella* spp. was higher than detection frequencies showed in several other countries (Ranjbar *et al*., 2013). Molecular epidemiologic studies have proposed that there is

spreading of strains producing extended spectrum β-lactamases among and within hospitals, in addition to transpose of plasmids encoding these enzymes among strains (Rice *et al*., 1990; Naumovski *et al*., 1993; Sader *et al*., 1994).

Sequencing and cloning of the genes encoding ESBL manifested that these genes varied from those encoding familiar plasmid-borne enzymes with more confined activity by replacements of only a few nucleotides (Sougakoff *et al*., 1988).

1.6. Bacterial genotyping

Bacterial genome is commonly thought to be streamlined, and yet varied families of short (30–150bp) interspersed repetitive sequences have been defined in bacteria (Tobes and Ramos, 2005; Wilson and Sharp, 2006).

Few is recognized concerning the origins, development, generation mode, or probable functions of these repeats. Majority of families are limited to single species or very closely relevant species, whereas several different species seem to own no such elements. This proposes that if these elements have any functions they have been achieved recently, may not employ to all the members of family, and are improbable to affect fundamental features of bacterial growth, replication and survival. Therefore, while few of repetitive sequences have been noted to act as binding sites for a different of proteins, involving DNA gyrase and DNA polymerase (Gilson *et al*., 1991), this could be incidental. Majority of short bacterial repetitive sequences are imperfect palindromes, with the capability to make secondary structures, that may increase the stability of mRNA. Instead, most repetitive elements may be nonfunctional junk (Newbury *et al*., 1987).

1.6.1. Enterobacterial repetitive intergenic consensus (ERIC)

Enterobacterial repetitive intergenic consensus (ERIC) sequences, further characterized as intergenic repetitive units, vary from most other bacterial repeats in being spreading among a broad scope of species. ERIC sequences were first defined in *Escherichia coli*, *Salmonella typhimurium* (now *Salmonella enterica* serovar *Typhimurium*), and other relations of the Enterobacteriaceae, as well as *Vibrio cholera* (Sharples and Lloyd, 1990; Hulton *et al*., 1991). The ERIC sequence is an imperfect palindrome of 127bp. Additionally, shorter sequences generated by internal deletions have been characterized (Sharp and Leach, 1996), moreover insertions of nearly 70 bp at specific internal sites generate longer sequences (Cromie *et al*., 1997; Sharp, 1997). ERIC sequences are located exclusively in intergenic regions, clearly exclusively inside transcribed regions. The quantity of the ERIC sequence copies differ among species, it was firstly predicted by extrapolation that there may be nearly 30 copies in *E. coli* K-12 and may be 150 in *S. enterica Typhimurium* LT2 (Hulton *et al*., 1991), whereas the genome sequence of *Photorhabdus luminescens* has been revealed to involve over 700 copies (Duchaud *et al*., 2003).

Chapter two

Materials and Methods

2. Materials and Methods

2.1.Materials

2.1.1.Laboratory Equipments and Instruments

The Table (2-1) shown the laboratory equipments and instruments used in this work.

Table (2-1) : Laboratory equipments and instruments.

Equipment	Model	Origin
Autoclave	HIRAYAMA	Japan
Automatic Micropipette	CYAN	Belgium
Camera	Samsung	China
Class II safety cabinet	BAKER	USA
Deep Freezer	ARISTON	Italy
Densicheck	BIOMERIEUX	France
Disposable Petri dishes	Al-Hani company	Lebenon
Distillator	CFL	Germany
Electrophoresis	Bioneer	Korea
Eppendorf tubes	Sterellin Ltd.	UK
High Speed Cold centrifuge	Eppendrof	Germany
Incubator	BINDER	Germany
Nanodrop spectrophotometer	THERMO	USA
PCR Thermocycler	Bioneer	Korea
Refrigerator	ARCTIKO	Denmark
Sensitive balance	Startorius	Germany
Sterilized cotton swabs	Sterile EO.	China
Sterile disposable cup		
Test tubes	PLASTILAB	Lebanon
Tips	Sterellin Ltd.	UK
U.V transilluminator	Wised	Korea
Vitek2 system	BIOMERIEUX	France
Vortex	CYAN	Belgium
Vortex centrifuge	Exispin	Korea
Water bath	MEMMERT	Germany

2.1.2. Culture Media

Several types of culture media are used to identify and isolate *Shigella* species. These media are shown in Table (2-2).

Table (2-2) : Culture media used in identification and isolation of *Shigella* spp.

Culture media	Purpose	Company/Origin
Brain heart infusion broth	Enrichment medium	Accumix^{TM}/India
Cary Blair transwab	Transport medium	mwe/England
Hektoen Entric agar	Selective medium	HIMEDIA/India
MacConkey agar	Selective medium	BIOMARK/India
Mueller Hinton agar	Antibiotic susceptibilty	HIMEDIA/India
Nutrient broth	Preserved medium	Accumix^{TM}/India
Salmonella Shigella agar	Selective medium	Accumix^{TM}/India
Xylose Lysine Dextrose agar	Selective medium	HIMEDIA/India

2.1.3. Chemical and biological agents

The chemical and biological agents used in current study were shown in Table (2-3). The diagnostic kits that used in current study were shown in Table (2-4).

Table (2-3):The chemicals which used in this study.

chemical	Companies	Origins
10XTBE (Tris-Boric acid EDTA)	BioBasic	USA
Absolute ethanol	bdh	England
Agarose gel	Bio Basic	USA
Barium chloride ($BaCl_2$)	Fluka	Switzerland
Ethidium Bromide	Bio Basic	USA
Free nuclease water	Bio Basic	Korea
Glycerol ($C_3H_8O_3$)	Jumana	Jorden
Oxidase reagent	Difco	USA
Sulfuric acid (H_2SO_4)	BDH	England

Table (2-4) Diagnostic Kits

Kit	Origins	Companies	Informations
Api20 E	Biomerieux	France	
Vitek 2	Biomerieux	France	Card type: GN , AST-N222

2.1.4. Antibiotic disks

The antibiotics used in this study were listed in Table (2-5)

Table (2-5): Antibiotics used in this study

Class	Antibiotic	Symbol	Concentration	Company/Origin
Cephalosporin	Cefotaxime	CTX	30 µg	MASTDISKS™/UK
	Cefepime	Cpe		Vitek2
	Ceftriaxone	CRO	30 µg	MASTDISKS™/UK
	Ceftazidime	CAZ	30 µg	MASTDISKS™/UK
	Cefoxitin	FOX	30 µg	MASTDISKS™/UK
β-lactams/β-lactamase inhibitor combinations	Pipracillin-tazobatam	PTZ	30 µg	MASTDISKS™/UK
	Augmentin	AUG	30 µg	MASTDISKS™/UK
Monobactam	Aztreonam	ATM	30 µg	MASTDISKS™/UK
Penicillin	Ampicillin	Amp	30 µg	MASTDISKS™/UK
	Ticarcillin	Ti		Vitek2
	Pipracillin	Pi		Vitek2
Polymyxin	Colistin			Vitek2
Tetracycline	Tetracycline	Te	30 µg	Bioanalyse/Turkey
	Minocycline			Vitek2
Folate pathway inhibitors	Trimethoprim-Sulfamethoxazole	STX		Vitek2
Carbapenem	Imipenem	IMP		Vitek2
	Meropenem	MEM		Vitek2
Quiolones	Ciprofloxacin	CIP	30 µg	MASTDISKS™/UK
	Levofloxacin	LEV	30 µg	MASTDISKS™/UK
	Pefloxacin	PEF		Vitek2
	Nalidixic acid	Na	30 µg	Bioanalyse/Turkey

2.1.5. Molecular study

2.1.5.1. Polymerase chain reaction materials

Table (2-6) illustrated the polymerase chain reaction materials which used in this study.

Table (2-6):PCR system materials

Materials	Company	Origin
Genomic DNA Extraction Kit	Gene aid	USA
GT buffer		
GB buffer		
W1buffer		
Wash buffer		
Elution buffer		
GD column		
2ml Collection tube		
Maxime PCR PreMix (*i*-Taq)	iNtRON Biotechnology	Korea
Taq DNA polymerase		
dNTPs dATP, dCTP, dGTP, dTTP		
Tris- HCl pH 9.0, KCl, and MgCl2		
Stabilizer and Tracking dye		
DNA ladder (100-1500)bp	Bio Basic	Korea

2.1.5.2. Primers

In this study all primers were provided from Bioneer company, Korea.

2.1.5.2.1. *Shigella* diagnostics primers

The diagnostic primers for *Shigella* genus and species which used in this study are shown in the Table (2 -7).

Table (2-7): Specific primers of *Shigella* genus and species.

Primer		Primer sequences (5'- 3')	Target gene	Product size (bp)	Target identity	Reference
SgenD	F	TGCCCAGTTTCTTCATACGC	*invC*	875	*Shigella* genus	(Ojha *et al.*, 2013)
	R	GAAAGTAGCTCCCGAAATGC				
SflexD	F	TTTATGGCTTCTTTGTCGGC	*rfc*	537	*S. flexnrei*	(Ojha *et al.*, 2013)
	R	CTG CGT GAT CCG ACC ATG				
SsonD	F	TCTGAATATGCCCTCTACGCT	*wbgZ*	430	*S. sonnei*	(Ojha *et al.*, 2013)
	R	GAC AGA GCC CGA ACC G				
SdysD	F	TCTCAATAATAGGGAACACAGC	*rfpb*	211	*S.dysentriae*	(Ojha *et al.*, 2013)
	R	CATAAATCACCAGCAAGGTT				
SboyD	F	TCTGATGTCACTCTTTGCGAT T	Conserved hypothetical protein	248	*S. boydii*	(Ranjbar *et al.*, 2014)
	R	GAATCCGGTACCCGTAAGGT				

2.1.5.2.2. β-lactamases primers

The specific primers for β-lactamases genes used in this study were designed by using NCBI Gene sequence data base and primer 3 plus design. Table (2-8).

Table (2-8): β-lactamases primers

Primer		Primer sequences (5' _ 3')	Amplicon size (bp)	Target gene	Gene bank
CTX-M	F	ACG GAT AAC GTG GCG ATG AA	247	$bla_{CTX\text{-}M}$	JN411912.1
	R	TCA TCC ATG TCA CCA GCT GC			
TEM	F	GGT GCA CGA GTG GGT TAC AT	531	bla_{TEM}	JN037848.1
	R	TGC AAC TTT ATC CGC CTC CA			
AmpC	F	AAA CGA CGC TCT GCA CCT TA	670	bla_{AMPC}	AY533245.1
	R	TGT ACT GCC TTA CCT TCG CG			
SHV	F	CCG CCA TTA CCA TGA GCG AT	410	bla_{SHV}	FJ668798
	R	AAT CAC CAC AAT GCG CTC TG			

2.1.5.2.3. Enterobacterial repetitive intergenic consensus primers

The ERIC primers used in this study are shown in the Table (2-9).

Table (2-9) ERIC primers

Primer	Sequence (5’ – 3’)		Reference
ERIC	ERIC 1	ATG TAA GCT CCT GCG GAT TCAC	(Ranjbar and Ghazi, 2013)
	ERIC 2	AAG TAA GTG ACT GGG GTGAGCG	

2.2 .Methods

2.2.1. Patients

A total of 282 stool samples were collected from different patients ages (adults, teenagers and children) and included males and females. Patients ages range from 4 days to 70 years. The patients group covered 144 females and 138 males. Stool samples were obtained from diarrheal patients who were admitted to Al-Hussain children hospital , Feminine and children teaching hospital and Al-Diwaniyah teaching hospital during the period from November 2017 to May 2018 . Each patient had a case sheet containing name, age, gender, sample appearance and symptoms as a questionnaire (Appendix 1)

2.2.2. Samples collection

Under safety handling conditions , a total of 282 diarrheal stool samples were collected from patients by a sterile containers and well labeled with patients information and transported to the laboratory

immediately. Sometimes the samples preserved in transport medium according to the time of collection until reaching to the laboratory.

All samples were streaked according to standard methods on differentiation and selective media (MacConkey, XLD, Hektoen and SS agar) for detection of *Shigella* species and incubated aerobically at 37^0 C for 24 hours (Murray *et al*., 1995). The samples processing steps were clarified in Figure (2-1).

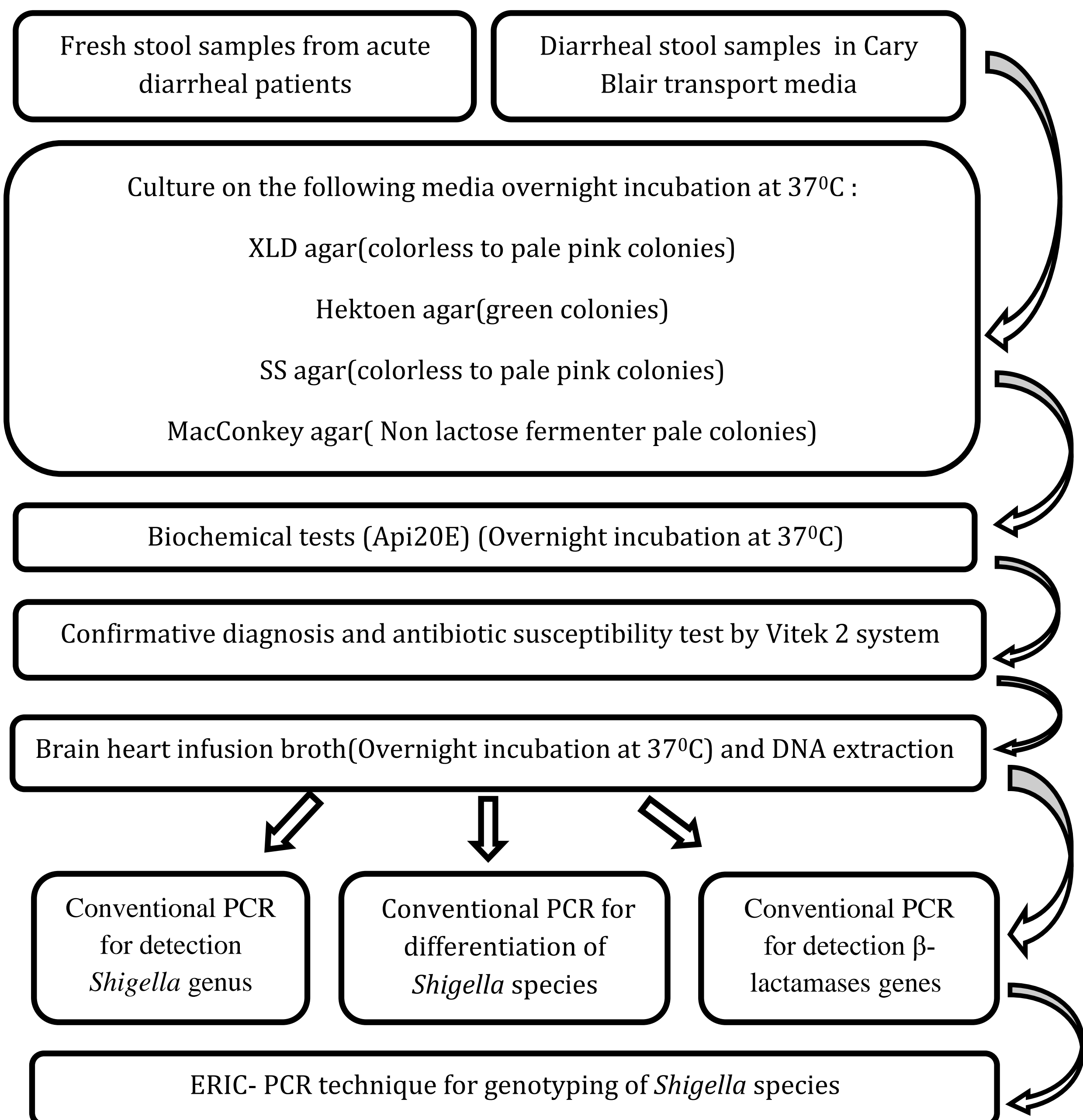


Figure 2-1 :Study design

2.2.3. Preparation of culture media

2.2.3.1. Ready-prepared media

Ready-prepared media including Xylose Lysine Deoxycholate agar, MacConkey agar, Hektoen Enteric agar, Salmonella Shigella (SS) agar, Brain Heart Infusion broth, Mueller Hinton agar and Nutrient broth were prepared according to the manufacturing companies instructions , which are usually fixed on the container of the medium. They were kept at 4^0C until being used.

2.2.3.2. Long term preservation medium

Nutrient broth is the basal medium of this medium and supplemented with 25% glycerol. After autoclaving at121˚C for15 min., and cooling to 56˚C in a water bath, 5 ml apportioned in sterile test tubes, and maintained at4˚C up to used. These media were used to preserve the isolates of bacteria at deepfreeze for long term storage (Thomas, 2008).

2.2.4. McFarlande's Turbidity Standard (0.5) preparation

A barium-sulfate turbidity standard solution equal to a 0.5 McFarlande's standard prepared as follows:

A 0.5 ml of 1.175% (w/v) $BaCl_2$.2H2O solution was added to 99.5 ml of 1% (v/v) H_2SO_4. The McFarland standard tube closed by parafilm to prevent evaporation and kept in the dark at room temperature for up to 6months. The fitness of the density of a prepared 0.5 McFarlands standard equivalent 1.5 $x10^8$ cells per ml was tested by a spectrophotometer. The wavelength of 625 nm absorbance must be 0.08 - 0.1 (Cockerill *et al*., 2012).

2.2.5. Confirmatory Tests

2.2.5.1. API 20E system

API 20E is a system of standard identification for Enterobacteriaceae and other non-fastidious Gram- negative bacilli.

The API 20E strip composed of 20microtubes involving dehydrated substrate. The bacterial suspension are inoculated into the micro tubes leading to reconstitutes the media. During incubation period , bacterial metabolism generates changes in color of the media which are either spontaneous or detected by the addition of indicator reagents.

The reactions are read in accordance with reading table and identification is got by the Analytical Profile Index.

According to the manufacturer's instructions (Biomerieux), the following steps were performed:

- Preparation of incubation box (tray and lid) by distributing about 5ml of D.W. into the wells of the tray to produce a humid atmosphere.
- The test strip was put in the incubation tray.
- By using a pipette, a single young (18-24 hours old) isolated colony was picked up from an isolation culture plate and emulsified carefully in 5ml ampule of API Nacl 0.85% medium to obtain a homogeneous bacterial suspension.
- With a sterile pipette, the bacterial suspension was distributed into the micro tubes of the test strip according to certain instructions.
- The incubation tray was closed and incubated at $36^0C \pm 2^0C$ for 18 to 24 hours.

- After the incubation period, the test strip was read by referring to the reading table. The spontaneous reactions were reported on the result sheet and then revealed the tests which require the addition of reagents.

  *The TDA reagent was added to the TDA cupule.

  *The VP cupule requires two reagents: VP1 was firstly added and then VP2 was added .

  *The JAMES (Kovacs reagent) was added to the IND cupule.
- The results was obtained with numerical profile and matched with analytical profile index.

2.2.5.2.Oxidase test

Oxidase reagent was ready to use (o.5ml hermetically sealed dropper of 1% aqueous solution of N,N,N,N tetramethyl-para-phenyl diamine dihydrochloride. It was used for detection the ability of bacteria to produce oxidase enzyme.

A strip of filter paper was soaked with a little of oxidase reagent, then a bacterial colony to be tested was picked up with a sterile wooden stick and smeared on the filter paper. Development of a dark purple color within 5-10 sec reveals a positive result (MacFaddin, 2000).

2.2.5.3. Vitek2 system

Vitek2 is fully automated advanced colorimetry system that achieves bacterial identification and the antibiotics susceptibility.

According to the manufacturer's instructions (Biomerieux, Card type GN, AST-N222), the following steps were performed:

- Two Khan tubes were prepared for each sample, one for identification ID and another for antibiotics susceptibility test AST. The tubes were labeled and arranged in special Vitek rack. Then, 3 ml. of diluents (normal saline) was added to each tube.
- Bacterial suspension from young growth culture plate(18-24 hours) was prepared by adding 2-3 pure *Shigella* colonies to each identification tube and mixed well. Each ID tube was tested for colony standardization to McFarland's standard solution 1.5 $x10^8$ cell / ml by dens check apparatus. The density of bacterial suspension must be ranged 0.53-0.63.
  If the density lower than limit , more colonies was added .
  If the density higher than limit , more diluent was added .
- By sterilized pipette ,145 µl of bacterial suspension was transferred from ID tube to AST tube for each sample.
- In the same Vitek rack, ID and AST cards were placed in front of ID and AST sample tubes respectively .
- The rack was placed in the specified Vitek chamber , and the process was continued according to certain instructions .
- The results were obtained after 8 hours.

2.2.6. Antibiotics Susceptibility Testing

The antimicrobial susceptibility testing was performed by the agar discs diffusion method as that described by (Bauer *et al*., 1966) as follows:

2.2.6.1. Bacterial Inoculum preparation

About 3-5 isolated bacterial colonies were suspended in 4-5 ml brain heart infusion broth. The broth culture was incubated at 37˚C for 2-4 hrs.

The turbidity of the effectively growing broth culture was compared with sterile broth to get turbidity optically comparable to the 0.5 McFarlands standards tube (growth corresponding to1.5 x10^8 cell/ml).

2.2.6.2. Streaking of test plates

- ∗ A sterile cotton swab was dipped into the comparable suspension, then rolled firmly several times on the insider wall of the tube above the suspension level to eliminate excessive inoculum from the cotton swab.
- ∗ The entire surface of a Mueller Hinton agar plate streaked with the dipping swab. The plate streaking was repeated more than two times and the plate was rotated about 60°each time to certify an equal distribution of inoculum. Finally, the margin of the agar was swabbed.
- ∗ The agar plate was permitted to dry for 15 to 20 min. at room temperature prior the application of the antimicrobial discs.

2.2.6.3. Antimicrobial Discs Application

1. The antimicrobial discs were distributed onto the surface of the inoculated Petri plate by aiding of flamed and cooled forceps.. Each disc was squeezed gently to ensure perfect contact with the agar plate surface.

2. The agar plates were incubated in an inverted position at 37 C° for an overnight.

3. After overnight incubation. By using clear ruler, the growth inhibition zones diameters were measured.

4. The results were compared with the inhibition zone diameter supported by the CLSI (2016).

2.2.7. Preservation and Maintenance of Bacterial Isolates

Shigella isolates cultured propagated were preserved on nutrient agar slant at4˚C. Weekly sub-culturing of preserved isolates was done on new media for maintenance. For long preservation, nutrient broth with 25% glycerol was used and the *Shigella* isolates were kept deep frozen at -70˚C for several months medium-term maintenance (Thomas, 2008).

2.2.8. Detection of Extended spectrum β- lactamase production

All *Shigella* isolates were tested for ESBLs production. ESBLs production was screened by disc approximation method that carried out as modified by (Coudron *et al*., 1997) as follows:

An overnight agar culture of the test *Shigella* isolate was inoculated into Muller-Hinton agar plate as recommended for standard disc diffusion susceptibilities test. Discs containing 30 µg cefotaxime, ceftazidime, ceftriaxione, and azetreonam were placed 30mm (edge to edge) from a disc of augmentin 20 µg amoxicillin plus 10 µg clavulanate. Incubation followed for16 to 20 hours at 35˚-37˚C. Any enhancement of the inhibition zone between aβ-lactam disc and augmentin disc was indicative of ESBL presence.

2.2.9. Polymerase Chain Reaction Amplification

2.2.9.1. Bacterial DNA extraction (Template preparation)

The following steps were done to extract the DNA of *Shigella* isolates according to the manufacturer instructions (Gene aid, USA):

- 1 ml of *Shigella* brain heart infusion broth was transferred to labeled micro-centrifuge .
- The micro-tubes were placed in the micro centrifuge at 15 x g for 1min. to obtain pellet. Then the supernatant discard.
- 200 µl of GST (lysing) Buffer added to the tubes and then mixed well by vortex to re-suspend the pellet .
- The tubes were stood 5 min at room temperature.
- 200 µl of GB (binding) Buffer added to the tubes and mixed well via vortex.
- The tubes were placed in the water bath at 60^0C for 3 min. The tubes were inverted each 3 min.
- 200 µl of absolute ethanol added to the tubes and mixed well. Then, the tubes were transferred to GD column and centrifuged at 15xg for 1 min. .
- The 2 ml collection tubes including the flow through were discarded and replaced by new 2ml collecting tubes.
- 400 µl of W1 Buffer added to the GD column and centrifuged at 15 x g for 1 min. .
- The flow through was discarded and the GD column was return back to the 2ml collecting tubes.
- 600 µl of Wash Buffer added to the GD column and centrifuged at 15 x g for 3 min.
- The GD column was placed in new micro-centrifuge tubes.
- 100 µl of preheated Elution Buffer (pH 8.5) to the GD column and waited for 5 min. at room temperature .
- The GD column was centrifuged at 15 x g for 1min.
- The GD column was discarded and the micro-centrifuge tube was obtained.

- The purity and concentration of DNA for each sample were measured by Nonodrop instrument.
- The micro-centrifuge tubes were preserved at deep freeze temperature for PCR technique.

2.2.9.2. Estimation of DNA extracts

The extracted DNA was tested by using Nanodrop that measured DNA concentration (ng/µL) and checked the DNA purity by reading the absorbance at 260 /280 nm.

2.2.9.3. Amplification procedure

2.2.9.3.1. PCR master mix preparation

Polymerase chain reaction master mix prepared by using (Maxime PCR PreMix Kit) according to instructions of company as in Table(2-10):

Table (2-10): PCR master mix preparation

PCR Master mix	Volume
DNA template	5µL
Forward primer (10pmol/µL)	1.5µL
Reveres primer (10pmol/µL)	1.5µL
PCR water	12µL
Total volume	20 µL

These PCR master mix components that mentioned in Table (2-10) were placed in standard Maxime PCR PreMix tubes that containing all other elements which needed to PCR reaction like Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2, and loading dye. Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000 rpm for 3minutes, and then put in PCR Thermocycler (My Gene, Bioneer. Korea).

2.2.9.3.2. Preparing the Primers Suspension

The primers were re-suspended by dissolving the lyophilized product, after spinning down concisely, with 250μL PCR water (free nuclease) according to manufacturer instructions to reach to 100 pmol/μL as a stock primer suspension. Working primer equal to 10 pmol/μL concentration was prepared by dilution of 10 μL of stock primer with 90 μL of PCR water, then mix well by vortex.

2.2.9.3.3. Polymerase Chain Reaction Thermocycling Conditions

The PCR tubes were put into the thermocycler and the right PCR cycling program parameters conditions were adjusted according to each primer as following tables:

Table (2-11): Program of PCR thermocycling conditions for *Shigella* genus gene (*invC*) detection.

Temperature (^{o}C)/Time					Cycle number
Initial denaturation	Cycling condition			Final extension	
	Denaturation	Annealing	Extension		
95/3.0	95/0.30 sec.	57.2/0.30 sec.	72 / 1.30 min.	72 / 5.0 min.	30

Table (2-12): Programs of PCR thermocycling conditions (Touchdown PCR) for detection of *Shigella flexneri* gene (*rfc*).

Step	Temperature	Time	Cycle
Initial denaturation	95 ^{0}C	2 min.	1
Denaturation	"95 ^{0}C"	30sec.	15
Annealing	60.8 ^{0}C decrease 0.5^{0}C per cycle	30sec.	
Extension	72 ^{0}C	50sec.	
Denaturation	95 ^{0}C	30sec.	20
Annealing	53.8 ^{0}C	30sec.	
Extension	72 ^{0}C	50sec.	___
Final extension	72 ^{0}C	5min.	
Hold	4 ^{0}C	Forever	

Table (2-13): Programs of PCR thermocycling conditions (Touchdown PCR) for detection of *Shigella sonnei* gene (*wbgZ*).

Step	Temperature	Time	Cycle
Initial denaturation	95 ^{0}C	2 min.	15
Denaturation	95 ^{0}C	30 sec.	
Annealing	63.4 ^{0}C decrease 0.5^{0}C per cycle	30 sec.	
Extension	72 ^{0}C	50 sec.	
Denaturation	95 ^{0}C	30 sec.	20
Annealing	56.4 ^{0}C	30 sec.	
Extension	72 ^{0}C	50 sec.	
Final extension	72 ^{0}C	5 min.	___
Hold	4 ^{0}C	Forever	

Table (2-14): Programs of PCR thermocycling conditions (Touchdown PCR) for detection of *Shigella dysenteriae* gene (*rfpB*).

Step	Temperature	Time	Cycle
Initial denaturation	95 ^{0}C	2 min.	15
Denaturation	95 ^{0}C	30 sec.	
Annealing	58.7 ^{0}C decrease 0.5^{0}C per cycle	30 sec.	
Extension	72 ^{0}C	50 sec.	
Denaturation	95 ^{0}C	30 sec.	20
Annealing	51.7 ^{0}C	30 sec.	
Extension	72 ^{0}C	50 sec.	
Final extension	72 ^{0}C	5 min.	___
Hold	4 ^{0}C	Forever	

.

Table (2-15): Programs of PCR thermocycling conditions for detection of *Shigella boydii* (Conserved hypothetical protein).

Temperature (^{o}C)/Time					Cycle number
Initial denaturation	Cycling condition			Final extension	30
	Denaturation	Annealing	Extension		
95/5.0 min.	95/0.45 sec.	56/0.35 sec.	72/0.45 sec.	72/5.0 min.	

Table (2-16): Programs of β- lactamases gene thermocycling conditions

Temperature (°C)/Time					Cycle number
Initial denaturation	Cycling condition			Final extension	30
	Denaturation	Annealing	Extension		
95/3.0 min.	95/0.30 sec.	58.3 /0.30 sec.	72/1.30 sec.	72/5.0 min.	

Table (2-17): Programs of ERIC-PCR thermocycling conditions for genotyping of *Shigella* spp.

Temperature (°C)/Time					Cycle number
Initial denaturation	Cycling condition			Final extension	
	Denaturation	Annealing	Extension		30
95/7.0 min.	90/0.30 sec.	52/1.0 sec.	65/3.0 min.	65/16.0 min.	

2.2.9.3.4. Agarose Gel Electrophoresis

The PCR products were analyzed by agarose gel electrophoresis according to manufacturer instructions (Bio Basic/ USA) as the following steps:

- 1.5 % Agarose gel was prepared by dissolving the agarose gel powder in 1X TBE buffer and putting in water bath at 100 °C for15 minutes for complete dissolving, after that, allowed to cool to about 50°C.
- 3μL of ethidium bromide stain were added into melted agarose gel.

- The comb was fixed in appropriate position into the tray and then the melted agarose gel was poured in tray after that allowed to solidified for about 15 min. at room temperature, then the comb was detached carefully from the tray that result in formation of several wells within gel.
- 10µl of PCR product were added in to each well and 5µl of (100bp Ladder) in first one well.
- The agarose gel tray was located in electrophoresis chamber and fill with 1X TBE buffer. Then the electric current was supplied at 70 volts for1 hour.
- The products of PCR were visualized by using UV Transilluminator.

2.2.10. Statistical Analysis

Statistical analysis was performed by Social Science Statistics and the Statistical Package For Social Sciences version 23 for Windows Software and Microsoft Excel 2010. Chi-square test was used for the estimation of association between the studied variables. The p-value of less than 0.05 was statistically significant (Field, 2005).

2.2.11. Ethical Management of the Study

The present study has been managed according to recommendations guide gained from the College of Medicine, University of Al-Qadisiyah.

The study did not include forbidden biological materials or genetically modified organisms. All the *Shigella* isolates included in present study were obtained from hospitalized patients specimens without any additional substances.

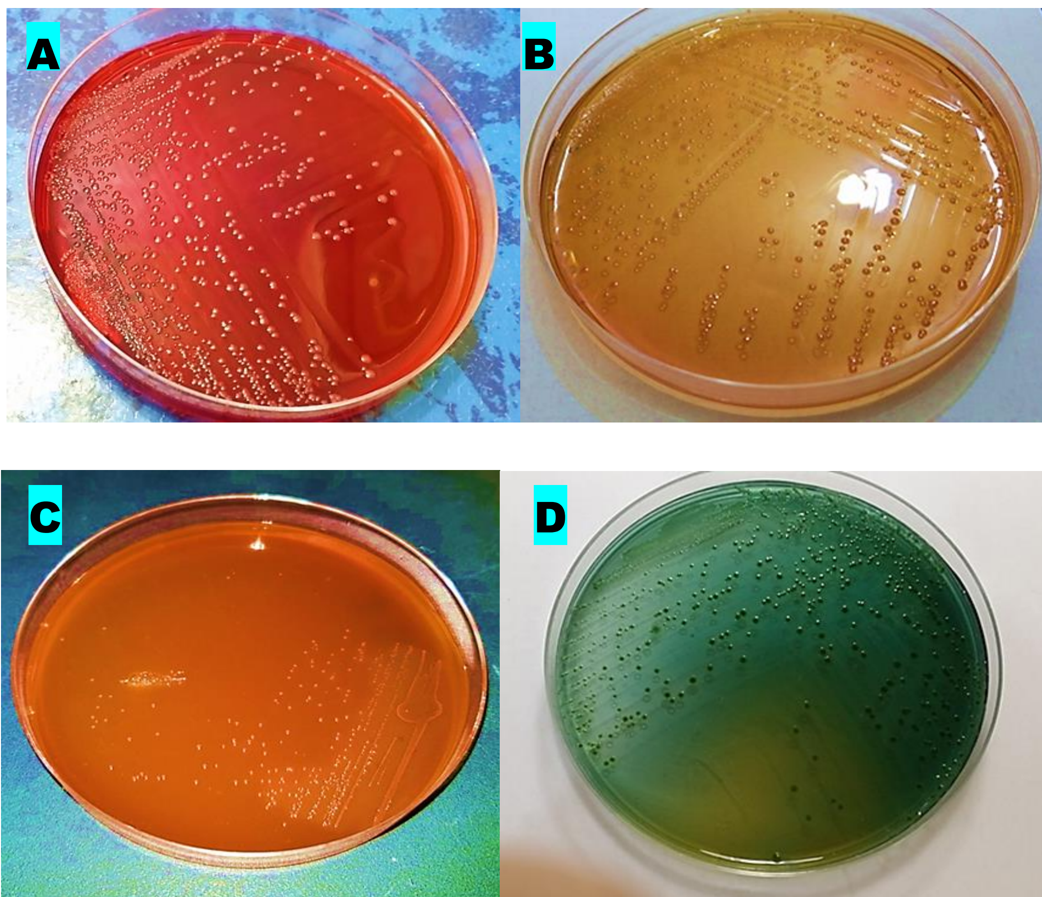
Chapter Three

Results and Discussion

3. Results and Discussion

3.1. Isolation and Identification of *Shigella* isolates

The *Shigella* isolates were identified by examination of colonial morphology on each culture media. *Shigella* isolates on XLD were appeared as translucent, convex and colorless to pale pink colonies without H2S production (Figure3-1A). On MacConkey agar were appeared as non-lactose fermenter translucent pale colonies (Figure3-1B). On SS agar were appeared as small, pale and colorless colonies (Figure3-1C). On Hektoen agar were appeared as translucent, convex green colonies (Figure3-1D).



Figure(3-1) A: *Shigella* isolates on XLD agar ; B: *Shigella* isolates on MacConkey agar; C: *Shigella* isolates on SS agar; D: *Shigella* isolates on Hektoen agar.

The usual method to examine the agent of dysentery is culture and performing specific biochemical tests to identify the genus and species of bacteria, which is a time consuming and laborious method. Nowadays, DNA-dependent molecular methods, especially the PCR method, are widely used in scientific and research centers for the detection of shigellosis (Farfán *et al*., 2010; Alipour *et al*., 2012).

An Iraqi study in Al-Ramadi City was observed that molecular methods has numerous advantages over the conventional methods for the diagnosis of shigellosis such as safety, high sensitivity and specificity (NoorAl-huda, and AL-Ouqaili, 2017).

In this study out of 282 patients only 19(6.73%) of them were confirmed diagnosed with Shigellosis Table(3-1). The positive cases comprised 6 (31.5%) males and 13 (68.4%) females. The age of positive cases comprised 15(78.9%) ≤ 10 years, 3 (15.7%) between 11 to 20 years and 1 (5.2%) between 41 to 50 years.

Table (3-1) *Shigella* isolation rate in diarrheal patients

Result	Total samples	No.	Percentage%
Positive samples	282	19	6.73
Negative samples		263	93.26
P –value	<0.0001*		

*** Significant association at p < 0.05**

Shigella isolation rate in this study appeared as significant with P-value. This percentage may due to the majority of collection period occurred during winter months in which *Shigella* infection is rare.

A recent local study in Baghdad by Ibraheem, (2016) was revealed that *Shigella* spp. the second (11.96%) of most common microorganisms isolated from 92 diarrhoeal children patients.

The *Shigella* isolation rate (6.73%) in this study was lower than one reported in Iran by MoezArdalan *et al*. (2003) that represented (16.8%), while it was higher than another reported by Ashkenazi *et al*. (2003) that represented (2.5%).

Table (3-2) Distribution of clinical samples according to the sex .

Sex	No. of patients		Percentage %
Males	138	6	31.57
Females	144	13	68.42
total	282	19	100
P value	0.023*		

*** Significant $p < 0.05$**

According to the current results (Table 3-2), the distribution of *Shigella* spp. in females more than in males that represented 68.42% and 31.57% , respectively. However, a study observed that there was no sex difference in the incidence of diarrhea, but the environmental and sociodemographic factors are relevant to diarrhea rather than biological factors (Parashar *et al*., 1998).

In this study, the symptoms of the patients with shigellosis were reported as follows: all patients suffering from abdominal pain (100%, 19 / 19), vomiting (73.6%, 14 / 19), fever (36.8%, 7 / 19), nausea (21%, 4 / 19) and fatigue (10.5%, 2/19). Figure (3-2).

The clinical manifestations of *Shigella* infection range from mild diarrhea to severe dysentery, depending on the *Shigella* serotype provoking infection, infection dose, the age and immunity of the host (Lampel and Maurelli, 2007). Earliest manifestations involve liquid diarrhea, fever and fatigue. Patients can develop dysentery in more severe cases (described as recurrent, painful stools comprising blood and mucus), abdominal pain, nausea and vomiting (Niyogi, 2005; Nygren *et al*., 2013). All *Shigella* spp. can provoke acute bloody diarrhea (FDA 2012).

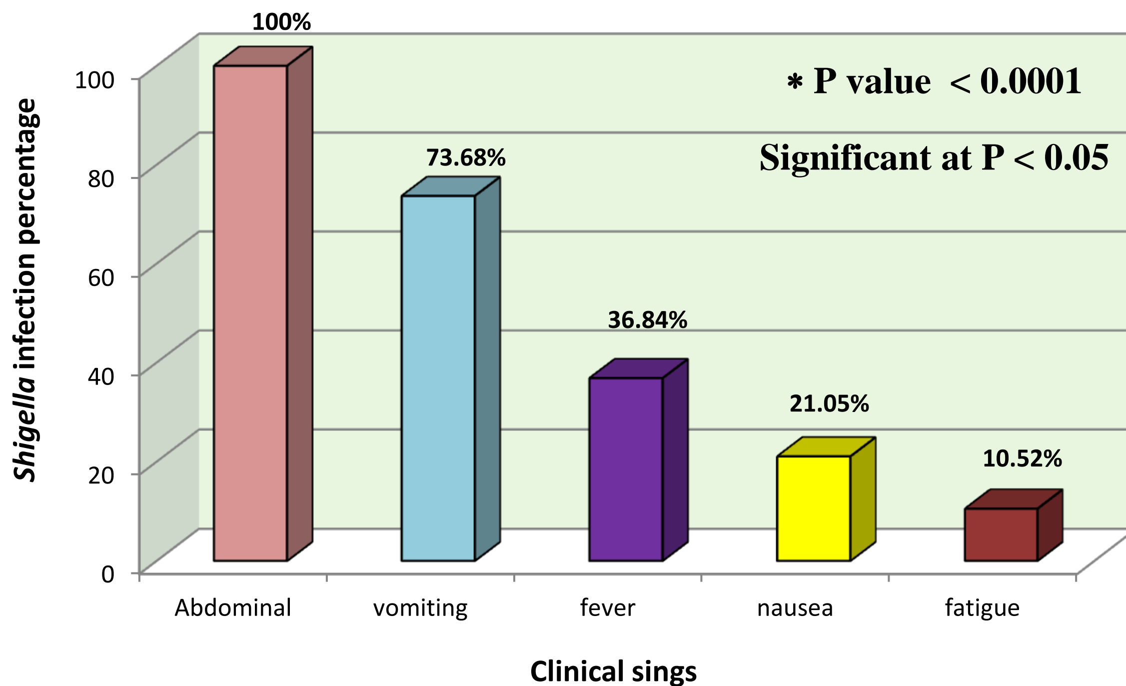


Figure (3-2) Clinical signs of infected patients with shigellosis

The results of present study were nearly agreed in some data reported by Saeed *et al*. (2010) who observed that fever was presenting (90.9%), vomiting (63.6%) and abdominal pain (18.8%).

The specimen characteristics collected from patients with shigellosis in present study were mucoid bloody stool (36.8% ,7 / 19), liquid mucoid stool (5.2%, 1 / 19), semiliquid bloody, semiliquid mucoid and liquid bloody stool each of which (15.7%, 3 / 19). (Figure 3-3).

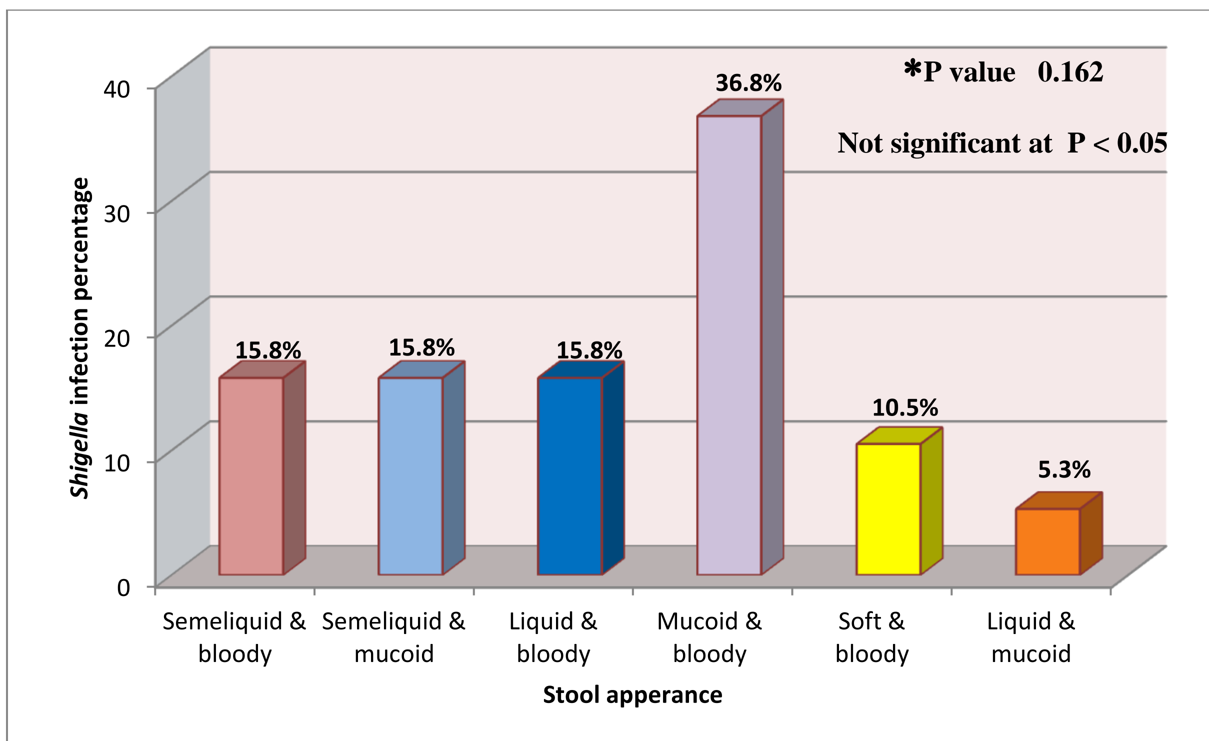


Figure (3-3) The characteristics of stool samples of patients with shigellosis.

As soon as ingested, *Shigella* spp. must persist the acidic medium of the stomach and invade the colonic epithelial layer to facilitate the infection. *Shigella* spp. multiply within the colonic epithelial cells and spread to neighboring cells, causing the destruction of the infected cells. The colon develops inflammation and ulceration and the destroyed mucoid cells are shed, resulting in the bloody mucoid diarrhea frequently characteristic of Shigellosis (Hocking, 2003; Warren *et al.*, 2006; Montville and Matthews, 2007).

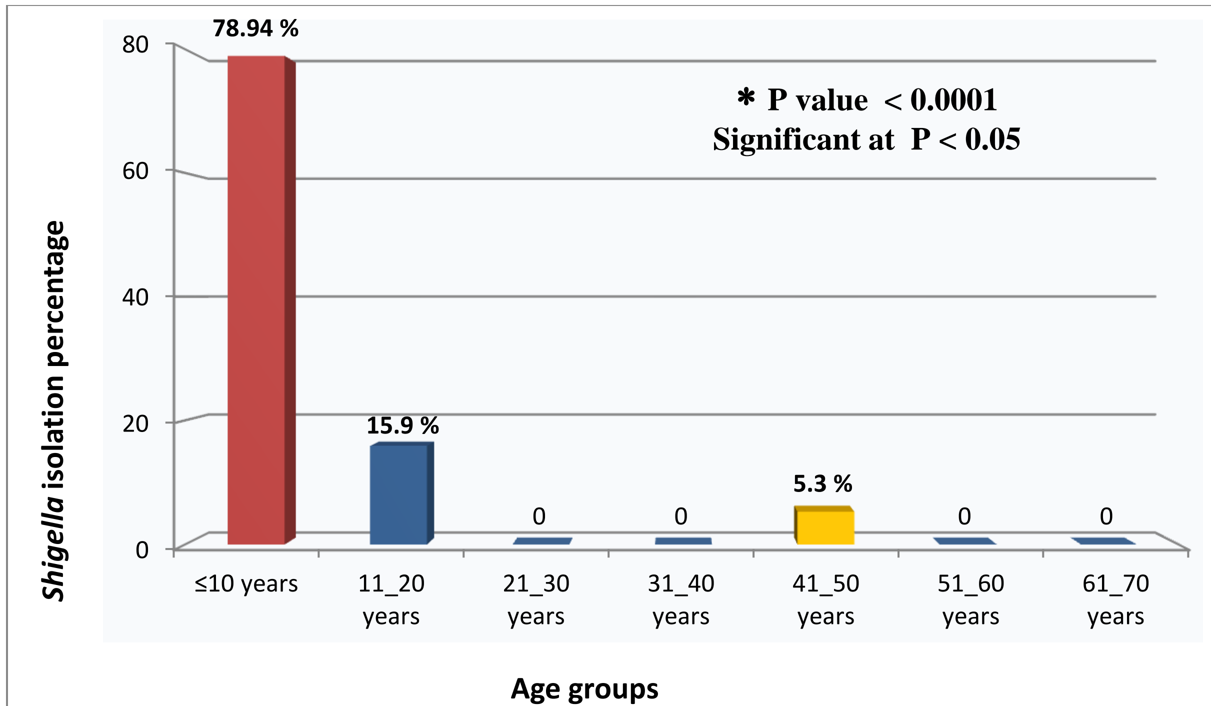


Figure (3-4) Distribution of *shigella* isolation according to age groups.

It is clear from Figure (3-4) the results observed that majority of shigellosis was obtained from children under 10 years (78.9%) ,while obtained in low rate in age group between 11 to 20 years (15.7%) and age group between 41 to 50 years (5.26%). The high susceptibility in children may be due to several factors conferred by artificial feeding, immunologically naivety, they more frequently come into contact with the contaminated agents such as toys and diaper touching and their coexistence in crowded areas like childcare settings and schools.

However, this rate among children was higher than one obtained in Basra by Mohammed *et al*. (2004) who observed that the percentage of shigellosis among children with diarrhea which was (1.4%).

Human of all ages are susceptible to *Shigella* spp. infection. However, children, the aged and immune-compromised persons are most at risk (FDA, 2012).

A local study by Saeed *et al*. (2010) showed that out of 316 diarrheal patients 22(6.9%) of them with *Shigella* positive. Out of 22 diarrheal patients with *Shigella* positive 12(54.5%) of them were less than 2 years.

The present results are similar to that reported by Abdulrahman *et al*. (2015) who observed that the *Shigella* isolation rate was high among children under 10 years and low in teenagers and adults. The former study observed that the isolation rate of *shigella* was 14 % , while in this study was (6.73%) (Table 3-1). Ali *et al*. (2010) revealed high incidence of *Shigella* in age group of 1 month -10 years, while the lowest incidence was in 10 to 20 years and only one isolate obtained in 60 to 70 years. Additionally, another similar results by Orrett (2008) observed that 88.8% were recovered from children aged ≤10 years with 8% isolation rate with *Shigella*.

In 2016, a quantitative molecular analysis from the Global Enteric Multi-center Study (GEMS) identified an increased burden of Shigellosis and reported it as the leading pathogen among the top six attributable pathogens causing childhood diarrhea (Zhang *et al*., 2014).

Bhattacharya *et al*. (2005) showed that the majority (79%) of *Shigella* spp. were isolated from children less than 5 years in a study in Eastern Nepal.

Again, higher *Shigella* isolation percentages in pediatric age group, have been reported, 9.5% in Bay of Bengal islands and 11.5% in Kolkata (Nandy *et al*., 2010; Bhattacharya *et al*., 2014).

3.2. Diagnosis by biochemical tests using Api20E system

All *Shigella* isolates were tested for further biochemical tests by using Api20E strips to confirm the diagnosis. The isolates were showed different results according to metabolism activities of each spp. of *Shigella* genus (Figure 3-5). These biochemical differences occurred especially in carbohydrate fermentation reactions, ornithine decarboxylase and ONPG reaction. All *Shigella* isolates were observed negative results with H2S production, Gel diffusion, indol production, urease and VP test. According to the obtained results, the Api20 E system is specific and sensitive for diagnosis, but Vitek2 system and molecular method are more specific and sensitive.

Figure(3-5) Api20 E system represented positive result for *Shigella* species. From left to right: ONPG(-ve), ADH(-ve), LDC(-ve), ODC(-ve), CIT(-ve), H2S(-ve), URE(-ve), TDA(-ve), IND(-ve), VP(-ve), GEL(-ve), GLU(+ve), MAN(+ve), INO(-ve), SOR(-ve), RHA(-ve), SAC(-ve), MEL(-ve), AMY(-ve), ARA(+ve).

All *Shigella* isolates were tested for oxidase test as a recommended step in Api20 E test. All the isolates were showed negative results.

3.3. Conformational diagnosis by VITEK2 system

The conformational diagnosis of bacterial isolates was performed by Vitek2 system. All suspected *Shigella* isolates were identified by this technique. Twenty one isolates were confirmed as *Shigella* spp.(Table 3-3) and these isolates were investigated for antibiotic susceptibility test by this technique.

3.4. Molecular identification of *Shigella* genus by PCR

All the *Shigella* isolates which identified by Api20 E system and Vitek2 system were submitted to DNA extraction. The obtained extracted DNA samples were submitted to conventional PCR to amplify of specific *invC* gene.

Table (3-3) Identification of *Shigella* spp. by Api20E, Vitek2 system and PCR technique

No.of samples	Positive by Api20 E system	Positive by Vitek2 system	Positive by PCR method
282	23	21	19

The results of gel electrophoresis observed that out of 23 samples, only 19 sample produced the target DNA fragment (875 bp) when compared with DNA ladder, Figure (3-6).

In present study the highly specific *invC* gene (invasion protein gene C) was used for *Shigella* genus identification because it is existent among all of the *Shigella* spp. In contrast, the conventional PCR, that frequently target the invasion plasmid H (*ipaH*) gene, the 16S rRNA gene and O antigen synthesis genes for identification of *Shigella* spp. In these cases, the detection is frequently based on genetic differences or sequence polymorphisms rather than on the presence or absence of a gene (Houng *et al*. 1997; Thong *et al*. 2005).

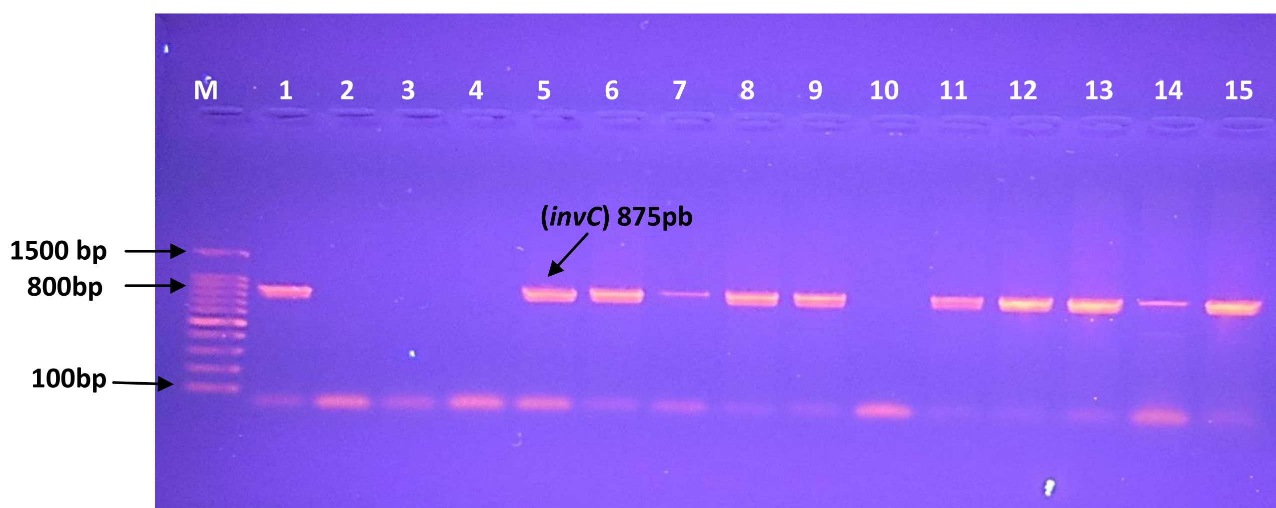


Figure (3-6) Ehidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of *invC* genes (875bp) of *Shigella* genus for 1 hrs. at 70 volts Lane(1 , 5, 6, 7, 8, 9, 11, 12, 13, 14 and 15) Amplifying of *invC* gene in clinical *Shigella* isolates. (2, 3, 4 and 10) No amplifying of *invC* gene in clinical isolates.

Shigella spp. are delicate organisms that are discharged in vast numbers in the feces, but they die rapidly due to stool acidity (Khalil *et al*., 1998). Therefore, routine microbiological strategies used to identify *Shigella* spp. from stool specimens are relatively inefficient, time exhausting, and labor

intensive, and the diagnosis usually continues obscure because the low quantities of causative microorganisms, competition with other normal flora , and improper sample collection. In addition, if the specimens are collected following antibiotics treatment, growth of the pathogens maybe impaired.

Moreover, Dutta *et al*. (2002) and Islam *et al*. (1998) observed that the sensitivity of the culture methods to be 54% and 74%, respectively, compared to the conventional PCR method.

Many researchers such as Ojha *et al*., (2013); Fatahi *et al*., (2015) in Iran and Das *et al*., (2016) in India also used the *invC* gene as a specific identification target for *Shigella* genus detection.

The molecular technique can identify a small quantity of culturable and non-culturable pathogens. Such identification is particularly crucial for *Shigella*, because they are able to cause disease via as few as 10-100 microorganisms. The PCR can be preferred as an alternative to the culture method, and also useful for diagnosing asymptomatic carriers, who are serving as potential reservoirs of *Shigella* and silently transmitted the illness among communities (Alipour *et al*., 2012).

3.5. Molecular differentiation of *Shigella* species

The DNA extracted from all isolates that identified as *Shigella* genus by PCR technique were differentiated to *Shigella* species also by PCR technique by using specific target sequences for each species. *Rfc* gene (537 bp), *wbgZ* gene (430 bp), *rfpB* gene (211 bp), and conserved hypothetical protein (248 bp) were used to identified *S. flexneri*, *S. sonnei*, *S. dysenteriae* and *S. boydii*, respectively. (Figures 3-7, 3-8, 3-9).

The concentration and purity of DNA that extracted from *Shigella* isolates were measured by Nanodrop spectrophotometer. The obtained concentration of DNA for amplification process ranged from (20.9 – 49.5) ng/µl. While the purity of extracted DNA ranged from (1.48 – 1.98) ng/µl. (Table 3-4).

Table(3-4) Concentration and purity values of extracted DNA from *Shigella* isolates.

No. of isolates	Concentration of DNA (ng/µl)	Purity of DNA ng/µl (ratio of A_{260}/A_{280})
1	25.3	1.78
2	23.6	1.94
3	49.1	1.98
4	39.1	1.86
5	24.8	1.80
6	26.0	1.68
7	31.5	1.56
8	24.7	1.93
9	30.5	1.48
10	20.9	1.89
11	44.6	1.75
12	35.2	1.48
13	42.1	1.58
14	25.9	1.74
15	49.5	1.86
16	49.0	1.97
17	48.2	1.88
18	35.9	1.90
19	35.9	1.82

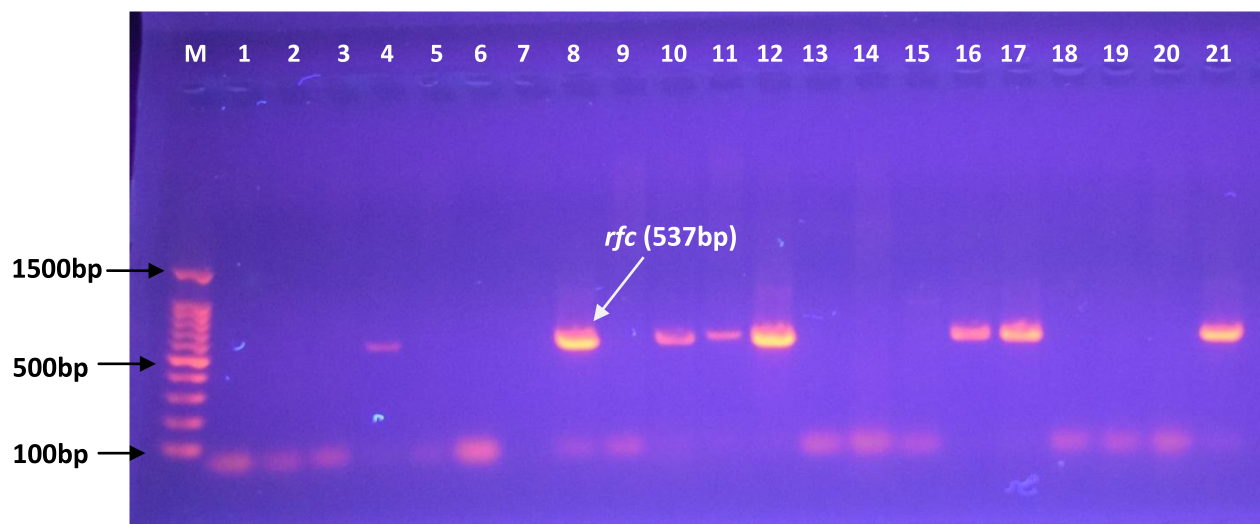


Figure (3-7) Ehidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of *rfc* gene(537 bp) of *S. flexneri* for 1 hr. at 70 volt. Lane M: DNA marker. Lane(4, 8, 10, 11, 12, 16, 17, 21) Amplify of *rfc* gene of *S. flexneri* isolates.

The *rfc* gene is chromosomal gene that encoded for O- antigen of lipopolysaccharide of *S. flexneri* that considered a virulence determinants (Morona *et al*., 1994).

Similar to this study, Alizadeh-Hesar *et al*. (2014); Bakhshi *et al*. (2018); Fatahi *et al*. (2015); Ojha *et al*. (2013) were used *rfc* gene for specific molecular identification of *S. flexneri*.

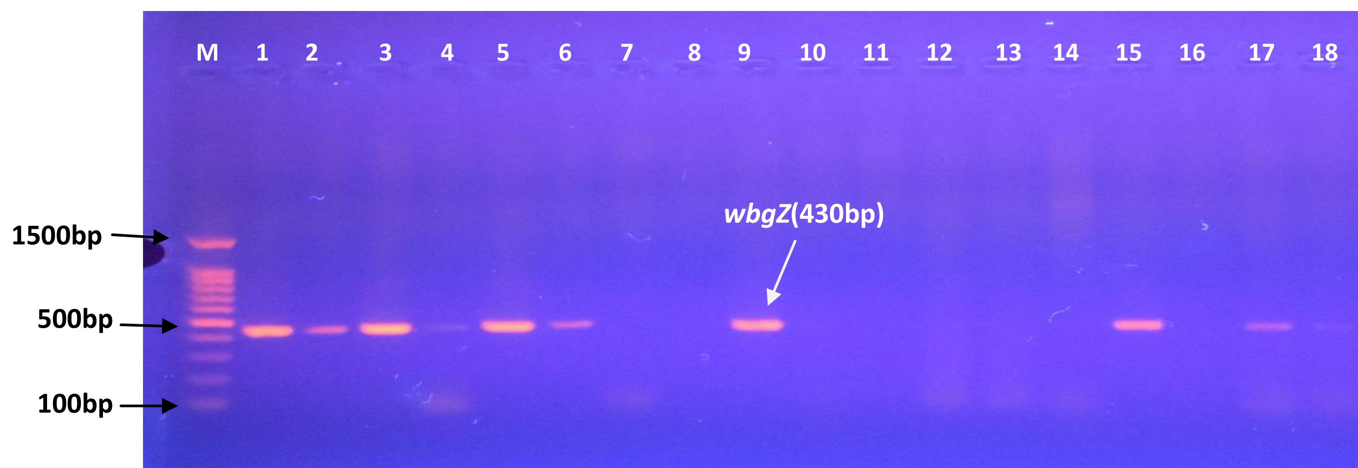


Figure (3-8) Ehidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of *wbgZ* gene(430 bp) of *S. sonnei* for 1 hr. at 70 volt. Lane M: DNA marker. Lane (1, 2, 3, 5, 6, 9, 15, 17) amplify *wbgZ* gene of *S. sonnei* isolates.

The *wbgZ* (putative epimerase /dehydratase) gene is highly specific plasmid gene used for detection of *S. sonnei*. Radhika *et al*. (2014) who detected *S.sonnei* from human clinical specimens , food and environmental sources by molecular method using *wbgZ* specific gene.

Bakhshi *et al*. (2018) were identified *S. sonnei* in children with diarrhea by using specific primer of *wbgZ* gene. Additionally, Fatahi *et al*. (2015) and Alizadeh-Hesar *et al*. (2014) were depended on *wbgZ* specific gene for *S. sonnei* molecular identification.

In this syudy, Amplification of *rfpB* gene for *S. dysenteriae* was showed no bands on agarose gel electrophoresis. Similar to the current study, other researchers such as Fatahi *et al*. (2015) who used *rfpB* specific gene to detect *S. dysenteriae* from fecal specimens of the patients with diarrhea and Ojha *et al*. (2013) who amplified *rfpB* specific gene for *S. dysenteria*

identification from hospitalized diaarrhoeal patients by pentaplex PCR assay .

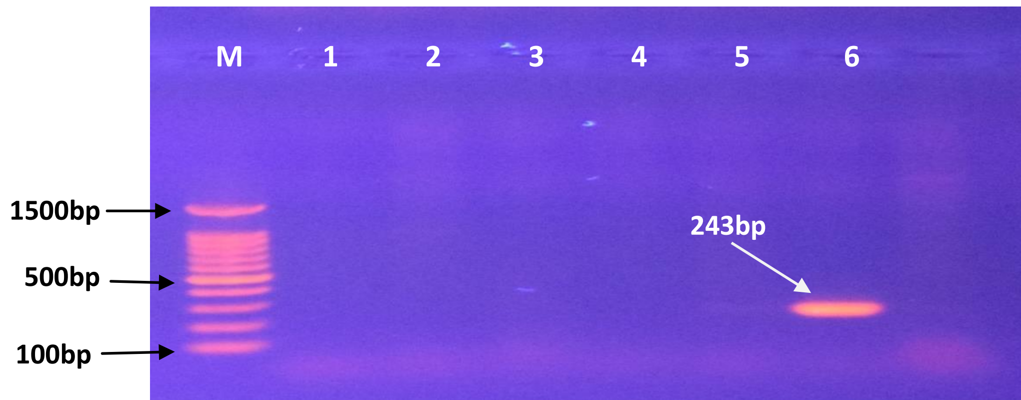


Figure (3-9) Ehidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of conserved hypothetical protein gene(248 bp) of *S. boydii* for 1 hr. at 70 volt. Lane M: DNA marker. Lane (6) Amplifying of conserved hypothetical protein gene of *S. boydii*.

The conserved hypothetical protein primer for specific identification of *S. boydii* molecularly was used in this study. This protein is exist in organisms from several phylogenetic progenies but have not been functionally described (Galperin and Koonin, 2004).

Other studies done by Nave *et al*. (2016) and Ranjbar *et al*. (2014) similar to this study in using the conserved hypothetical protein primer as target gene to differentiate *S. boydii* from other *Shigella* spp.

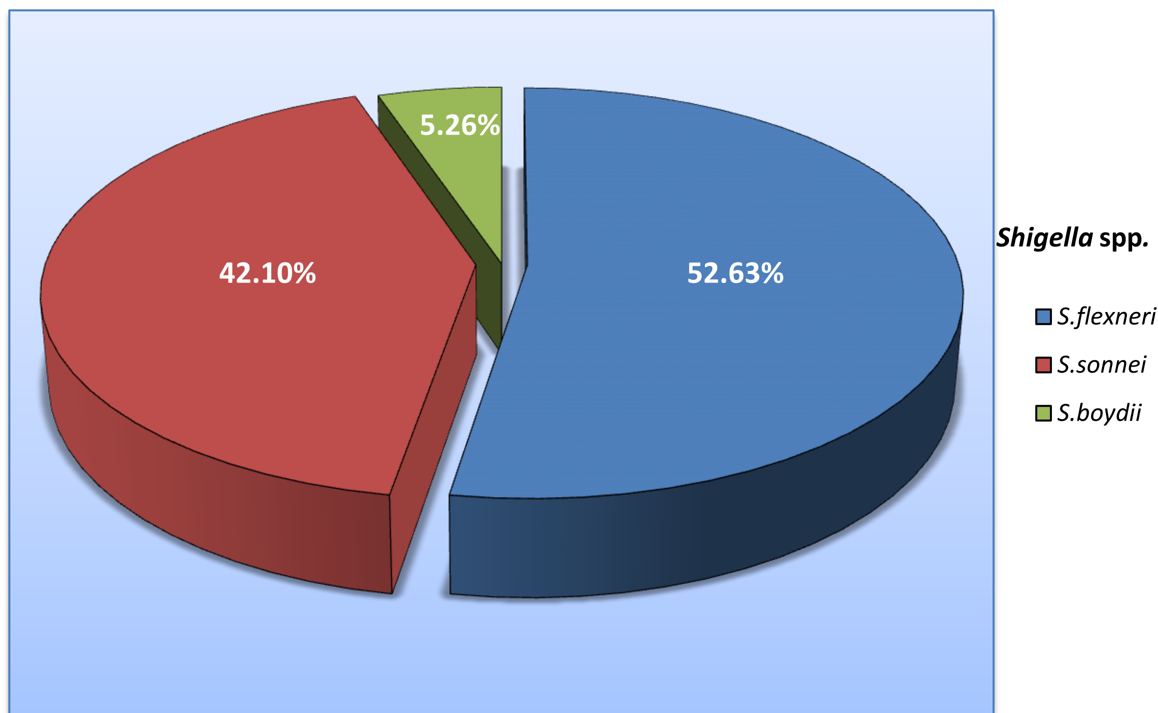


Figure (3-10) The occurrence rate of *Shigella* spp. using PCR technique.

According to Figure (3-10), the obtained results in current study showed the most common spp. among *Shigella* isolates was *S. flexneri* (52.63%) and followed by *S. sonnei* (42.10%), then *S. boydii* (5.26%) and no *S. dysenteriae* was detected. These observation agreed with other previous results reported by local study by Mohammed (2009) who reported that the predominant species was *S. flexneri* (66.6%, 6 / 9) followed by *S. sonnei* (33.3%, 3 / 9).

The present results was closely similar to results gained by Qiu *et al*. (2015) who showed the most common species isolated from diarrheal patients in China was *S. flexneri* (55.3%), followed by *S. sonnei* (44.1%).

There is a progressing tendency toward *S. sonnei* predominance, which is reflective of a present global shift in the epidemiologic distribution of this species (Thompson *et al*., 2015).

Iraqi study by Abdulrahman *et al*. (2015) was reported that *S. flexneri* (54.2%) with highest percentage followed by *S. sonnei* (37.3%) then *S. dysenteriae* (8.5%),while no *S. boydii* was found. The occurrence rate of *S. flexneri* and *S. sonnei* are nearly similar to those of the present study, while no *S. dysenteriae* was obtained . However, Ali *et al*. (2010) were reported *Shigella flexneri* was the most prevalent serogroup (72.7%) then *Shigella boydii* (18.2%) and *Shigella dysentriae* was the least common serogroup (9.1%).

In Iran, MoezArdalan *et al*. (2003) showed that the isolation rate of each spp. of *Shigella* was as follow: 7.5% *S. flexneri*, 5.2% *S. sonnei*, 2.6% *S. dysenteriae*, and 1.5% *S. boydii*. Also, Thiem *et al*. (2004) who recovered (80%) of *S. flexneri*, (19%) 0f *S. sonnei* and (1%) of *S. boydii* , this agreed with current study in that *S. dysenteriae* was not detected.

In India, *Shigella* infection turned endemic in Kolkata and *S. flexneri* (58%) was the most dominant serogroup, followed by *S. sonnei* (28%), *S. boydii* (9%), and *S. dysenteriae* (5%) (Dutta *et al*. 2002).

In developing nations, the prevalent serogroup is *S. flexneri*, which is described by long term persistence of sub lineages in regions of endemic shigellosis with insufficient hygienic conditions and unsafe water supplies (Connor *et al*., 2015). More seldom isolated are *S. dysenteriae*, causative agent of large epidemics in the past, and *S. boydii* (Livio *et al*., 2014).

Shigellosis happens predominantly in developing countries due to overcrowding and poor sanitation conditions., Children, non-breast fed infants, recovered children from measles, malnourished children, and adults more than 50 years are more susceptible and have a more severe disease and a greater risk of mortality (Schroeder and Hilbi, 2008).

In contrast to this study, Ranjbar *et al*. (2013) observed that *S. sonnei* was the most prevalent (60.7%) followed by *S. flexneri* (31.5%). It has been proposed that factors included in natural selection may have been the principle cause for these conflicts (Farshad *et al*., 2006).

In other study in India done by Pazhani *et al*. (2005) reported the distribution of *Shigella* spp. that showed *S. flexneri* was the most prevalent serogroup, followed by *S. sonnei*, *S. dysenteria* and *S. boydii*.

Shigella flexneri is the major species exist in developing countries (median 60% of isolates), with *S. sonnei* is the next most common (median 15%). *S. dysenteriae* and *S. boydii* occur with equal percentage (median 6%) (Kotloff *et al*., 1999). As well as, *S. flexneri* was reported to be the dominant species (68%) in a multi-centric study from six Asian nations involving China, Thailand, Vietnam, Bangladesh, Indonesia and Pakistan, except in Thailand where *S. sonnei* was the dominant (84%), while *S. dysenteriae*, which is most frequent isolated in Southern Asia and sub Saharan Africa, comprised only 4 % of the isolates (Taneja and Mewara, 2016).

In contrast, data from Spain, Israel and the United States consistently illustrate that *S. sonnei* is the most prevalent serogroup existed in industrialized nations (median 77%), then *S. flexneri* (median 16%), *S.boydii* (median 2%) and finally *S. dysenteriae* (median 1%) (Kotloff *et al*., 1999).

3.6. The antimicrobial susceptibility patterns

In present study, the antimicrobial susceptibility testing for *Shigella* isolates was performed by performing the Kirby-Bauer disk diffusion method and the obtained results were interpreted depending on CLSI (2016). In addition ,Vitek2 system was used to performed the susceptibility testing.

All confirmed 19 *Shigella* isolates were screened for their antibiotic resistance against 20 antimicrobial agents of different classes that recommended for *Shigella* infection according to (CLSI, 2016).(Table 3-5).

Table (3-5) Antibiotic susceptibility pattern of *Shigella* spp. isolates

Isolate No.	Kirby-Bauer method									Vitek2 system										
	AMP	LEV	CIP	CRO	CTX	PTZ	TE	NA	FOX	ATM	Cpe	CAZ	CS	IMP	MEM	Min	Pef	Pi	Ti	SXT
*1	R	S	S	R	R	S	R	I	S	S	S	S	S	S	S	I	S	R	R	R
*2	R	I	S	R	R	S	R	S	S	I	S	S	S	S	S	S	S	R	R	R
*3	R	S	S	S	R	S	I	R	S	S	S	S	S	S	S	I	S	R	R	R
*4	R	S	S	R	R	S	I	R	S	S	S	S	S	S	S	S	S	S	S	R
*5	R	I	S	R	R	S	R	I	S	S	S	S	S	S	S	I	S	R	R	R
6	I	S	S	I	R	S	I	S	S	I	S	S	S	S	S	S	I	S	S	S
*7	R	S	S	I	R	I	R	S	S	S	S	S	S	S	S	S	S	S	R	S
*8	R	I	S	R	R	S	R	I	S	S	S	S	S	S	S	S	S	R	R	R
*9	R	S	S	R	R	S	R	I	S	I	S	S	S	S	S	S	S	R	R	R
*10	R	S	S	I	R	S	R	S	S	S	S	S	S	S	S	S	S	S	R	R
*11	R	S	S	R	R	S	R	I	S	R	R	R	S	S	S	S	S	R	R	R
*12	R	R	R	R	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R
*13	R	I	S	S	R	S	R	R	S	I	S	S	S	S	S	S	I	S	S	S
*14	S	S	S	S	R	S	R	R	S	S	S	S	S	S	S	S	S	S	S	R
*15	R	S	S	R	R	S	R	S	R	R	R	R	S	S	S	S	S	R	R	R
*16	R	I	S	R	R	S	R	I	S	S	S	S	S	S	S	I	S	R	R	R
*17	R	S	S	R	R	S	R	R	S	R	S	R	S	S	S	S	I	R	R	R
*18	R	S	S	I	R	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S
*19	R	I	S	S	I	S	R	r	S	R	R	R	S	S	S	S	S	R	R	R

*** MDR *Shigella* isolate. R: Resistant, I: Intermediate, S: Sensitive**

The (Table3-6) revealed that there are high resistance rates among isolates to cefotaxime (94.7%), ampicillin (89.4%), tetracycline (84.2%) and moderate resistance trimethoprim-sulfamethoxazole (78.9%) and ticarcillin (68.4%), pipracillin (57.8%), while were showed resistance in lesser degree to ceftrixone (57.8%). All the isolates were sensitive to colistin (100%), imipenem (100%) and meropenem (100%). In addition, the isolates were showed high level of sensitivity to ciprofloxacin (94.7%), cefoxitin (94.7%), pipracillin-tazobactam (89.4%), cefepime (84.25). Resistance to cefotaxime was distributed as 90% of of *S. flexneri* isolates and 100% of *S. sonnei*.

Resistance to ampicillin was distributed as 100% of *S. flexneri* isolates and 87.5% of *S. sonnei*. Resistance to tetracycline was distributed as 100% of *S. flexneri* isolates, 75% of *S. sonnei* and 100% of *S. boydii*.

Table (3-6) Percentage of antibiotic susceptibility of each *Shigella* spp.

Anti-biotics	*S.flexneri* (10isolates)			*S.sonnei* (8 isolates)			*S.boydii* (1 isolate)			Total		
	R%	I%	S%	R%	I%	S%	R%	I%	S%	R%	I%	S%
AMP	100	0	0	87.5	12.5	0	0	0	100	89	5.26	5.26
LEV	10	40	50	0	25	75	0	0	100	5.26	31.57	63.15
CIP	10	0	90	0	0	100	0	0	100	5.26	0	94.73
CRO	60	20	20	62.5	25	12.5	0	0	100	57.89	21.05	21.05
CTX	90	10	0	100	0	0	0	0	100	94.73	5.26	0
PTZ	0	10	90	12.5	0	87.5	0	0	100	5.26	5.26	89.47
TE	90	10	0	75	25	0	100	0	0	84.21	15.78	0
NA	40	30	30	25	37.5	37.5	100	0	0	36.84	31.57	31.57
FOX	0	0	100	12.5	0	87.5	0	0	100	5.26	0	94.73
ATM	30	10	60	12.5	37.5	50	0	0	100	21.05	21.05	57.89
CPE	20	0	80	12.5	0	87.5	0	0	100	15.78	0	84.21
CAZ	30	0	70	12.5	0	87.5	0	0	100	21.05	0	78.94
CS	0	0	100	0	0	100	0	0	100	0	0	100
IMP	0	0	100	0	0	100	0	0	100	0	0	100
MEM	0	0	100	0	0	100	0	0	100	0	0	100
MIN	0	10	90	0	37.5	62.5	0	0	100	0	21.05	78.94
PEF	0	20	80	0	12.5	87.5	0	0	100	0	15.78	84.21
PI	50	0	50	75	0	25	0	0	100	57.89	0	42.10
TI	70	0	30	75	0	25	0	0	100	68.42	0	31.57
SXT	80	0	20	75	0	25	0	0	100	78.94	0	21.05

The highest resistance rate to ampicillin and trimethoprime-sulphamethoxazole observed in this study also detected in *Shigella* spp. isolated from diarrheal patients by Nave *et al*. (2016) in Kerman, Iran that represented (92.9%) and followed by tetracycline (78.6%) and nalidixic acid (21.4%). Additionally, the former researcher observed *Shigella* spp. resistant to the third generation cephalosporins that comprised (58.9%) of resistant to cefotaxime and (48.2%) of resistant to ceftriaxone , respectively, and *S. sonnei* showed to be highly resistant to these antibiotics, so that (88.9%) of its isolates were cefotaxime resistant and (72.2%) isolates were ceftriaxone resistant. These observation lower than the current obtained results that exhibited (94.7%) and (57.8%) of resistant *Shigella* isolates to cefotaxime and ceftriaxone , respectively, whereas (100%) of *S. sonnei* were cefotaxime resistant and (62.5%) were ceftriaxone resistant, while *S. flexneri* exhibited (90%) and (60%) resistance rate to cefotaxime and ceftriaxone, respectively .

An Iraqi study done by Al-Rahman (2013) had approximate results to this study, in which showed high resistance rate to ampicillin (84.7%), tetracycline (84.7%) and cefotaxime (52.5%), moderate resistance to ceftriaxone (52.5%) and low resistance to ceftazidime (30.5%).

In this study majority of *Shigella* isolates were exhibited resistance to different antibiotics (94.7% , 18/19). A strain is described as a multidrug resistant (MDR) if an isolate is resistant to representatives of three or more classes of antimicrobial agents (Falagas and Karageorgopoulos, 2009).

Multidrug resistance was exhibited in 10 (100%) of *S. flexneri*, 7 (87.5%) of *S. sonnei* and 1 (100%) of *S. boydii*.

The high rate of MDR isolates in this study was closely similar to another high rate which represented (95.1%) of total *Shigella* isolates achieved by Zamanlou *et al*. (2018) in Iran.

On the other hand, current result is higher than one obtained in Rural Mozambique by Mandomando *et al*. (2009) who recorded (65 %) of the *Shigella* isolates were multidrug resistant.

The continuous usage of even if a single antibiotic agent over a time of weeks or months will make pathogens resistant to various kinds of antimicrobial agents as well as the one in use (Livermore, 2007).

In the present study, *Shigella* spp. exhibited nearly equal percentage against nalidixic acid (31.5% susceptible , 31.5% intermediate and 36.8% resistant). However, the presence of considerable rate of intermediate resistant to nalidixic acid can be result in the possible increase in *Shigella* resistance to this antimicrobial drug. On the other hand, in Switzerland, nalidixic acid resistance was showed in all species obtained during 2004–2014 (Nüesch-Inderbinen *et al*., 2016).

Poramathikul *et al*. (2016) in Cambodia, reported that 10 (91%) of the 11 *Shigella* isolates were resistant to ampicillin, tetracycline, trimethoprim-sulfamethoxazole, and nalidixic acid.

The development of antibiotics resistance in these isolates is frequently related to the misuse and overuse of the antibiotics appointed. Iraq is one of the developing countries where antibiotics sold over the counter leading to encouraging self-medication (Al-Charrakh, 2005).

Notably, the present study observed that all *Shigella* isolates were susceptible (100%) to colistin, imipenem and meropenim and high susceptibility rate to ciprofloxacin (94.7%), cefoxitin (94.7%) and levofloxacin (63.1%). This results was very similar to the results reported

from Iranian study by Zamanlou *et al*. (2018) showed no resistance was found to carbapenems including (imipenem and meropenem) and high susceptibility percentage to ciprofloxacin, cefoxitin and levofloxacin.

The carbapenem antibiotics like meropenem and imipenem are most effective and reliable which has highly active *in vitro* against Enterobacteriaceae including organisms that produce ESBLs (Samaha-Kfoury and Araj, 2003; Paterson *et al*., 2005).

However, Nüesch-Inderbinen *et al*. (2016) observed the emergence of ciprofloxacin resistance among *Shigella* spp. isolates. Fortunately, this study observed very low resistance rate to ciprofloxacin (5.26 %) which consistent with those achieved in Belgium by Vrints *et al*. (2009) who observed that all *Shigella* isolates were susceptible to ciprofloxacin during a period of 18 years from 1990 to 2007.

Colistin was active against Gram-Negative bacteria, which firstly used therapeutically during the 1950s in Japan and in Europe and in the United States in the form of colistimethate sodium in 1959 (Reed *et al*., 2001). However, the colistin was gradually rejected in most parts of the world in the early 1980s due to the observed high incidence of nephrotoxicity (Nation and Li, 2009). However, the emergence of resistant bacteria to majority of classes of commercially usable antibiotics and the inadequacy of new antimicrobial drugs with efficiency against Gram-negative bacteria have resulted in the reconsideration of colistin (polymyxins) as a valuable therapeutic option (Boucher *et al*., 2009).

Dwipoerwantoro *et al*. (2005) in Indonesia showed that100% of *Shigella* isolates were sensitive to colistin and this agreed with present study, but the other antibiotics patterns disagreed in that the isolates were completely resistant (100%) to ceftriaxone and nalidixic acid.

In United States, first national surveillance to determine of antimicrobial resistance among isolates of *Shigella* was carried out in 1999. It was a part of National Antibiotic Resistance Monitoring System (NARMS) for enteric bacteria (Sivapalasingam *et al*., 2006).

A local study by Abdulrahman *et al*. (2015) was reported that *S. flexneri* and *S. sonnei* isolates were resistant to ciprofloxacin and nalidixic acid in percentage reached to 49.2% and 54.2%, respectively.

Twenty-eight *Shigella* strains were isolated from children with diarrhea only. AMP and SXT showed very low activity against *Shigella* strains with one strain was susceptible to all tested antibiotics. There were 22 of 28 (78.6%) multi-antibiotic-resistant *Shigella* strains. One *S. boydii* strain was resistant to all tested antibiotics. The most common multi-resistance pattern was Amp^r Ctx^s Nal^s Cip^s Ipm^s Sxt^r with a prevalence of 35% (Nguyen *et al*., 2005).

Another local study by Mohammed (2009) who determined the drug sensitivity patterns of 9 isolates of *Shigella* spp. Resistant to ampicillin was found to be 78%, and 89% to trimethoprim-sulphamethoxazole, these are closely similar to present results . The same study was showed that all isolates were susceptible to ceftriaxone, ciprofloxacin and nalidixic acid, while the current study was reported 11 of 19 *Shigella* isolates were resistant to ceftriaxone and 7 of 19 isolates were resistant to nalidixic acid.

The irregular use of broad spectrum antibiotic drugs causes the bacteria more resistant to these antibiotics that lead to an increase in morbidity rate, (Zhanel *et al*., 2000). The broadly spread use of this way has criticized on the ground of drug toxicity and the risk of an increase spread of antibiotic resistance (Aiyegoro *et al*., 2007).

3.7. Detection of Extended-Spectrum β-Lactamases

The detection strategies for ESBL can be partitioned into two kinds: phenotypic strategies, which identify the capacity of the ESBL enzymes to hydrolyze diverse cephalosporins, and genotypic (molecular techniques) strategies, which identify the genes responsible for the ESBL production.
The phenotypic strategies are widely utilized by clinical analytic laboratories because these methods are simple to operate and they are also cost effective. In addition, the consolidation to automated sensitivity systems, have made them simply accessible (Wiegand *et al*., 2007).

3.7.1. Disk Approximation Method

This method was the first detection test described in 1980's; it is also known as double-disk test (Jalier *et al*., 1988). In this test, a disk containing amoxicillin clavulanate is locate in proximity to disks incorporated with oxyimino-β-lactam and aztreonam antibiotics. The zone enhancement of inhibition of the oxyimino-β-lactam is a positive result (Bradford, 2001).

In this study the test was performed by putting cefotaxime, ceftazidime, ceftriaxone, and aztreonam antibiotic discs (30 µg for each one) on Muller Hinton agar at a similar distances (30 mm from edge to edge) from the amoxicillin/clavulanic acid (Augmentin) disc which put in the center of the agar plate. (Figure 3-11 A and B).

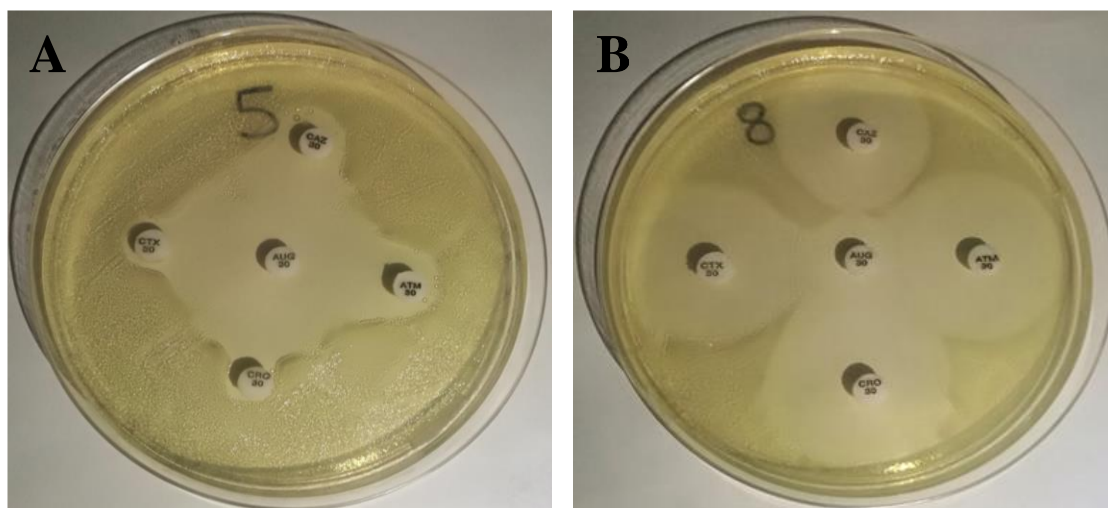


Figure (3-11) Disk approximation test for detection of ESBL in *Shigella* spp.(AUG, Augmentin; ATM, Aztreonam; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime); A: Positive result ; B: Negative results.

The clavulanic acid is separated and diffused around disc, this component can penetrate the bacterial cell walls and can inactivate extracellular enzymes (Bedenic *et al.*, 2001; Livermore and Brown, 2001). The synergy effect seen depended on the diffusion from side to side the agar plate. An obvious enhancement of the inhibition zone was seen on sides of the amoxicillin/clavulanic acid disc against other discs, these obvious enhancement revealed some synergistic effect between the clavulanic acid and other an oxyimino-cephalosporin (third generation cephalosporins) and monobactam antibiotics which denote that these bacteria are ESBL producers, while the isolates without synergistic effect considered non-ESBL producers (Al-Jasser, 2006) .

All the 19 *Shigella* isolates were investigated for ESBL production by disk approximation test. The results revealed that the three species of *Shigella* obtained in this study had ESBL-positive phenotype.

Table (3-7) The rate of ESBL production among *Shigella* spp. by disk approximation method

***Shigella* spp.**	**Total isolates**	**Disk approximation method for ESBL production**
S. flexneri	10	7
S. sonnei	8	7
S. boydii	1	1
Total	**19**	**15**

The results of current study observed that 7 of 10 *S. flexneri* isolates , 7 of 8 *S. sonnei* isolates and 1 of 1 *S. boydii* isolate were ESBL producers isolates. (Table 3-7).

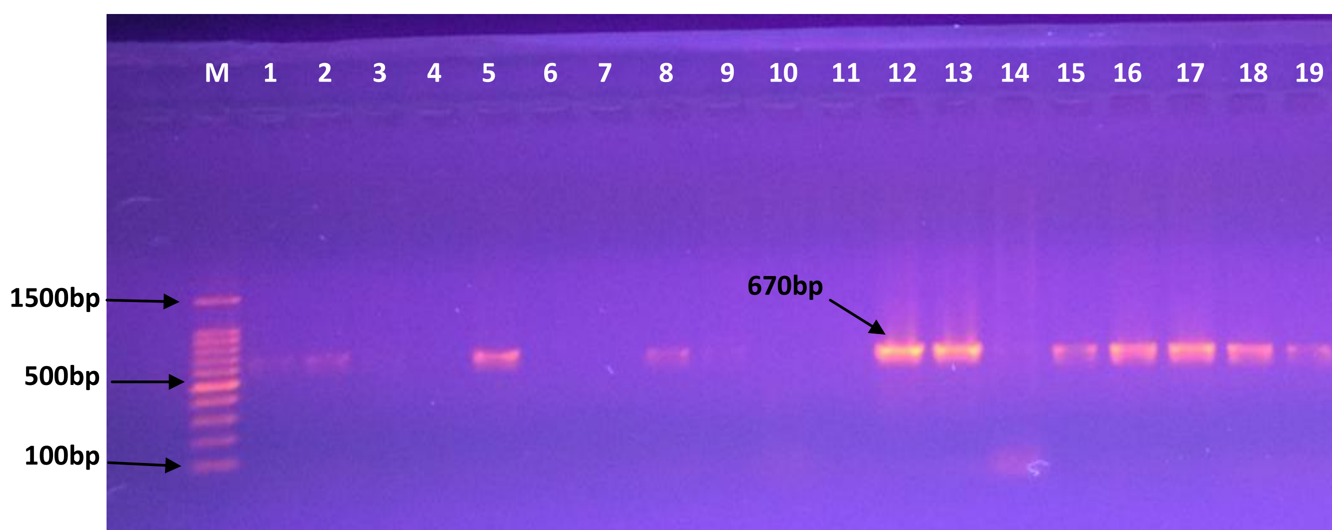
Although the β-lactamases absolutely play a crucial role in the β-lactam antibiotics resistance, the high rate of resistance to ampicillin was not only attributable to the production of β-lactamase enzymes. The other mechanism conferring resistance to these compounds is caused by lowering of the activity of β-lactam antibiotics in a resistant cell due to several factors such as the sensitivity of the antibiotic to β-lactamases, the penetration through the outer membrane, the affinity for the target (PBPs), the amount of β-lactamase, and the affinity of the antibiotic for the β-lactamase (Stapleton *et al*., 1999; Philippon *et al*., 2002; Jacoby and Munoz-Price, 2005).

In this study, the ESBL production among *Shigella* spp. (15 among 19) were high if compare with results achieved in Iran by Ranjbar *et al*. (2013) who reported that 4 among 55 *Shigella* spp isolates, involving three *S. sonnei* and one *S. flexneri,* exhibited an ESBL positive phenotype.

3.7.2. Molecular detection of β-lactamases genes

The best efficiency method for ESBL detection is PCR because it is more rapid than phenotypic detection method and also detect the presence of inadequately or non-expressed genes difficult to determine by phenotype (Chiangjong, 2006). Moreover, plasmid of a pathogen may harbor more than one gene that may encode for different types of ESBL enzyme. Additionally, the spread of most broad spectrum β-lactamases is facilitated by transferable and trans-conjugable plasmids, which often convey other resistance genes by means of their integrin design (Jacoby and Munoz-Price, 2005).

All the *Shigella* isolates were investigated genotypically for harboring β-lactamases genes including bla_{AmpC}, $bla_{CTX\text{-}M}$, bla_{TEM}, and bla_{SHV} by PCR technique. The target amplicon size were 670bp, 247bp, 531bp and 410bp, respectively.



Figure(3-12) Ethidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of bla_{AmpC} gene (670bp) for 1 hours at 70 volts. Lane M: DNA marker Lane (1, 2, 5, 8, 12, 13, 15, 16, 17, 18 , 19) Amplified bla_{AmpC} gene(670bp).

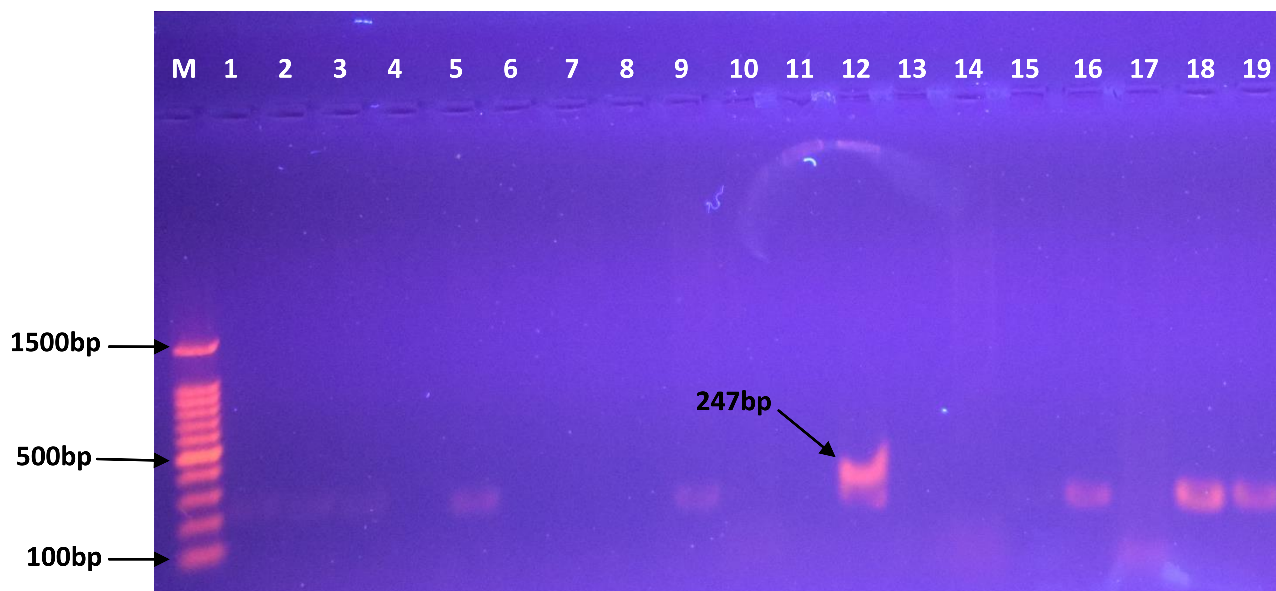


Figure (3-13) Ethidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of *bla*$_{CTX-M}$ gene (247bp) for 1 hours at 70 volts. Lane M : DNA marker Lane (1, 2, 3, 5, 9, 12, 16, 18 and 19) Amplified *bla*$_{CTX-M}$ gene (247bp).

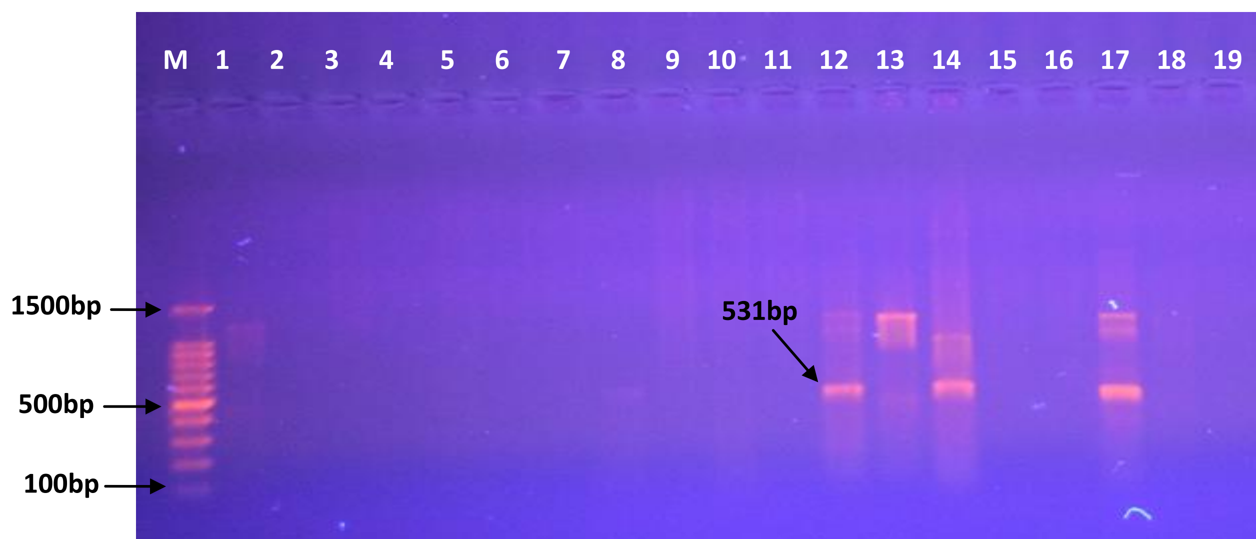


Figure (3-14) Ethidium bromide stained agarose gel electrophoresis (1.5%) of PCR amplified of *bla*$_{TEM}$ gene (531bp) for 1 hours at 70 volts. Lane M: DNA marker Lane (12, 14, 17) Amplified of *bla*$_{TEM}$ gene (531bp).

In this study, regarding to PCR results showed that *bla*$_{AmpC}$ gene and *bla*$_{CTX\text{-}M}$ gene were the most present in investigated bacterial isolates from than *bla*$_{TEM}$ gene, while *bla*$_{SHV}$ was not detected. *S. flexneri* isolates differentiated into (6/10) harbored *bla*$_{AMPC}$ gene, (3/10) harbored *bla*$_{CTX\text{-}M}$ gene and (2/10) harbored *bla*$_{TEM}$ gene. *S. sonnei* isolates differentiated into (5/8) harbored *bla*$_{AmpC}$ gene and (6/8) harbored *bla*$_{CTX\text{-}M}$ gene. *S. boydii* (1/1) was harbored *bla*$_{TEM}$ gene only.

An increasing concern around the world is the bacterial resistance to broad spectrum β-lactams which mediated by extended spectrum β-lactamase (ESBL), and AmpC beta-lactamases (AmpC) (Rizvi *et al*., 2009). These enzymes usually cause inappropriate therapy rising to increase infections and mortality, medical cost and elongated residence in hospital Bacterial isolates that possess such genes are usually resistant to various antimicrobial drugs and can confront treatment, making therapeutic choices are few (Tseng *et al*., 2014).

The ESBLs frequently locate on plasmids and the common members are CTX, TEM and SHV families. Plasmids involving encoded genes for ESBLs usually accommodate resistance determinants for several classes of antimicrobial drugs by possessing various resistance mechanisms and are simply transmitted from strain to strain and among particular species of enteric Gram-negative rods (Mehrgan *et al*., 2009). Another resistance mechanism includes overproduction of chromosomal or plasmid-derived AmpC beta-lactamases (Pfaller and Segreti, 2006).

The AmpC overexpression in clinical isolates is mostly due to a mutation in *ampD* causing AmpC hyperinducibility or significant hyperproduction (Schmidtke and Hanson, 2006).

Organisms producing adequate AmpC β-lactamase will typically yield a positive ESBL screening test but these organisms are unsuccessful in the confirmatory test including increased susceptibility with clavulanic acid (Tenover *et al*., 2003; Bell *et al*., 2007).

The CTX-M types ESBLs are plasmid-mediated β-lactamases having higher hydrolytic action against cefotaxime. CTX-M-15 has been observed as common genotype of ESBL among *Shigella* isolates (Parajuli *et al*., 2017).

The SHV (sulfydryl variable) and TEM enzymes are another family of β-lactamases. The SHV-1 is the originator of the SHV enzymes and it is first described in *Klebsiella pneumonia*. The SHV mediates resistance to broad-spectrum penicillins. TheTEM-1 was first manifested in 1965 in an *Escherichia coli* isolate from Athenian patient, named Temoneira (designated TEM) (Datta and Kontomichalou, 1965). Mutants of the TEM derivatives (CMT-1, CMT-2, CMT-3 and CMT-4) have been detected that are able to hydrolyze third generation cephalosporins and β-lactamase inhibitors (Fiett *et al*., 2000; Neuwirth *et al*., 2001).

In Iraq, Al-Rahman (2013) who observed that bla_{CTX-M} genes were identified in (74.19%) *Shigella* isolates whereas (45.16%) of *S. flexneri* and (29.03%) of *S. sonnei*, while no gene was detected in ESBLs *S. dysenteria* isolates. Nevertheless, this study reported high occurrence of bla_{CTX-M} genes harboring *S.sonnei* and lower in *S. flexneri*.

The present results were approximate to results of Chinese study by Zhang *et al*. (2014) that observed the majority of *Shigella* isolates carried $bla_{CTX\text{-}M}$ genes and with less number of isolate carried bla_{AmpC} genes and bla_{TEM}, while no isolate carried bla_{SHV}, but Akhi *et al*. (2016) showed different results that bla_{SHV} and bla_{TEM} genes were the most dominant in *Shigella* isolates from than $bla_{CTX\text{-}M}$ and AmpC plasmid mediate (*cmy*) gene. Among all the isolates, (25.9%) isolates had only single gene, which (18.5%) were bla_{SHV} and (7.4%) were bla_{TEM}.

In Nepale, Parajuli *et al*. (2017) who firstly reported two *S. flexneri* isolates hosting ESBL genotype CTX-M associated with acute dysentery in two siblings.

The CTX-M enzymes appear to processes a superior capacity to diffuse and result in outbreaks. To date, there are more than 50 variants of CTX-M, and they have been related to several outbreaks of infections in the hospitals and in the community (Dhillon and Clark, 2012).

Molecular demonstration of resistant to third generation cephalosporin has revealed CTX-M type ESBLs, especially CTX-M-15, as the most frequent ESBL determinants in different nations like India (Taneja *et al*. 2012), China (Zhang *et al*., 2011) and Vietnam (Nhu *et al*., 2010), whereas in Korea, TEM-19, TEM-15, TEM-52 and CTX-M-14 beta-lactamases were appeared to be prevalent ESBL types (Kim *et al*., 2004).

Spanu *et al*. (2002) in Italy and Aggarwal *et al*. (2016) in India had recorded production of ESBL (TEM/SHV-type) in Enterobacteriaceae to be associated with resistance to aminoglycosides and ciprofloxacin. In this

study, the low occurrence of TEM type and absence of SHV type may be due to ciprofloxacin-susceptible *Shigella* spp. isolated in current study.

From North India, Taneja *et al*. (2012) reported (9/20) of *S. flexneri* were ESBL producers and four of them were AmpC positive.

In this study 6 from 8 (75%) of *S. sonnei* isolates hosted $bla_{CTX\text{-}M}$ gene, but bla_{TEM} gene was not exhibited, while Sabra *et al*. (2009) in Lebanon who found 4 from 5 of *S. sonnei* harbored the $bla_{CTX\text{-}M\text{-}15}$ gene and 3 of them harbored the $bla_{TEM\text{-}1}$ gene.

The clinical and trade tension to use β-lactams, likewise the international traveling of people, animals and food products ensure the continuous spreading of β-lactamase genes .

3.8. Genetic diversity among *Shigella* spp. isolates

In this Study, a method for investigation of the genetic correlation among 19 *Shigella* isolates utilizing PCR primers derived from within ERIC sequences was employed. The amplicon was electrophoresed on 1.5% ethidium bromide agarose gel at 70 volts for 1.5 hours, if the positions and numbers of copies vary among different isolates, the amplification products yield each with a unique fingerprint.

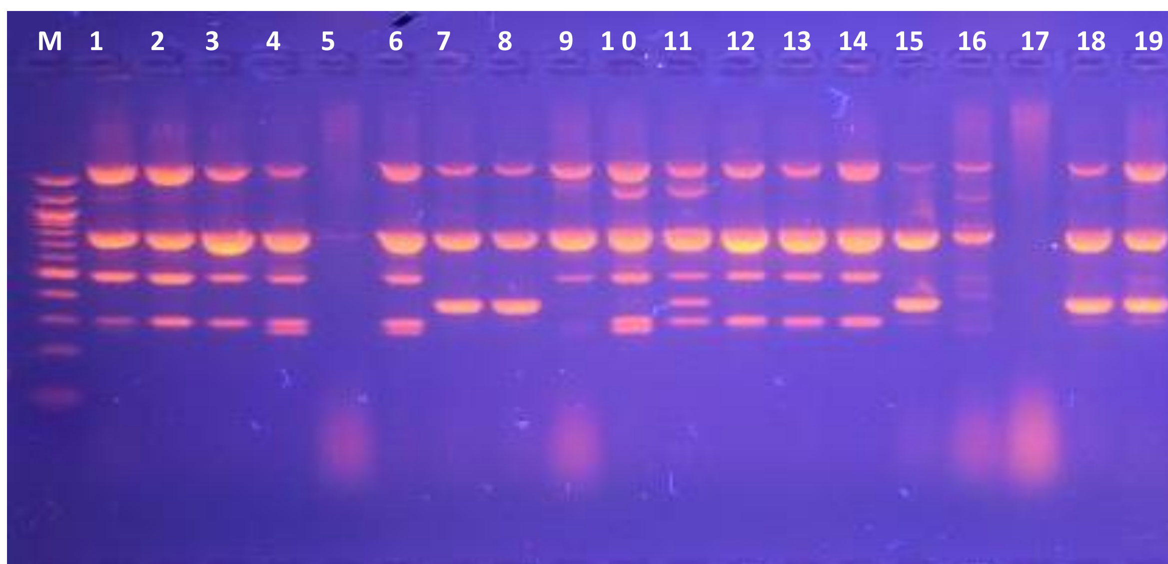


Figure (3-15) Ethidium bromide stained agarose gel electrophoresis (1.5%) of ERIC- PCR results of 19 *Shigella* isolates for 1.5 hours at 70 volts. Lane M : DNA marker; Lane (1 to 19) ERIC bands profiles of *Shigella* spp. isolates.

Amplification 1 to 6 bands ranging with different sizes were produced (Figure 3-15). DNA banding patterns revealed considerable diversity of genetic types of 19 *Shigella* spp. isolates in Al-Diwaniyah hospitals. No banding patterns were obtained from one isolate may be due to DNA auto-digestion. The *Shigella* isolates were separation into 11 clusters (E1–E11) in the current study. Attractively, *S. flexneri* isolates in this study observed a standardized band pattern with the pattern given by *S. sonnei* isolates, which can proposed that interspecies variance do not essentially influence the ERIC-PCR band profiles of *Shigella* strains.

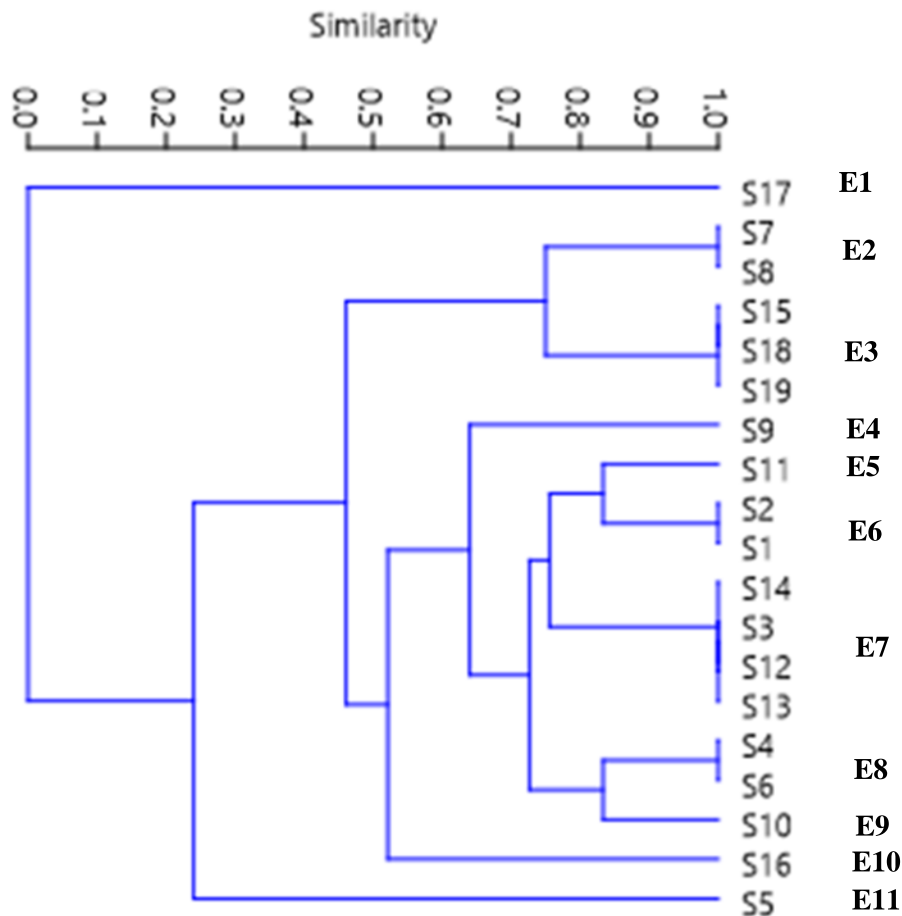


Figure (3-16) Dendrogram UPGMA generated from (ERIC-PCR) band pattern of 19 *Shigella* spp. isolated from diarrheal patients. The scale at the top of the figure shows the percentage similarity. E1(S17, *S.flexneri*), E2(S7, *S.flexneri* ; S8, *S.flexneri*), E3(S15, *S.sonnei*; S18, *S.sonnei*; S19, *S.flexneri*), E4(S9, *S.sonnei*), E5(S11, *S.flexneri*), E6(S2, *S.sonnei*; S1, *S.sonnei*), E7(S14, *S.boydii*; S3, *S.sonnei*; S12, *S.flexneri*; S13, *S.flexneri*), E8(S4, *S.flexneri*; S6, *S.sonnei*), E9(S10, *S.flexneri*), E10(S16, *S.flexneri*), E11(S5, *S.sonnei*).

It is clear from Figure (3-16) that 10 isolates of *S. flexneri* grouped into 8 different patterns (E1, E2, E3, E5, E7, E8, E9, E10), and 8 *S. sonnei* isolates grouped into 6 different patterns (E3, E4, E6, E7, E8, E11), that mean there

is considerable genetic diversity among the predominant *Shigella* serotypes, which are *S. flexneri* and *S. sonnei* in our region.

The stable and genetically related *Shigella* clones in a given area are the principle cause of *Shigella* outbreaks in this area (Shokoohizadeh *et al*., 2017).

Bakhshi *et al*. (2018) in Iran, who differentiated 50 isolates into 5 ERIC types, which were grouped into five clusters (ET1–ET5) by using ERIC-PCR profiles and showed a high degree of similarity among the isolates strains.

Kosek *et al*. (2012) were done a comparative study of molecular typing methods for *Shigella* and showed that ERIC-PCR is a credible alternative approach to pulsed field gel electrophoresis (PFGE). Additionally, in United states, Finger *et al*. (2006) who revealed that the longer length of the ERIC-PCR (22 bp) contrast to 10–12 bp in standard RAPD primers appears to increase the efficiency of primers and the power of the ERIC-PCR compared with RAPDs.

An Iranian study by Ranjbar *et al*. (2013) was revealed five ERIC patterns (E1-E5) appeared between *S. sonnei* isolates and because the half of these isolates were grouped in E4 pattern confirming the epidemic nature of the *S. sonnei* strain resulting in many outbreaks per year in Iran.

In China, Zhang *et al*. (2014) obtained ERIC-PCR results which indicated that two and three principle genotypes were identified in *S. flexneri* and *S. sonnei,* respectively.

3.9. Distribution of β-Lactamase genes, ESBLs production and pattern of ERIC-PCR among 19 *Shigella* isolates.

Table (3-8) illustrates the summary for the distribution of β-Lactamase genes including CTX-M, AmpC and TEM , ESBLs production and pattern of ERIC-PCR among 19 *Shigella* isolates.

Table (3-8) Distribution of β-Lactamase genes(CTX-M, AmpC and TEM), ESBLs production and pattern of ERIC-PCR among 19 *Shigella* isolates.

No. of isolates	*Shigella* genus (PCR)	*Shigella* species (PCR)	β-Lactamase genes (PCR)	ESBLs production	ERIC-PCR Pattern
1	+	*S. sonnei*	CTX-M , AmpC	+	E6
2	+	*S. sonnei*	CTX-M , AmpC	+	E6
3	+	*S. sonnei*	CTX-M	-	E7
4	+	*S. flexneri*	__	+	E8
5	+	*S. sonnei*	CTX-M , AmpC	+	E11
6	+	*S. sonnei*	__	+	E8
7	+	*S. flexneri*	__	+	E2
8	+	*S. flexneri*	AmpC	-	E2
9	+	*S. sonnei*	CTX-M	+	E4
10	+	*S. flexneri*	__	-	E9
11	+	*S. flexneri*	__	-	E5
12	+	*S. flexneri*	CTX-M, AmpC, TEM	+	E7
13	+	*S. flexneri*	AmpC	+	E7
14	+	*S. boydii*	TEM	+	E7
15	+	*S. sonnei*	AmpC	+	E3
16	+	*S. flexneri*	CTX-M , AmpC	+	E10
17	+	*S. flexneri*	AmpC, TEM	+	E1
18	+	*S. sonnei*	CTX-M , AmpC	+	E3
19	+	*S. flexneri*	CTX-M , AmpC	+	E3

Conclusions and Recommendations

Conclusions and Recommendations

Conclusions:

1- *Shigella* species contributed to 6.73% of diarrhoeal cases as a causative agents in in Al-Diwaniyah hospitals .

2- The predominant species among *Shigella* species is *Shigella flexneri* followed by *Shigella sonnei* with simple disparity. *Shigella boydii* had lowest occurrence rate and *Shigella dysenteriae* was not detected

3- The majority of *Shigella* isolates are multidrug resistant and this is a matter of concern resulting in threating of shigellosis treatment in this region.

4- Carbapenems (imipenem and meropenem) and colistin antibiotics are fully effective against shigellosis. However, there are precautions about using colistin as a drug of choice for treatment. Ciprofloxacin, cefoxitin and pipracillin-tazobactam are highly effective treatment against shigellosis in this region.

5- Alarming occurrence rate of β-lactamases producing *Shigella* isolates was revealed, especially CTX-M and AmpC which are the most common among investigated isolates.

6- The advanced techniques like PCR should be dependable in our hospitals to confirm diseases diagnosis and to decrease time consuming paid in conventional methods.

7- There is a considerable genetic diversity among *Shigella* isolates in Al-Dewaniyah city.

Recommendations

1- Continued studies should be carried on *Shigella* drug resistance to recognize timely adaptation of therapeutic recommendations to be made.
2- Saving and documentation of the information about the prevalence of a diarrhoeal diseases and even other diseases in our country. This leading to facilitate the control of the diseases in the country.
3- Continuous wakefulness of the food safeness, travellers control, health instructions of food handlers, and tight attention to personal hygiene and sanitary conditions can supply an effective barrier against the shigellosis spreading.
4- Reduction of randomly antibiotic usage and commitment of physician prescription resulting in decreasing of β-lactamases producing bacteria.
5- Study of the association between *Shigella* virulence factors and antimicrobial drugs.
6- Further researches must be employed to make continued surveillance of β-lactamases-producing bacteria in Al-Diwaniyah city and other parts in Iraq, which will be helpful in monitoring antimicrobial resistance and reduce its occurrence.

References

References

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Appendix

Questionnaire

- **Name : --**
- **Age: --**
- **Gender : --**
- **Stool appearance :------------------------------**

- **Symptoms:--**

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- **Date : --**

الخلاصــة

تعد أنواع بكتريا *Shigella* من بين المسببات البكتيرية الشائعة لأمراض الإسهال. و التي تسبب داء الشيغلات بجرعة معدية منخفضة، وهي عدوى معوية حادة تتسم بأسهال مائي مصحوب بالدم والمخاط ومعروف أيضا بأسم الزحار العصوي .

هدفت الدراسة الحالية الى الكشف عن تنوع و نمط مقاومة المضادات الميكروبية لأنواع بكتريا الشيغيلا المعزولة من مرضى الإسهال الحاد الذين دخلوا مستشفيات الديوانية، العراق وتحديد العلاقة الجينية لهذه الأنواع المعزولة.

جُمعت ٢٨٢ عينة براز من المرضى ومن مختلف الفئات العمرية الذين يعانون من الإسهال في مستشفى الحسين للأطفال ومستشفى النسائية والأطفال والمستشفى التعليمي في الديوانية أثناء الفترة الممتدة من تشرين الثاني لسنة ٢٠١٧ و لغاية أيار لسنة ٢٠١٨.

تم أستعمال أربعة أوساط زرعية انتقائية و تفريقية لتحديد أنواع بكتريا *Shigella* و ذلك لزيادة فرصة العزل، وهي XLD agar , MacConkey agar, Hektoen enteric agar و agar Sallmonella Shigella . المستعمرات المشتبه فيها تم تشخيصها بنظام Api20 E و أعطى نتائج أيجابية ل ٢٣ عزلة. كذلك تم اختبار تأكيدي لهذه العزلات بواسطة نظام Vitek2 التشخيصي و أعطى نتائج أيجابية ل ٢١ عزلة فقط .

تم أستخدام تفاعل سلسلة البلمرة المتعدد لتحديد جنس بكتريا *Shigella* و ذلك بأستخدام جين *invC* كبادئ متخصص، و كانت ١٩ عزلة إيجابية. و كذلك تم أستخدام جينات مختصة مستهدفة للتمييز بين أنواع الشيغيلا وهي (*rfc*, *wbgZ*, *rfpB* and conserved hypothetical protein) لتشخيص *Shigella flexneri*، *Shigella sonnei* ، *Shigella dysenteriae* و *Shigella boydii* على التوالي. كشفت النتائج التي تم الحصول عليها بأن معدل عزل *Shigella* (٦,٧٣%، ٢٨٢/١٩). وشملت ٦ (٣١,٥%) من الذكور و ١٣ (٦٨,٤%) من الأناث. وأن أرتفاع معدل الإصابة (٧٨,٩%، ١٩/١٥) قد ظهرت في الفئة العمرية ١٠ سنوات أو أقل، بينما أظهرت انخفاض في المعدل (١٥,٧%، ١٩/٣) في من تتراوح أعمارهم بين ١١ و ٢٠ سنة وأظهرت معدل (٥,٢ في المائة، ١٩/١) في الأعمار ٤١ إلى ٥٠ سنة. كانت الأنواع المعزولة الأكثر شيوعاً *Shigella flexneri* بنسبة (٥٢,٦٣%، ١٩/١٠) تليها *Shigella sonnei* بنسبة (٤٢,١٠%، ١٩/٨) و *Shigella boydii* بنسبة (٥,٢٦%، ١٩/١).

تم أختبار قابلية مقاومة عزلات *Shigella* للمضادات الحيوية بواسطة طريقة Kirby-Bauer ونظام Vitek2 ضد ٢٠ مضاد ميكروبي. عزلات الشيغيلا أظهرت معدلات مقاومة عالية إلى سيفوتاكسيم (٩٤,٧%)، الأمبيسلّين (٨٩.٤%)، التتراسيكلين (٨٤,٢%)، وترايميثوبريم سالفاميثاكزازول (٧٨%) . و أظهرت معدلات مقاومة معتدلة إلى تيكارسيلين (٦٨,٤ %)، سيفترياكسون (٥٧,٨ %) و بيبراسيلين (٥٧,٨%). وكانت حساسة تمامًا (١٠٠%) للإميبينيم والميروبينيم والكوليستين وحساسة للغاية للسيبروفلوكساسين (٩٤.٧%)، (٩٤,٧%) سيفوكزتين، (٨٩,٤%) بيبراسيلين تازوباكتام، (٨٤,٢ %) سيفيبيم و (٨٤,٤ %) بيفلوكساسين. و كانت العزلات متوسطة الحساسية للمضادان سيفتازيديم و المينوسايكلين بنسبة (٧٨,٩٤) , ليفوفلوكساسين (٦٣,١٥) و أزتريونام (٥٧,٨٩) بينما أظهر العزلات نسبة مقاومة و حساسية متساوية للمضاد ناليدكسك أسد. وكانت غالبية عزلات الشيغيلا متعددة المقاومة للأدوية بنبسة (٩٤.٧%، ١٩/١٨) لأنها أظهر مقاومة لفئات مختلفة من المضادات الحيوية.

بالإضافة إلى ذلك، تم فحص عزلات *Shigella* جزيئيا لمعرفة تواجد جينات β-lactamases و التي شملت (bla_{CTX-M} , bla_{TEM} , bla_{SHV} and bla_{AmpC}). كان توزيع الجينات β-lactamases كالآتي: عزلات *Shigella flexneri* تأوي bla_{CTX-M} بنسبة (٣٠% ، ١٠/٣) ، bla_{AmpC} بنسبة (٦٠% ، ١٠/٦) ، bla_{TEM} بنسبة (٢٠% ، ١٠/٢) ، *Shigella sonnei* تأوي bla_{CTX-M} بنسبة(٧٥% ، ٨/٦) ، bla_{ampC} بنسية (٦٢,٥% ، ٨/٥) و *Shigella boydii* تأوي فقط bla_{TEM} بنسبة (١٠٠% ، ١/١) بينما لم يظهر الجين bla_{SHV} في أياً من العزلات.

وأخيراً، تم دراسة العلاقة الجينية لعزلات الشيغيلا بواسطة أستخدام تقنية التوافق الناسخ الجيني البيني للبكتريا المعوية - تفاعل البوليميراز المتسلسل. نتجت هذه التقنية عن ١ إلى ٦ حزم بأحجام جزيئية مختلفة ، و أعطت عزلات *Shigella* ١١ نمط مختلف (E1-E11) وهذا يفسر وجود تغاير جيني بين العزلات المستحصلة في هذه المنطقة.

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فرع الأحياء المجهرية

التوصيف الجزيئي و الحساسية للمضادات الحيوية لبكتريا الشيغيلا (*Shigella*) المعزولة من مرضى الأسهال

رسالة مقدمة إلى

مجلس كلية الطب – جامعة القادسية

كجزء من متطلبات نيل درجة الماجستير

في علم الأحياء المجهرية الطبية

من قبل

شيماء شاكر جواد

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ذو الحجة ١٤٣٩ هـ   آب ٢٠١٨ م