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Molecular Study of Some Toxins and Antibiotic Resistance Genes Among Clinical Isolates of *Shigella* Spp.

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(بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ)

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صدق الله العظيم

المجادلة الآية-11

Supervisor's Certificate

I certify that this thesis entitled "Molecular Study of Some Virulence and Antibiotic Resistance Genes Among Clinical Isolates of *Shigella* Spp." was prepared under my supervision at the Department of Biology, College of Science/ University of Babylon, as a partial fulfillment of the requirement for the degree of Doctor of Philosophy in Biology/ Biotechnology.

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Saja

Summary

The current study was conducted to investigate the molecular characterization of some virulence and resistance genes among clinical isolates of *Shigella* spp. A 200 stool specimens were collected from different ages (adults, teenagers and children) of male and female patients. The samples came from patients with diarrhea who were hospitalized to general hospitals, teaching hospitals for women and children, and Al-Diwaniyah teaching hospitals between November 2020 and May 2021. A case sheet for each patient listed their name, age, gender, typical look, and symptoms. There were only 20 patients (20%), out of 200, diagnosed with shigellosis. The specimens included 85 males (42.5%) and 115 females (57.5%) which divided into three patient age groups; **I**. Less than 11 years, 107 person (53.5%), **II.** 11-20 years, 48 person (24%), and **III**. 21-30 years, 45 person (22.5%). An important differences among patients according to the symptoms were found. The patients with shigellosis showed different symptoms; all of them suffering from abdominal pain (100%) 200/200, fever (51%) 102/200, vomiting (69%) 138/200, nausea (47%) 94/200 and fatigue (16%) 32/200.

A total of 20 isolates of *Shigella* were obtained, then identified according to morphological characteristics and biochemical tests, in addition to use the polymerase chain reaction (PCR) technique. The percentage of patients suffering from mucoid bloody (50%) 10/20, soft bloody (20%) 4/20, liquid bloody (20 %) 4/20, and liquid mucoid (10%) 2/20. The *Shigella* isolates were named as IQ. No. 1 to IQ No. 20.

The whole DNA was extracted, then, DNA concentration and purity were measured. The concentration of DNA was 20.9 – 49.5 ng/µl and the purity was

1.48 – 1.98. The *Shigella* spp. isolates were divided into (9/85) for males (10.58 %) and (11/115) for females (9.56 %). An important differences among species, *S. flexneri* (15%) 3/20, *S. Sonnei* (20%) 4/20, *S. dysenteriae* (65%) 13/20, *S. boydii* (0%) 0/20 . The results, upon examination by PCR method where half (50%, 10/20) of patients with shigellosis suffering from abdominal pain (3 *S. Sonnei*, 2 *S. flexneri*, 5 *S. dysenteriae*), fever (25%) 5/20, vomiting (20%) 4/20 and fatigue (5%) 1/20. In this study, there were significant differences ($p \leq 0.05$) according to the symptoms (diarrhoea conditions) of the patients with shigellosis as follows; all patients suffering from mucoid bloody (55%) 11/20, soft bloody (20%) 4/20 only (*S. dysenteriae*), liquid bloody (15%) 3/20)(1 *S. flexneri*, 2 *S. dysenteriae*) and liquid mucoid (10%) 2/20 only (*S. dysenteriae*). In this study, it was shown that *Shigella* isolates were resistant to ampicillin (100%), ceftriaxone (55%), erythromycin (55%), chloramphenicol (60%), ceftazidime (65%), ciprofloxacin (15%), gentamicin (40%), cefixime (60%), nalidixic acid (45%). About 30 % of the specimens examined were susceptible to ceftriaxone, 10% to nalidixic acid, and 60% to ciprofloxacin. Some virulence genes were studied and the results showed that there were no significant differences among species and virulence genes. For *S. flexneri*, *Stx*1 (33.33 %,1/3), *Stx*2 (0 %, 0/3), *ShET*-1 (0%, 0/3) and *ShET*-2 (0%, 0/3), while for *S. Sonnei* *Stx*1 (25 %,1/4), *Stx*2 (0%, 0/4), *ShET*-1 (50%, 2/4) and *ShET*-2 (0%, 0/4). For *S. dysenteriae Stx*1 (46.15 %, 6/13), *Stx*2 (38.46 %, 5/13), *ShET*-1 (23.07 %, 3/13) and *ShET*-2 (23.07 %, 3/13). In order to identify the some genes responsible for β-lactam resistance by molecular method using PCR technique, the following results were obtained; For *S. flexneri*, *blaCTX-M* (100 %, 3/3), *blaTEM* (66.66%, 2/3), *blaSHV* (33.33%, 1/3) and *blaAMPC* (66.66%, 2/3).

For *S. Sonnei*, the results were *blaCTX-M* (100 %, 4/4), *blaTEM* (50%, 2/4), *blaSHV* (33.33 %, 1/3) and *blaAMPC* (66.66%, 2/3). For *S. dysenteriae*, the results were *blaCTX-M* (69.23%, 9/13), *blaTEM* (53.84%, 7/13), *blaSHV* (30.76%, 4/13) and *blaAMPC* (38.46%, 5/13). The *blaCTX-M* gene isolates showed the highest resistance rate to most antibiotics, where the isolates that have *blaCTX-M* gene was completely resistant to all antibiotics (100%). The isolates that have *blaTEM*, *blaSHV* and *blaAMPC* genes were resistance to ciprofloxacin (66.66 %) and the isolates that have *blaSHV* genes was resistance to chloramphenicol (25%), ceftriaxone (27.27%).The phylogenetic tree analysis revealed that IQ-No.1-IQ-No.20 isolate have bolted genetics with *Shigella* spp. strain SK8 India isolate (MW600518.1), (0.0040-0.0010%; genetic change). The homology sequence identity between local *Shigella* spp. (IQ-No.1-IQ-No.20) isolates and NCBI BLAST *Shigella* spp. strain SK8 India isolate showed genetic homology sequence identity ranging from 99.31-99.78%. The genetic variation (substitution mutations) analysis in 16S ribosomal RNA gene between local *Shigella* spp. isolates and NCBI BLAST *Shigella* spp. strain SK8 India isolate found 1-3 substitution mutations at total genetic variation percentage ranged (0.22-0.69%). Finally, the local *Shigella* spp. (IQ-No.1-IQ-No.20) isolates were submitted into NCBI Genbank and identified by accession numbers (OK036604.1 into OK036623.1). In conclusion, *Shigella* spp isolates showed high levels of resistance to almost all antibiotics understudy and *S. dysenteriae* was the main cause of shigellosis and that may be due to *blaCTX-M* gene which was responsible for the resistance of the antibiotics used in this study.

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List of Abbreviations

Abbreviation	Meaning
AMP	**Ampicillin**
ANOVA	**Analysis of Variance**
ATP	**Adenosine Triphosphate**
bp	**base pair**
CAM	**Chloramphenicol**
CAZ	**Ceftazidime**
CCCP	**Carbonyl Cyanidem-Chlorophenylhydrazone**
CFM	**Cefixime**
CIDT	**Culture-Independent Diagnostic Assay**
CIP	**Ciprofxacin**
CLDI	**Clinical and Laboratory Standards Institute**
CRO	**Cetriaxone**
DNA	**Deoxyribonucleic Acid**
EHEC	**Enterohemorrhagic *Escherichia coli***
ERY	**Erythromycin**
ESBLs	**Extended-Spectrum *β*-lactamases**
GEMS	**Global Enteric Multicentre Study**
GEN	**Gentamicin**
GT	**Guanidium Thiocyanate**
GTDB	**Genome Science Taxonomic Database**
HGT	**Horizontal Gene Transfer**
HUS	**Hemolytic Uremic Syndrome**
Ial	**Invasion-associated locus**
ISs	**Insertion Sequences**
MDR	**Multidrug Resistance**
MFS	**Major Facilitator Superfamily**
MIC	**Minimum Inhibitory Concentration**
NA	**Nalidixic acid**
NADH	**Nicotinamide Adenine Dinucleotide**
NCBI	**National Center for Biotechnology Information**
PAI	**Pathogenic Islands**
PAN	**Phe-Arg—Naphthylamide**
PCR	**Polymerase Chain Reaction**
PFGE	**Pulsed-Field Gel Electrophoresis**

PMNs	**Polymorphonuclear Leukocytes**
RNA	**Ribonucleic Acid**
Stx	**Shiga toxin**
TBE	**Tris/Borate/ EDTA**
WGS	**Whole Genome Sequencing**
WHO	**World Health Organization**

وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية العلوم / قسم علوم الحياة

دراسة جزيئية لبعض جينات السموم والمقاومة للمضادات الحيوية في بعض العزلات السريرية لبكتريا الشيكيلا *Shigella* spp.

أطروحة مقدمة الى
مجلس كلية العلوم/ جامعة بابل
وهي جزء من متطلبات نيل درجة الدكتوراه فلسفة في العلوم
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1444 ه 2023 م

1.1.Introduction

Shigella spp. are facultative anaerobes, nonflagellated, rod, nonspore-forming bacteria. They can lead to mucoid diarrhea (bloody), also named as shigellosis, which is acute diarrhea that can progress (Kahsay and Muthupandian, 2016). Every year, *Shigella* continues to be a key microbe accountable for rising rates of dysentery illness and death, particularly in young children in developing nations. The most typical source of diarrhea is *Shigella* (Puzari *et al*., 2018; Mukhopadhyay *et al*., 2020). According to serological features, *Shigella* grouped into 4 spp: *S. dysenteriae*, *flexneri*, *boydii*, and *sonnei*. Each species grouped into numerous sero-types based on the *LPS* layer's 'O' antigen, with *S. dysenteriae* having fifteen sero-types, *S. flexneri* having six sero-types and fifteen sub-types, *S. boydii* having eighteen sero-types, and *S. sonnei* having only one sero-type (Kotloff *et al*., 2018).

Shigellosis can occur in pandemics, epidemics, or as a random disease. It is a serious public health concern due to its low infectious dosage of 10–100 organisms, compared to 105–108 for *Salmonella* and *Vibrio* (Kotloff *et al*., 2018). Also, it was identified as the instant important cause of death related with diarrhea across all age groups in an estimate of the global disease burden (Khalil *et al*., 2018). Shigellosis is more prevalent in children who lack admission to safe consumption water, have poor cleanliness and cleanness, and have insufficient nutrition (Saha *et al*., 2009).

Shigella attacks the gastric epithelium, causing infection and ulceration of the large intestine with distinct symptoms (mild watery diarrhea, acute dysentery, bloody stools, abdominal pain, and high fever) (Mukhopadhyay *et al*., 2020). *Shigella* species secrete a number of

virulence factors that generate considerable inflammation and mild enterotoxic effects in the colons, culminating in the early onset of characteristic watery diarrhea. As a result, a given isolate's pathogenicity is determined by the expression of several virulence genes involved in colonization, invasion/penetration, and toxin-mediated illness (Zhang *et al*., 2014).

*Shigell*a species with a secretion system (Type III) inject virulence agents into epithelial cells, wreaking havoc on the host cell's structure and function. This allows for epithelial cell invasion and unequal epithelial cell damage in the colon. Additionally, *Shigella* species produce compounds that have an effect on inflammation and the innate immune system. These compounds promote infection by inhibiting the adaptive immune response, exposing the host's cells to infection. These virulence variables may also contribute to the distinct infection capacities and symptoms of *Shigella* species, as well as their diverse transmission patterns (Mattock and Blocker, 2017).

The chromosomal or large pathogenic *inv* plasmids contained a variety of virulence factors that produced shigellosis (Shen *et al*., 2013). *Shigella* also yields toxins encoded on the chromosomal DNA or other DNA, such as *ShET*-1, *ShET*-2 and shiga toxin, in addition to virulence factors expressed on the virulence plasmid (Stx). *Stx*-1 and *stx*-2 are two distinct shiga toxins expressed by chromoso-genes and generated exclusively by *S. dysenteriae* sero-type 1. They are homologous to the enterohemorrhagic *Escherichia coli* (EHEC) shiga-like toxins. These toxins trigger the expansion of vascular lesions in a range of cell types in the central nervous system, kidney, and colon (Kim *et al*., 2020).

Drug-resistance of bacteria is one of the most important public health concerns. resistant to 2,3,4 or more different classes of medications, this is referred to as multidrug resistance (MDR). Numerous studies have highlighted MDR human and animal pathogens (Abolghait *et al*., 2020; Algammal *et al*., 2020 a, b). Antimicrobial selection is debatable, and no single antibiotic has emerged as clinically superior (Tickell *et al*., 2017). However, *Shigella* appears to rapidly gain drug resistance. Resistance develops over time as a result of the triple mutations on chromosomes *gyrA* and *parC*. Horizontally transferred components may also contribute to the shape and establishment of resistant clones in development.

1.2. Aim and Objectives of the Study

The aim of this study was to investigate the molecular characterization of some virulence and resistance genes among clinical isolates of *Shigella* spp. with the following objectives:

1. Primary isolation and culture identification *of Shigella* spp. isolates from clinical specimens.

2. Molecular identification of the isolates using universal 16S rRNA.

3. Investigation of the antibiotic susceptibility tests and determine the resistance patterns of the isolates.

4. Molecular study of some virulence genes (*shET*-1, *sh.ET*-2, *stx-1*, *stx*-2) and some antibiotics resistance genes using PCR.

5. Determination of the phylogenetic tree of the isolates to investigate the relationships and the origin of these isolates.

6. Study the relationship between virulence genes and antibiotic genes.

2. Literature Review

2.1. General Characteristics of *Shigella* spp.

Shigella is a slimy Gram-negative rod that produces coccobacillary forms in newly cultured bacteria. It is a non-motile enterobacterales bacteria (Morales-López *et al*., 2019). *Shigella* spp. are non-spore producing bacteria that thrive aerobically which consisting distinct colonies with convex, spherical, transparent and intact edges of around 2 mm in diameter in about 24 hours. All *Shigella* strains ferment glucose. Except for *S. sonnei*, they do not digest lactose. Additionally, with rare exceptions, they do not create gas from carbohydrates. *Shigella* isolates grown on a variety of culture media are differentiated by their inability to digest lactose. *Shigella* bacteria synthesize acid from carbohydrates, although they produce gas infrequently. *Shigella* can be classified into two types of fermenters: mannitol fermenters and non-mannitol fermenters (Jawetz, 2013).

2.2. *Shigella* species

Shigella is a genus of enterobacteriaceae family belonging to the proteobacteria phylum. *S. dysenteriae*, *S. flexneri*, *S. sonnei*, and *S. boydii* are the four species that comprise this genus (Muthuirulandi *et al*., 2017). Despite sharing comparable pathogenic goods and a widespread distribution pattern over many geographical locations, each species exhibits distinct epidemiological characteristics. Each species is grouped into several sero-types based on biochemical features and chemical structure of LPS. Thus, group A (*S. dysenteriae*) has 15 sero-types with two provisional sero-types, group B (*S. flexneri*) has six sero-types and fourteen subsero-types, group C (*S. sonnei*) has just one sero-type (Sethuvel *et al*., 2016), and group D (*S. boydii*) has twenty sero-types

(Taneja and Mewara, 2016). *S. sonnei* can be classified into five biotypes based on biochemical characteristics (Sethuvel *et al*., 2016). Six serotype-converting phages for serotype Y conversion have been found. Serotypes X, 4a, and Y have been reported to be transformed to Xv, 4av, and Yu following phosphoethanolamine modification of the O-antigen by plasmid-encoded phosphor ethanol amine (Sethuvel *et al*., 2016). However, *S. dysenteriae*, particularly the serotype (type 1), is regarded the most harmful species because to its ability to produce STX, a potent cytotoxic toxin. Because the other three species are incapable of producing the Shiga toxin, shigellosis is moderate in these three species. *S. dysenteriae* is epidemic in emerging and developed areas, while *S. flexneri*, *S. sonnei*, and *S. boydii* are endemic. Type 1 *S. dysenteriae* is particularly well-known for its endemic and epidemic-causing capabilities, as well as its high rate of infection with a high risk of mortality and sequelae (WHO, 2016).

2.3. Shigellosis

Shigellosis is a severe invasive enteric infection that causes bloody diarrhoea (Ranjbar and Farahani, 2019) and is one of the leading causes of diarrhoeal disease-related fatalities globally, particularly in children (Sati *et al*., 2019). It continues to be a prominent cause of children death (<5 years), where it accounts for up to 21% of deaths (Omona *et al*., 2020). According to a quantitative molecular analysis, shigellosis was identified as the primary pathogen among participants in the Global Enteric Multicentre Study (GEMS) in 2016. It is the sixth most common infections responsible for diarrhoea in children (Thompson *et al*., 2016). Shigellosis, caused by *Shigell*a bacteria, is a well-known clinical illness that causes fever and hemorrhagic diarrhea throughout the world (Chen *et*

al., 2020). Shigellosis symptoms begin one to two days after bacteria invade the terminal ileum, epithelial lining, colon, and rectum, resulting in an acute gastrointestinal illness (Al-Dahmoshi *et al*., 2020). According to Khalil *et al*. (2018), shigellosis caused a total of 212,438 fatalities worldwide, accounting for 13.2% of all diarrheal deaths worldwide each year. Additionally, shigellosis has been associated to stunted linear growth and weight gain in children aged 2–5, possibly contributing to global stunting (Rogawski *et al*., 2018). The diarrhea may appear bloody and lasts between five and seven days. *Shigella* spp. was identified as the pathogen most frequently associated with diarrhoea in children, and numerous asymptomatic children were identified as carriers, allowing the enteropathogen to persist and spread (Sethuvel *et al*., 2016). In both high- and low-income countries, *S. sonnei* is the most often isolated shigellosis agent (Kotloff *et al*., 2018). Complications may include reactive arthritis, convulsions, sepsis, and hemolytic uremic syndrome, and patients may develop further secondary complications such as septicemia and pneumonia in the absence of appropriate treatments (Afroze *et al*., 2017).

Shigellosis sickness severity is determined by factors other than the genetic variation of the infecting bacterium. Specific genes or gene segments isolated from patient isolates are unsuitable for outcome prediction and cannot be used to define, prioritize, or optimize shigellosis or infection control guidelines (Hendriks *et al*., 2020).

2.4. Epidemiology and Distribution

Kotloff *et al*. (2017) assessed that the worldwide problem of diarrhoeal disease about 700 million people in 2015, with about 500 thousand deaths. Shigellosis has been a significant source of illness and mortality in children (Taneja and Mewara, 2016). *S. flexneri* is prevalent in the

majority of underdeveloped nations and is associated with a higher mortality rate than other serotypes (Chung and Baker, 2018).

Shigellosis is typically an epidemic disease that has been recorded in a number of Asian countries, including the Maldives, Bhutan, Bangladesh, Sri Lanka, Myanmar, and Nepal (Sethuvel *et al*., 2016). In underdeveloped nations, *S. flexneri* accounts for 60% of shigellosis cases, while *S. sonnei* accounts for 77% of cases in established affluent countries and 15% in the underdeveloped countries. However, *S. dysenteriae* is a frequent cause of dysentery epidemics, particularly in densely populated areas such as slums, prisons, and refugee camps (WHO, 2016). According to Chang *et al*. (2016) *S. flexneri* **:** the most predominant species responsible for bacillary dysentery disease in China (63.86%) followed by *S. sonnei* (34.89%). However, from 2004 to 2014, the disease's incidence rate decreased significantly.

Yazdi *et al*. (2020) conducted a study in Iran on infected children with *Shigella* and their results indicated that *S. sonnei* was the most abundant (79%) followed by *S. flexneri* (20%) and *S. boydii* (2%). In India, a high incidence of *S. flexneri* has been recorded, a study conducted between 2001-2004 on 193 *Shigella* strains isolated from youngsters with severe diarrhea (60%) revealed that this strain is the most prevalent *Shigella* strain (Taneja, 2007).

Despite the fact that shigellosis is associated with less medical problems, effective treatment of this illness with *Shigella* may help alleviate the global burden of diarrhea (Tickell *et al*., 2017). In many underdeveloped countries, a lack of adequate food supply and inadequate health care contribute to a high risk of sickness and death.

2.5. Diagnosis and *Shigella* Growth Culture

Shigellosis is diagnosed from feces or rectal swabs. Due to the similarity of the symptoms of *Shigella* infection to those of a wide variety of other diseases, including the majority of foodborne infections, laboratory testing should be undertaken to confirm the diagnosis. Due to the contamination of the exemplary fecal samples, it is recommended to use a discerning medium. Hektoen enteric agar, xylose-lysine-dextrose, are all examples of inoculated media. Lactose, on the other hand, is not digested by bacteria, and hence all of these media encourage the establishment of chromic colonies. Blood cultures should be conducted to rule out shigellamia in patients with acute febrile gastroenteritis (Morduchowicz *et al*., 1987).

The triple sugar iron agar (TSI) slant inoculation is acidic and alkaline in nature, but does not create gas or H2S. cultivation on the suspension incubation method (SIM) card, the colonies appear fixed and do not release H2S. When the Kovac reagent is applied to SIM tubes following growth, it frequently suggests that indole is not generated, although other sero-types produce indole. Mannitol testing results are negative. Additionally, the ornithine decarboxylase test results are negative. Diagnosis of *Shigella* species or *Shigella*/enteroinvasive *E. coli* (EIEC) in clinical samples can be made by CIDT assays. *Shigella* shares a hereditary resemblance with EIEC, a pathogen identified in CIDT (Germani and Sansonetti, 2006).

2.6. Pathology and Complications

Shigellosis is characterized by bloody diarrhoea, stomach pains, and fever. The pathogen infects the colon mucosa, killing epithelial cells, and

then spreads laterally causing mucosal ulceration, bleeding, and inflammation (Schnupf and Sansonetti, 2019). Although the gastro intestinal tract accounts for the bulk of infections, extra intestinal infections, such as bloodstream infections, have been known to have a 46% mortality rate (Sethuvel *et al*., 2016). Additionally, *Shigella* infection can result in haemolytic-uremic syndrome, hepatic dysfunction (Shogbesan *et al*., 2017), hypoxia (Arena *et al*., 2017), and neurological disorders. Additionally, hyponatremia and pneumonia are rare but potentially fatal consequences (Zaidi *et al*., 2013).

Around 3% of *S. flexneri* infections can result in Reiter's syndrome, which is characterized by eye irritation, joint discomfort, and painful urination and can last from a month to many years. As a result of this, reactive arthritic illness can occur (Gaston and Lillicrap, 2003). Reactive arthritis is a late consequence of *S. flexneri* infection that is more likely to develop in those who carry the Human Leukocyte Antigen B27 (HLA-B27) gene. Sero negative spondyloarthropathies are an autoimmune illness category associated with *HLA-B*27. Hemolytic uremic syndrome (HUS), on the other hand, is a rather uncommon consequence of *S. dysenteriae* infection. Convulsions in children are a symptom of HUS. They are produced by a sudden rise in temperature caused by metabolic abnormalities. Additionally, it is associated with the formation of shiga toxin (Ram *et al*., 2008).

2.7. *Shigella* Toxins

Shigella generates toxins (chromosome or virulence plasmid) as well as virulence factors produced on the virulence plasmid, including shiga toxin, *shigella* enterotoxin 1, and *Shigella* enterotoxin 2. (*Stx*). The latter are AB5 cytotoxins that prevent the synthesis of eukaryotic proteins,

ultimately leading to the death of the host cell (Khan *et al*., 2013). *S. dysenteriae* type 1 strains produce the extremely toxic shiga toxin. Cytotoxicity, enterotoxicity, and neurotoxicity are the toxin's three biological effects. It is composed of two polypeptide chains: A (32,225 molecular weight) and B (32,225 molecular weight) (7,691 MW). Holotoxin is created when these two peptides are combined in a ratio of one A subunit to five B subunits. The biochemical action of the holotoxin is mediated by the A chain, whilst the binding of the toxin to cell surface receptors is mediated by the B chain. Due to its structural and functional resemblance, shiga toxin is the representative toxin compound of a class known as shiga-like toxin compounds (Luna-Gierke *et al*., 2014). The *stxA* and *stxB* operons include the toxin-coding genes.

Hemorrhagic colitis is caused by bacterial infections that produce *Stx* and can lead to major consequences such as hemolytic uremic syndrome (HUS) (Butler, 2012). Although four *Shigella* species induce bacillary dysentery, only *S. dysenteriae* 1 has historically caused HUS as a result of infection (Lanata *et al*., 2013).

2.8. Virulence Factors of *Shigella* spp.

Numerous virulence factors expressed by *Shigella* species result in considerable intestinal inflammation and mild enterotoxic effects, which early in the course of the infection lead to the development of the usual watery diarrhea. As a result, the pathogenicity of an isolate is defined by the expression of several virulence genes associated with colonization, invasion/penetration, and toxin-mediated disease (Zhang *et al*., 2014). *Shigella* species with a secretion system (Type III) inject virulence agents into epithelial cells, causing structural and functional harm to the host

cell. This enables epithelial cell invasion and uneven epithelial cell injury in the colon.

These elements promote infection, dampen the adaptive immune response, and leave the host's cells vulnerable to re-infection. These virulence traits may also be responsible for the various *Shigella* species' infection capabilities, symptoms, and transmission patterns (Mattock and Blocker, 2017). pH, iron availability, and osmolarity are all factors that affect virulence factor expression and secretion. At 37°C, transcriptional activators such as *VirF* and *VirB* are induced. *VirF*, in turn, induces the expression of the actin nucleator. The adhesin *IcsA* promotes actin remodelling. *VirB* promotes transcription of the components of the T3SS, such as the *mxi* and *spa* genes, which encode the initial set of effectors essential for cellular invasion (Ferraria *et al*., 2019).

Shigella infection's cellular pathogenesis and clinical symptoms are the result of intricate interactions between a number of *Shigella* virulence factors. *Shigell*a possesses the potential to colonize and infiltrate intestinal cells via plasmidial and chromosomal sequences. *Shigella*'s pathogenicity plasmid is approximately 220kb in length. It encoded for between 50-60 virulence-genes on a 31-kb entry region known as the ipamxispa region.

Numerous insertion sequences can be found in the virulence plasmid (ISs). Additionally, it has genes related to conjugation. The virulence plasmid can mobilize bacteria by gaining access from one bacterial cell to another via the pilus with the aid of additional plasmids with conjugation capacity (Kotloff, 2017). Virulence plasmid sequences have come from a variety of places. Each form encoding is comparable such as *pSS* of *S. sonne*i, *pC*301 of *S. flexneri* 2a, *pWR*501 of *S. flexneri* 5a, and *pWR*100 of

S. flexneri 5a. *Shigella* effectors inhibit the host cell's innate immune response, allowing the infected cell to survive longer. *IpaH*, a *Shigella* effector protein featuring E3 ubiquitin ligase activity, targets a large number of proteins for degradation by host proteasomes, hence impairing the host defensive mechanism (Ashida and Sasakawa, 2017).

Numerous virulence factors encoded by plasmids and/or chromosomes have been found in *Shigella*, including epithelial cell penetration encoded by the invasion-associated locus (ial) (Johnson, 2018), and the invasion plasmid antigen H (*ipaH*), which facilitates pathogen cell-to-cell movement. In *Shigella* spp., virulence regulator (*VirF*), an AraC-tpe transcriptional regulator, controls the expression of all downstream virulence factors that control the intracellular invasion of intestinal cells (Hurt *et al*., 2010).

The *Shigella* virulence plasmid, which can reach a size of 220 kb, encodes key virulence genes that enable *Shigella* spp. to penetrate and spread within human macrophages and enterocytes (Kaas *et al*., 2012). The virulence plasmid contains the Mxi–Spa type III secretion system (T3SS), as well as genes encoding antigens specific for invasion plasmids (*Ipas*). T3SS is a molecular syringe capable of directly delivering effector proteins to host cells. commotion of the intestinal mucosa is caused by the bacteria's ability to engage intricately with the host cell through this secretion mechanism. As a result, the virulence plasmid is a critical molecular marker for *Shigella* spp. pathogenicity, as it is necessary for infection and manipulation of the host's immune response (Ashida *et al*., 2011).

The chromosomal or big pathogenic *inv* plasmids carried a number of virulence factors that resulted in the development of shigellosis (Shen *et*

al., 2013). *Shigella* produces toxins (chromosomal DNA-plasmid), including*: ShET*-1,*ShET*-2 and shiga toxin, as well as virulence factors expressed on the virulence plasmid (*Stx*). Along with their enterotoxic action, *ShET*-1 and *ShET*-2 play a critical role in the colon's electrolyte and water transport (Zaidi and Estrada-Garcia, 2014). *Set1A* and *Set1B* are chromosomal genes encoding *Shigell*a enterotoxin 1 (*ShET*-1) that are involved with the watery phase of diarrhea. *Shigella* enterotoxin 2 (*ShET*-2) is abundant in large pathogenic plasmids and contributes to their invasion (Schroeder and Hilbi, 2008). *Stx*-1 and *Stx*-2 are two distinct Shiga toxins expressed by chromos-genes and generated exclusively by *S. dysenteriae* sero-type 1. They are homologous to the enterohemorrhagic *E. coli* (EHEC) shiga-like toxins. These toxins trigger the expansion of vascular lesions in a range of cell types (Cherla *et al*., 2003).

Due to the great poisonousness of the shiga-toxin, infections with *S. dysenteriae* serotype 1 usually result in life-threatening worries. Along with virulence plasmids, it has been discovered that a distinct region on the chromosome contributes to infection. This region is referred to as PAI "pathogenic islands," which are mobile, detachable fragments that can be classified according to *Shigella* species and subtypes. The enterotoxin sigA, pic, which causes intestinal colonization, set1A, set1B, and *ShET*1 enterotoxin are all carried by the SH-1. The siderophores iucA-D, iutA, and shiA-G found in SHI-2 and SHI-3 have been linked to a reduction in the host's inflammatory response. GtrA-B, serotype conversion, and an altered O-antigen are all present in SHI-O b. (Al-Hasani *et al*., 2009; Navarro-Garcia *et al*., 2010). Invasion plasmid antigens (Ipas) are the proteins *Ipa*A, *Ipa*B, *Ipa*C, and *Ipa*D. *Shigella*'s Ipa proteins are crucial virulence factors that are necessary for the invasion process. Upon coming into contact with host cells, they are injected into the cells

through a type III secretion pathway (Mattock and Blocker, 2017). Three vinculin joining sites (VBSs) in the IpaA structure can connect to the vinculin head domain to activate vinculin. The adaptor protein vinculin is required for focal adhesion stability. *Ipa*A controls the inflow and disposal of actin monomers, hence regulating actin polymerization. In turn, these proteins can rearrange the cytoskeletal structure of the host cell, facilitating *Shigella*'s entry into cells during epithelial cell invasion (Lee *et al*., 2014). *Shigella* can escape the phagosome and start macrophage apoptosis thanks to the regulation of the secretion system by the IpaB and IpaC proteins, which are found close to the apex of the T3SS canal. *Ics*B is a T3SS-dependent effector virulence factor that inhibits autophagy, whereas *Ics*A (also known as *Vir*G) is a T3SS-independent protein found outside the membrane that functions as an autotransporter and adhesion molecule (Arizmendi *et al*., 2016; Du *et al*., 2016).

Sethuvel *et al*. (2019) analyzed sixty *Shigella* whole genome sequences for the presence of virulence genes, including 22 *S. flexneri*, 14 *S. sonnei*, 17 *S. boydii*, and seven *S. dysenteriae*. *Ipa*H was detected most frequently (90%) in this investigation, followed by *sig*A (83%) and *lpf*A (83%). In comparison to *S. sonnei*, *S. flexneri*, particularly serotype 2, has much more virulence genes. Surprisingly, the *sig*A gene has been linked to fever, whilst *sep*A and *sig*A have been linked to diarrhea. The presence of virulence genes was shown to be more prevalent in *Shigella* isolates resistant to more than three medication classes. In an Iranian investigation: 7 virulence-genes were found including *ipa*B, *ipa*C, *ipa*H, *ipa*D, *ipg*D, *vir*A, and *sen*, was determined using the polymerase chain reaction (PCR) technique. Prevalences of 92.9%, 95.7%, 100%, 94.3%,

80.8%, 93.6%, and 94.3%, respectively, were detected (Yazdi *et al*., 2020).

2.9. Antibiotic Resistant Bacteria

Bacteria that are not inhibited by unusually achievable systemic dose of a drug administered on a regular basis and/or fall within the range of minimal inhibitory levels are classified as resistant. Multiple drug resistance (MDR) is a term that refers to a bacteria's resistance to two or more medications or pharmacological classes (Puzari *et al*., 2018). Antibiotic resistance is a major concern to the world, as it renders antibiotic treatments ineffective and increases the risk of therapeutic failure. Resistance to *Shigella* species has been connected to a number of shigellosis epidemics. The World Health Organization (WHO, 2014) has now declared antibiotic resistance in bacteria a global crisis as a result of their evolutionary process. Nowadays, approximately half of *Shigella* serotypes are resistant to several antibiotics in several parts of the world. As a result, treatment failure rates for infectious diseases caused by bacteria have increased, as formerly effective therapies have become ineffective (Kotloff *et al*., 2018).

Antibiotic resistance occurs when bacteria mutate in such a way that medications, chemicals, or other agents designed to cure or prevent infection lose their potency. As a result, the bacteria live and multiply, resulting in increased harm. Antibiotic resistance spreads as a result of widespread usage of antibiotics. Bacterial sensitivity to antibacterial agents is determined by calculating the minimal inhibitory concentration that stops bacteria from growing (Huynh *et al*., 2015).

Bacteria may be natively resistant due to their inherent characteristics, or they may acquire resistance via mutations and gene transfer. Antibiotic resistance in *Shigella* spp. can occur through a variety of methods, including insufficient drug penetration, antibiotic efflux via efflux pumps, target alteration via mutation, overexpression of drug-modifying and -inactivating enzymes, and antibiotic hydrolysis (Blair *et al*., 2015).

Individuals develop serotype-specific protective immunity when they are exposed to a particular serotype (Sethuvel *et al*., 2016). The primary reason for treatment in mild illnesses is to prevent the spread of the bacteria. Sulfonamides, tetracycline, and chloramphenicol were the first drugs used to treat *Shigella* infection. *Shigella* become resistant to those drugs, necessitating ampicillin and cotrimoxazole as a form of treatment. Additionally, due to *Shigella* species developing resistance to the previously recommended drugs, treatment guidelines were updated to include nalidixic acid (Shahsavan *et al*., 2017).

After the discovery of resistance to nalidixic acid, fluoroquinolones were introduced, and resistant strains for fluoroquinolones were isolated from numerous sources until now. For the treatment of *Shigella* infections that are resistant to fluoroquinolones, the WHO suggests bifemesillinam, azithromycin, and ceftriaxone. However, in some cases, ceftriaxone- and azithromycin-resistant isolates have been identified, indicating that this issue has developed into a major concern and a threat to humanity. Antibiotic resistance is, nevertheless, spreading among *Shigella* species worldwide. According to Chang *et al*. (2016), *Shigella* isolates were extremely resistant to ampicillin (88.90%), nalidixic acid (89.13%), sulfamethoxazole (82.92%), and tetracycline (88.43%). They reported that over the study's duration, the percentage of isolates resistant to

ciprofloxacin and cefotaxime grew from 8.53 and 7.87% in 2005 to 44.65 and 29.94% in 2014. Resistance to sulfamethoxazole was connected to the *sul* genes, but resistance to trimethoprim was associated to the *dfr*A12 and *dfr*A17 genes (Phiri *et al*., 2021). Fluoroquinolones, cephalosporins, and sulfonamides are the medications of choice for the clinical therapy of acute diarrhea caused by *Shigella* spp. (Baker and The, 2018). However, *Shigella* bacteria are developing resistance to these antibiotics at an alarming rate, limiting treatment choices for *Shigella* infections (Hussen *et al*., 2019). In *Shigella* spp., resistance to fluoroquinolones is produced by mutations in the *gyrA* and *parC* genes. The most frequently seen mechanisms of resistance to β-lactam antibiotics are β-lactamases, which may hydrolyze penicillin as well as first-, second-, and third-generation cephalosporins (Hooper and Jacoby, 2015), with OXA-lactamases responsible for ampicillin resistance. The most common causes of sulfonamide resistance are the A genetic change in the chromosomal dihydropteroate synthase gene (*folP*) or the acquisition of sul-type determinants encoding enzymes with reduced sulfonamide binding capacity (Adelowo *et al*., 2018). In their antimicrobial susceptibility investigations, Pakbin *et al*. (2021) discovered that whereas clinical isolates were strongly resistant to imipenem, amikacin, and azithromycin, food isolates were extremely resistant to tetracycline. Further evidence showed that tetracycline, chloramphenicol, and nalidixic acid are more effective against clinical isolates. Three different *sul*-type resistance gene variants have been reported in the literature: *sul*1, *sul*2, and *sul*3. *Sul*1 is usually related with changes in class 1 integrons (Hammerum *et al*., 2006). All of these genetic markers are present in enterobacterales isolated from human sources, including feces. Due to these bacteria' increasing resistance to routinely used antibiotics, a great effort has been

made to develop vaccines to prevent infection. Antibiotic resistance was detected in *Shigella* spp. to cotrimoxazole (59%) tetracycline (40%) amoxicillin (38%) and ciprofloxacin (25%). Both *Salmonella* spp. and *Shigella* spp. were most resistant to nalidixic acid (Bastola *et al*., 2021). In a study conducted by Kahsay and Muthupandian (2016) *S. dysentriae* had 72.1, 69.4, 60, 44, 40, 17 and 7% resistance to ampicillin, tetracycline, cotrimoxazole, chloramphenicol, nalidix acid, gentamicin, and ciprofloxacin, respectively while *S. flexneri* had 75.8, 75.6, 74.5, 72.7, 51.7, 14.5, and 7% resistance to tetracycline, ampicillin, nalidix acid, cotrimoxazole, chloramphenicol, gentamicin, and ciprofloxacin, respectively. However, *S. boydii* had 64, 63, 48, 29, 10, 6 and 3% resistance to ampicillin, tetracycline, cotrimoxazole, chloramphenicol, ciprofloxacin, gentamicin, and nalidix acid, respectively.

S. sonnei resistance to tetracycline, cotrimoxazole, nalidix acid, and ampicillin, chloramphenicol, gentamicin and ciprofloxacin were 79, 71, 54, 47, 35, 16 and 0 % respectively. In addition, reported that all *Shigella* strains tested were resistant to ampicillin, sulfamethaxol-trimethoprime but they were susceptible to ciprofloxacin and mecillinam (Sharmin *et al*., 2021).

2.10. Antibiotic-Resistance Mechanisms in *Shigella* spp.

Bacteria possess remarkable genetic plasticity, which enables them to adapt to a wide variety of environmental challenges, including the presence of antibiotic chemicals that could jeopardize their survival. Bacteria that share an ecological niche with antimicrobial-producing organisms have evolved ancient mechanisms to withstand the antibiotic molecule's destructive activity, and as a result, their natural resistance enables them to thrive in its presence (Ranjbar and Farahani, 2019).

Bacteria adapt to drugs via two fundamental genetic mechanisms: gene mutations and horizontal gene transfer (HGT) of foreign DNA encoding resistance determinants (Jose *et al*., 2016). Bacteria have evolved advanced antimicrobial resistance mechanisms in order to avoid being killed by antimicrobial chemicals, a process that likely took millions of years. Generally, many metabolic pathways can be exploited to confer resistance to a single antimicrobial class. Antibiotic resistance pathways are classified as follows:

2.10.1. Role of outer-membrane permeability

Antibiotics used to treat *Shigell*a infections should be able to permeate the cell membrane and reach intracellular accumulation and target locations. Bacterial cell walls serve as the initial line of defense against drug penetration. Porin loss can occur as a result of changes in membrane permeability or membrane structure, resulting in an increase in the antimicrobial drug's minimum inhibitory concentration (MIC) (Kar *et al*., 1997).

Quinolone antibacterial drugs like nalidixic acid, ciprofloxacin and ofloxacin, for example, disrupt DNA replication. Streptomycin and spectinomycin are aminoglycoside antibiotics that suppress translation of mRNA by blocking ribosome . Antibiotics having a -lactam ring in their molecular structure, such as penicillin and cephalosporin, prevent the formation of cell walls by binding to penicillin-binding proteins. *Shigella* species' susceptibility to -lactam drugs like aztreonam and dianionic moxalactam, as well as the reduced permeability of hydrophilic drugs like penicillin and piperacillin, depend on the presence or absence of a 39 kDa porin in their membranes (Ranjbar and Farahani, 2019).

2.10.2. Efflux systems

Active efflux pumps are important in Gram-negative bacteria's antibiotic resistance phenotypes and in the removal of harmful chemicals from their cells. Efflux pumps can be divided into five families; major facilitator superfamily (MFS), small multidrug resistance (MDR) family, resistance–nodulation–division family, multidrug and toxic compound extrusion family and ATP-binding cassette superfamily (Sun *et al*., 2014).

Antibiotic resistance phenotype in *Enterobacter* spp., *Escherichia*, and *Shigella* isolates is mediated by the AcrAB–TolC pump. AcrAB–TolC is a member of the resistance–nodulation–division family of efflux pumps, which are involved in quinolone efflux and are one of the factors that contribute to the resistance development in *Shigella*. Certainly, over-expression of AcrAB–TolC causes a decrease in accumulation of quinolone inside bacterial cells, as well as a reduction in susceptibility to them (Yang *et al*., 2008). AcrAB has been linked to bile-salt resistance. *Shigella* was able to resist the bactericidal effects of bile by increasing their expression following exposure to bile salts. This behavior, may confer resistance to other antimicrobial drugs. Furthermore, in several gram-negative bacteria, overexpression of *Acr*B has been associated to different drug resistance phenotypes (Nickerson *et al*., 2017). In *Shigella* spp., enhanced expression of the MdfA efflux pump causes resistance to fluoroquinolones, according to Kim *et al*. (2008). Fluoroquinolone resistance caused by the MdfA efflux pump, a component of the MFS antibiotic efflux system, was initially identified in MDR *E. coli*. Gram-negative microorganisms like *Klebsiella* spp. and *Shigella* spp. the MFS expressed by different *tet* genes is linked to tetracycline efflux and resistance. The *tet*A and *tet*B, among the *tet* efflux systems, appear to be

mediated by tetracycline resistance in *S. sonnei* and *S. flexneri*, respectively (Shahsavan *et al*., 2017).

2.10.3. Resistance to β-Lactam antibiotics

A. Class A β-Lactamases

Narrow-spectrum penicillin can be hydrolyzed by class A -lactamases. Extended-spectrum -lactamases (ESBLs) are categorized as Strider class A enzymes. ESBLs that give resistance to third-generation cephalos-porins are present in several *Shigella* isolates. In 2004, Bangladesh was the first nation to publicly announce the discovery of *Shigella* strains that produce ESBLs. First discovered in *Shigella* spp. were 23 ESBLs (Rahman *et al*., 2004). *Shigella* isolates have been found to contain a variety of β - lactamases from the Ambler class A family, including :TEM/SHV/CTX-M. In 1995, France reported the first insulate of *S. flexneri* that produced an ESBL and had a plasmid containing the *blaSHV*-2 gene (Fortineau *et al*., 2001). Several publications from Argentina, Canada, Japan, South Korea, China, and other Asian countries have found *Shigella* spp. carrying various forms of ESBL genes. (Ranjbar *et al*., 2013).

B. Class B *β*-Lactamases

Conventional -lactamase inhibitors like tazobactam and clavulanic acid have no effect on the class B-lactamase enzyme, which can hydrolyze carbapenem and other -lactams except for aztreonam. In Japan, IMP-1 was discovered in a variety of gram-negative rods. In addition to -lactamase resistance, this plasmid also provided resistance to sulfonamide and kanamycin (Thamizhmani *et al*., 2015). Pediatric diarrhea patients in India's Andaman and Nicobar islands had isolates of *S. sonnei* and *S. flexneri* that were resistant to carbapenem because of the

blaVIM and *blaIMP* genes (Sambe-Ba *et al*., 2013).

C. Class C *β*-Lactamases

Class C-lactamases, sometimes referred to as amp C-type enzymes, provide bacteria high levels of resistance to cephalosporins. *S. sonnei* isolates obtained from a bacillary dysentery outbreak in Taiwan had the CMY-2-type (plasmid). Both plasmid and chromosomal genes encode AmpC-lactamase. Different pandemic strains from China/Taiwan/Costa Rica/Iran and India have been identified to contain CMY-2 enzymes (Zamanlou *et al*., 2018). Ceftriaxone is advised against *Shigella* that are resistant to ciprofloxacin. But some *Shigella* spp. strains now have a gene for cephalosporin resistance. Other studies conducted in Iran have revealed that *Shigella* isolates are becoming more resistant to extended-spectrum cephalosporins (Zamanlou *et al*., 2018). In a related Iranian investigation of cephalosporin-resistant *Shigella* isolates, 1 isolate (*S. sonnei*) from pediatric patients (12 years) included the gene CMY-59 (Ranjbar *et al*., 2013).

D. Class D *β*-Lactamases

Class D-lactamases, often referred to as OXA-type-lactamases, can hydrolyze oxacillin, cloxacillin, and benzylpenicillin and confer resistance to ampicillin and cephalothin, despite not being inhibited by tazobactam or other inhibitors (Cui *et al*., 2015a). *Shigella* spp. ampicillin resistance is mostly brought on by an OXA-type -lactamase (Cui *et al*., 2015b). *S. flexneri* isolates are blaOXA-type-most lactamase's likely host (Cui *et al*., 2015a).

2.11. Multidrug Resistance - ESBLs

Extended spectrum beta-lactamases (ESBLs) are enzymes produced by certain types of bacteria that are capable of hydrolyzing extended

spectrum cephalosporin. Therefore, they are effective against β-lactam antibiotics such as ceftazidime, cefotaxime, ceftriaxone, and oxyimino-monobactam (Ghafourian *et al*., 2015). ESBLs break down and render medicines from the penicillin and cephalosporin families. ESBLs are transmissible β -lactamases that can be inhibited by clavulanic acid, tazobactam, or sulbactam and are expressed by genes that can be passed from one bacterium to the next. CTX-M is the most frequent ESBL genetic variation nowadays. Even though there are numerous types of bacterial resistance toward β -lactamase antibiotics, the most important are β –lactamases enzymes which can hydrolyze the β -lactam ring of some antibiotics such as penicillins and other drugs to make them inactive (Shaikh *et al*., 2015). There are many different types of β -lactamases, each with its own substrate specificity and kind of the host. The majority of attempts to find new beta-lactam antibiotics have thus far been unsuccessful due to the emergence of bacteria that can manufacture beta-lactamases, which can destroy beta-lactam medicines. The chromosomal cephalosporinases from pseudomonas, enterobacter, and other taxa, as well as the major plasmid-produced Enterobacteriaceae enzymes, can cleave previous cephalosporins (such as cephalothin) into smaller molecules (Founou *et al*., 2018).

The widespread use of extended-spectrum cephalosporins is a result of their efficacy, safety, and excellent pharmacokinetics. Early 1980s Gram-negative rods with chromosomally encoded -lactamases first developed resistance to these medications, mostly as a result of transmutations that led to the rudimentary production of these obviously inducible enzymes. Western Europe was the first region to establish enteric Gram-negative rods with transportable extendedspectrum cephalosporin resistance in the mid-1980s (Knothe *et al*., 1983). These genes can be found on

transposable elements, which enables them to move between plasmids and propagate extended-spectrum lactamases among gram-negative rods. The most prevalent beta-lactamases identified in ¯ve bacteria are TEM/OXA/ SHV/ CTX-M. (Muthuirulandi *et al*., 2017). Due in part to antibiotic use practices, the incidence of ESBL production in gram negative rods differs between countries and between institutions within a country. An Iraqi investigation found that *Shigella* isolates produced -lactamases at a high rate of 75.6%. (Al-Rahman, 2013).

In a different study, researchers discovered that Iran had a higher prevalence of *Shigella* species that produce ESBLs than many other countries (Ranjbar *et al*., 2013). According to molecular epidemiologic research, plasmids encoding these enzymes are being transmitted from one strain to the next and extended spectrum -lactamases are spreading within and within hospitals (Sader *et al*., 1994). ESBL are usually encoded by genes found on chromosomes and/or mobile genetic elements such as transposons, integrons and plasmids, that carrying resistance genes to some other classes of antibiotics. This mobility is linked to a large number of genes that code for beta-lactamase. In humans, the isolation of ESBL-producing multi-resistant strains is a primary cause of therapeutic failure, resulting in a significant rise in bacterial infection morbidity (Silva and Lincopan, 2012).

2.12. Antibiotic Resistance in *Shigella* spp. and Gene Involvement

In a study conducted in India, the connection of multi-drug resistant with drug-resistant genes was discovered in 88 isolates from India. The TEM -lactamase genes were found to be related with ampicillin resistance in all of the isolates used, while the *aac*2 gene was found to be associated

with gentamicin resistance in 22% of the isolates (Taneja and Mewara, 2016). The existence of the *catA*1, *strA*, and *tetB* genes has been associated to *Shigella* spp. resistance to streptomycin, chloramphenicol, and tetracycline. Trimethoprim resistance is linked to the *dhfr*I*a* or *dhfr*III*c* genes (Kim *et al*., 2008), sulphonamide resistance associated to the *Sul*I-*Sul*II- *Sul*III genes (Sethuvel *et al*., 2016). *S. flexneri* strains, *S. flexneri* and *S. flexneri*, were all shown to be resistant to fluoroquinolones in a study conducted in Korea (Kim *et al*., 2008).

According to other study, the number of *Shigella* resistant strains to ciprofloxacin increased from 0% in 2004 to 44% in 2010. *S. flexneri* was discovered to be the most common and predominant (Azmi *et al*., 2014). The majority of the isolates tested were positive for amox and Co-trimoxazole resistance, but some isolates from other country tested were positive for cipro resistance (Seidlein *et al*., 2006). *S. flexneri* was also shown to be the most widespread and resistant strain in molecular research conducted in Pakistan's Faisalabad region (Tariq *et al*., 2012). However, in medical isolates of *S. flexneri* acquired from rustic hospitals in China, overexpression of the *acr*A gene was discovered to be the cause of multiple antibiotic resistance (Mar) phenotypes (Yang *et al*., 2008).

When carbonyl cyanidem-chlorophenylhydrazone was present, ciprofloxacin accumulation increased and the efflux pump had a role in the emergence of resistance (CCCP). PAN, an efflux pump inhibitor, reduced the MIC of ciprofloxacin for the isolates. *Shigella* species were found to have mutations in the *acr*A and *tol*C genes that play a critical role in antibiotic resistance. *ACR*B and *mdf*A genes have also been linked to efflux-modulated resistance (Kim *et al*., 2011). (Sethuvel *et al*., 2016). *S. sonnei* isolates from a bacillary dysentery outbreak in Taiwan also

included plasmid-encoded CMY-2-type AmpC-lactamases that were resistant to ceftriazone (Huang *et al*., 2005). In *S. dysenteriae* type I and *S. flexneri*, fluoroquinolone resistance has increased year after year (Pazhani *et al*., 2005). Antibiotic resistance in *S. dysenteriae* has been linked to the development of a proton-driven efflux pump. High fluoroquinolone resistance is thought to be caused by overexpression of the RND pump AcrAB (Li *et al*., 2015) as opposed to the MFS pump MdfA (Kim *et al*., 2011). Fluoroquinolone-resistant *Shigella* spp. are treated with cephalosporins. Nevertheless, the first ceftriaxone-resistant *S. flexneri* isolate was exposed in 2001, and the number of resistant isolates has grown since then (Taneja and Mewara, 2016). Ampicillin resistance is predominantly brought on by the presence of the *bla-TEM* gene in *S. sonnei* as opposed to the *bla-oxa* gene in *S. flexneri*. Mutations broaden the spectrum of *ESBL*s that cause resistance to third-generation cephalosporins, including TEM-1, TEM-2, and SHV-1 (Taneja and Mewara, 2016).

2.13. Genetics of *Shigella* spp.

Shigella genomes broadly correspond to *Escherichia coli* K12 genomes in terms of their fundamental backbone. A large virulence plasmid, measuring 181–214 kb, is present in all virulent *Shigella* species and is crucial for disease. The virulence plasmid and a succession of gene deletions and additions on the chromosome (Maurelli *et al*., 1998; Jin *et al*., 2002). Bacterial genomes of the same species or clinically isolated strains differ in size as a result of ongoing sequence fluctuation and rearrangement in response to environmental stimuli, which results in genome elasticity and species diversity (Schmidt and Hensel, 2004; Juhas *et al*., 2009). Two contigs comprising a 4.34 Mb chromosome and a 177

kb big virulence plasmid were produced using the combination of next-generation sequencing platforms and assembly. To further comprehend gene complexity and pathogenic elements, this genome sequence is compared to other *Shigella* genomes (Vongsawan *et al*., 2015).

Large virulence plasmids seen in *Shigella* strains are known to contain genes necessary and adequate for attack of epithelial cells (Sansonetti *et al*.,1982). Chromosomes with "pathogenicity islands" also play a direct role in the pathogenic process or help bacteria survive in the settings they encounter when infected, though (Al-Hasani *et al*., 2009). Despite the fact that *Shigella* species have been recognized as distinct from *E. coli* since 1972, DNA hybridization studies found that *Shigella* and *E. coli* are taxonomically indistinguishable at the species level. *Shigella* is categorically positioned within the genus *E. coli* and emerged multiple times independently, according to recent work by the Reeves group (Lan *et al*., 2001) based on multi locus enzyme electrophoresis and sequencing of genes. The exact genetic link between *S. flexneri* and *E. coli* K-12 is established by comparing the full genome sequences of the two organisms.

The comparison should also highlight significant genetic variations other than the presence or lack of the virulence plasmid that are likely to cause disease, given the significantly different lifestyles of internal *Shigella* and extracellular *E. coli* (Wei *et al*., 2003).

Shigella species' invasive phenotype, which infects epithelium and produces disease, is mediated by plasmid-genes (181-241 kb). Additionally, the *Shigella* chromosome must contain at least eight loci for virulence to fully express itself. The by-products of these genes fall into three groups: I cytotoxins that worsen disease, like stx and a supposed

analogue (flu); (ii) controlling loci that influence of p53 expression; and (iii) virulence determinants that directly influence *Shigella*'s capacity to survive in intestinal tissues. The pathogen's plasmid and chromosome contain the essential virulence components necessary for *Shigella*'s pathogenesis, which allow the infection to persist intracellularly. Shigellosis is often self-limited, although using antibiotics shortens the time patients experience symptoms and lessens the amount of pathogen shed, which lowers transmission (Kotloff *et al*., 2018). The majority of the virulence and resistance determinants are found in *Shigella* spp. mobile genetic elements (MGEs), which include plasmids/insertion sequences/ integrons/ pathogenicity islands/bacteriophages. These elements' horizontal gene transfer (HGT) is a key force in bacterial evolution (Juhas, 2015). The pathogen increases its ability to infect and forge resistance to outcompete other weak bacteria in the gut by exchanging genes with other locally circulating commensal and pathogenic bacteria (Ragupathi *et al*., 2019). A single complete chromosome was created via a hybrid assembly method for *S. flexneri* (FC906), while three plasmids with sizes of 8400 bp, 6016 bp, and 2691 bp were produced for *S. sonnei* (FC1653) (Muthuirulandi *et al.*, 2017). pic and sigA, ipaH, lpfA, and invasion plasmid antigen are among the virulence genes found in the *S. flexneri* genome (ipaH). The genome of *S. sonnei* also contained the serine protease autotransporter protein (sigA), enterotoxin *ShET*-2 (senB), long polar fimbriae (*lpfA*), and invasion plasmid antigen (ipaH) (Venkatesan *et al*., 1989).Furthermore, the toxin genes that are members of the SPATE family have typically been divided into two classes. While the class 1 gene *sigA* is harmful to epithelial cells, the class 2 gene *pic* is safe (Nave *et al*., 2016). The *S. flexneri* chromosome contains resistance genes such *aadA*1, *blaOXA*-1, *tetB*,

*dfrA*1, and *catA*1. *sul*II/*aph*(6)-Id/*aph*(3")-Ib/*tet*(A) were found in plasmid 1, which is now known as pSS1653. The *dfrA*1 gene was found in the chromosome of *S. sonnei* (Zhu *et al*., 2018; Gu *et al*., 2017). Bacteriophages typically play a role in the serotype conversion in Shigella (Parajuli *et al*., 2019). 15 phage areas in *S. flexneri* were identified using the hybrid assembly study (8 entire, 4 incomplete, and 3 dubious). Similar to *S. sonnei*, 15 phage areas were found there, with 5 entire, 6 incomplete, and 4 dubious. The phage sections of *S. flexneri* and *S. sonnei* each occupy 10% and 7% of the whole chromosome, respectively. A complete copy of the SfII bacteriophage, which confers the serotype 2a on *S. flexneri*, was found in the third phage region of the chromosome (Muthuirulandi *et al*., 2017).

2.14. Bacterial Genotyping

Genotyping is a laboratory test that looks for certain nucleotides or bases in an individual's germline DNA to see if certain mutations are present. Genotyping varies from sequencing in that it evaluates all of the nucleotides that make up a certain length of DNA (e.g., within a gene, exome, or genome). Genotyping is also the act of determining which genetic variants an individual carries, and there are a variety of methods for doing so, the most prevalent of which being microarrays. Using multiple genotyping approaches to analyze *Shigella* isolates can provide useful information for outbreak investigations (Reller *et al*., 2006), disease transmission pathways, illness monitoring (Swaminathan *et al*., 2006), and evolutionary research (Chiou *et al*., 2006).

Since the late 1980s, various genetic approaches for genotyping microorganisms have been developed. Several genotyping approaches for *Shigella* strains have been established including plasmid profiling.

Plasmid profiling, has a lower level of discriminatory power than PFGE (pulsed field gel electrophoresis) (Chiou *et al*., 2006). Plasmid profiling, on the other hand, is more beneficial than PFGE for investigating the genetic links among *Shigella* strains circulating over a longer time period, as well as for discriminating specific strains that PFGE could not separate. When compared to the genomes of mammals and single-cell eukaryotes, bacterial genomes are smaller and have less variation in size between species. Bacterial genomes can range in size from 130 kbp to 14 Mbp (Van Leuven *et al.*, 2014). Bacteria have a compact genome layout that differs from eukaryotes in two ways: their functional gene count is inversely correlated with genome size, and their functional genes are arranged into operons (Mahmoudi *et al*., 2019). The main reason why bacterial genomes are more abundant than eukaryotic genomes is because they contain noncoding DNA in the form of intergenic regions and introns (Valian *et al*., 2020). The identification of strains based on phenotypic features such as morphology of colonies in various culture conditions, biochemical assays, serology, pathogenicity, and antibiotic susceptibility is known as phenotyping (Pourakbari *et al*., 2018). Because of their excellent resolution, these approaches have proven popular in bacterial identification. However, a specialized genotyping method's identification genetic profile of any bacteria can be as distinctive as a fingerprint (Mahmoudi *et al*., 2019).

The research and surveillance of *S. sonnei* outbreaks are increasingly using whole genome sequencing (WGS). Researchers confirmed the intercontinental spread of highly resistant *S. sonnei* clones by using this method to analyze more than 4,000 *S. sonnei* isolates sequenced in public health labs in three countries. They also demonstrated how the genomic framework can help monitor the spread of resistant clones, including

those that have recently emerged, at both a local and global scale (Hawkey *et al*., 2021).

2.15. DNA Sequencing

Nucleotide sequencing is a method for figuring out the order of the nucleotides in DNA, or the nucleic acid sequence. Included is any technique or technology for figuring out the relative positions of the four bases adenine, guanine, cytosine, and thymine. Rapid advances in DNA sequencing have greatly accelerated biological and medical research and knowledge. Understanding DNA sequences is becoming more and more important for basic biological research as well as a range of applied fields like biotechnology, forensic biology, virology, and biological systematics (Abate *et al*., 2013). The human genome as well as the genomes of several other animals, plants, and microbiological species have all been sequenced in their entirety thanks to advances in modern DNA sequencing technology (Chmielecki and Meyerson, 2014). The Sanger technique, also known as the dideoxy or chain termination method, is based on the synthesis of DNA chains using dideoxynucleotides that stop DNA amplification at the elongation phase. Elongation is stopped when the polymerase enzyme inserts a nucleotide containing a 3' hydroxyl group into the chain. By separating the PCR products on an acrylamide gel electrophoresis, the dideoxy nucleotide terminated in the chain can be determined (Yildirim *et al*., 2011).

2.16. Phylogenetic Tree

A phylogenetic tree, also known as an evolutionary tree or phylogeny, is a tree diagram that illustrates the evolutionary histories or relationships of diverse biological groups or other categories based on the similarities

and contrasts in their physical or genetic traits (Hu *et al*., 2019). It is utilized in phylogenetics, a field of biology that focuses mostly on the analysis of morphological data matrices and molecular sequencing data to determine how diverse groups of animals have evolved over time. Since it has been used to study biodiversity, evolution, genetics, and ecology among groupings of organisms, the phylogenetic tree is crucial to phylogenetics.

A single phylogenetic tree that represents all life on earth reveals a common ancestor (Felsenstein, 2004). The phylogenetic tree illustrates phylogeny, or the similarities and differences in genetic makeup and morphology between several groups of animals (or taxa). It also shows relationships between taxa that suggest evolutionary relatedness. Additionally, it is possible to assume that they share a common ancestor (internal node). The phylogenetic tree is said to be rooted when the ancestral path is mentioned. Phylogenetic trees come in a variety of forms. A rooted phylogenetic tree is one in which the nodes point to the studied taxa's most recent common ancestor.

The discovery of the *Shigella* pathogenic plasmid proved to be an important development in the emergence of numerous *Shigella* spp. However, there has long been controversy around the plasmid's origin and interspecific interaction. It is now feasible to get a clearer picture of the evolutionary links between diverse *Shigella* spp. and their origin and relationship to *E. coli* thanks to the development of DNA sequencing and the accompanying phylogenetic analysis (The *et al*., 2016). Parks *et al*. (2021) address the problem related to *Shigella* genus members who have high genome homology to *E. coli* and are generally considered atypical members of the *E. coli*. In this matter, in an attempt to treat *Shigella*

species as recognizable substances, they were renamed as *Escherichia* species in the Genome Science Taxonomic Database (GTDB). This resulted in the reclassification of nearly 80% of the *E. coli* genome into new species, including the known laboratory strain of *E. coli* K-12 (to `*E. flexneri*`) as it is more closely related to the strain of *S. flexneri* than to the strain of *E. coli*.

Therefore, they treated *Shigella* species as the later heterologous synonym of *E. coli* (Parks *et al*., 2021). The *et al*. (2016) have demonstrated that *Shigella* genus are distributed over three major clusters or clades namely; C1, C2 and C3, showing the contrasting between the evolutionary history and the traditional nomenclature based on serology. Three subclusters (SC1, SC2 and SC3) are included in cluster C1 which contain a combination of serotypes from *S. boydii*, *S. dysenteria* in addition to *S. flexneri* serotype 6. In contrast to SC2, which mostly consists of S. boydii (serotypes 1, 3, 6, 8, 10 and 18) and *S. dysenteriae* serotype 5, SC1 contains solely *S. dysenteriae* (serotypes 3, 4, 6, 9, 11, 12, and 13). The *S. flexneri* serotype 6 and three more *S. boydii* serotypes (2, 4 and 14) make up the SC3 subcluster. However, *S. dysenteriae* serotype 2 and *S. boydii* serotypes 5, 7, 9, 11, 15, 16, and 17 are both present in cluster C2. The *S. flexneri* serotypes 1, 2, 3, 4, 5, X, and Y, as well as *S. boydii* serotype 12 are all present in cluster C3 with the exception of serotype 6. They came to the conclusion that clusters C2 and C3 shared a more recent common ancestor than cluster C1. Svad *et al*. (2017) investigated the evolutionary position of *S. sonnei* 75/02. Based on the core genome SNP-based phylogeny created earlier in silico SNP genotyping was performed on 439 *S. sonnei* genome sequences.

Using SNP data, researchers have proposed a phylogeny of the genomes that have been analyzed for the first time. Based on 97 SNP sites, the genomes might be classified into 20 different genotype groups. Groups I and IV represent the earlier European clones, Groups II and III represent the more modern global clones. A phylogenetic framework was utilized to ascertain the relationship of *S. sonnei* strain 866 within *Shigella*. To assess the degree of diversity among these four species, the framework includes representative genomes from *S. flexneri, S. sonnei, S. dysenteriae,* and *S. boydii*. Strain 866 is placed in clade S2 by the recently discovered whole genome-based phylogeny, which demonstrates the clonal character of *S. sonnei* segregates (Karaolis *et al*., 1994).

The hereditary homogeneity of this cluster was already established by whole genome comparison, as evidenced by the high degree of overall nucleotide arrangement similarity among the genomes. In light of what has previously been determined (Sahl *et al*., 2015).

As has already been demonstrated, the tree structure is divided into five separate phylogenetic groupings (S1–S5) (Sahl *et al*., 2015). The key point is that, aside from the monophyletic *S. sonnei* clade S2, the *Shigella* species assignment, which is often based on clinical findings, frequently disagrees with the phylogenetic grouping since clades S1 and S3-S5 are made up of a range of species (Alluue-Guardia *et al.*, 2018). In an investigation conducted from 2003 to 2016, Zhi *et al*. (2021) identified 26 *S. flexneri* strains among 366 clinical *Shigella* isolates in Alberta, Canada. The majority of the 26 *S. flexneri* strains that tested were positive for *stx*1 originated in the Dominican Republic, while genomic analysis suggests that three of them may have Haitian roots. These isolates were observed to have geographically specific transmission patterns as distinct clades

from Haiti and the Dominican Republic. However, given the potential for the *stx* quality on the phage to move to other strains and the increase in global travel, it could promote this spread and raise concerns about public health. For this reason, accurate detection of *stx*1 positive *S. flexneri* strains is of utmost importance.

Materials and Methods

3.1. Materials

3.1.1. Equipment and instruments

3.1.1.1. Bacterial isolation instruments and equipment

Table (3-1): list of company and country of equipment and instruments

No.	Equipment and Instruments	Company// Country
1	Beaker 100-1000ml	HLALAB/China
2	Bunsen burner	Shanghai Even/ China
3	Gradient glass cylinders 100-1000ml	HLALAB/China
4	Incubator	Benchmark/ USA
5	laminar flow cabinet	LabTech/Korea
6	loop	HiMedia/ India
7	Millipore syringe filter 0.42 nm and 0.22 nm in size	Microlab scientific/ China
8	Petridish 20ml and 10ml	Biozek/ Netherlands
9	Perks glass Flask 100-1000ml	HLALAB/China
10	Plain tube 10ml	Arth Al.Rafidain/ China
11	Plastic tube rack	Shanghai Even/ China
12	Portable autoclave	Allfine Medlab/ China
13	Refrigerator	Concord/Lebanon
14	Screw cap tubes 25ml	Shanghai Even/ China
15	Sterile cotton swab	Shanghai Even/ China
16	Sterile stick woods	Arth Al.Rafidain/ China
17	Syringe 1ml, 5ml, 10mm	BD Emerald/ Spain
18	Transport media swab	Shanghai Even/ China
19	Water distiller	Henan/ China

3.1.1.2. Molecular study equipment and instruments

Table (3-2): Molecular study equipment and instruments (companyand country of origin)

No.	Equipment and Instruments	Company/ Country
1	Cell Disruptor Genie vortex	Scientific Industries/USA
2	Centrifuge	Hettich /Germany
3	Cooling Centrifuge	Hettich / Germany
4	Electrophoresis	Bioneer/ Korea
5	Eppendorf tubes	BioBasic/ Canada
6	Micropipettes	Gibson/ France
7	Nanodrop spectrophotometer	THERMO/ USA
8	PCR thermocycler T100	BioRad/USA
9	PowerPac HC Electrophoresis Power Supply	BioRad/USA
10	Sensitive Balance	Ohaus /USA
11	U.V Transilluminator	Wised/ Korea
12	Vortex mixer	Talboys/USA
13	Water Bath	Polyscience/ USA

3.1.2. Chemicals materials and solutions

3.1.2.1. Chemicals and solutions

Table (3-3): Chemicals and solutions.

No.	Chemicals	Company/ Country
1	Absolute ethanol	CHEM-LAB/ Belgium
2	Agarose	iNtRON / Korea
3	DNA Marker ladder 1500-100bp	iNtRON / Korea
4	Ethidium bromide 10mg/ml	BioBasic/ Canada
5	Nuclease free water	BioLabs/ UK
6	TBE buffer 10X	iNtRON / Korea

3.1.3. Bacterial media and chemical materials

Table (3-4): Bacterial media (company and country of origin).

No.	Media	Company/ Country
1	BHI agar	Hi media / India
2	BHI broth	Hi media / India
3	Luria-Bertani agar	Hi media / India
4	Luria-Bertani (LB) broth	Hi media / India
5	McConkey agar	Hi media / India
6	Muller-Hinton agar	Hi media / India

3.1.4. Antibiotics disc

The antibiotic discs that used in this study for antimicrobal susceptibility test were done by disc diffusion method on Mueller Hinton agar according to CLSI guide 2022 are listed in table 3-5.

Table (3-5): Antibiotic discs with their concentrations and manufacture company

Antibiotic	Symbol	Disk conc.	Company
Ampicillin	AMP	10µg	Mast/ UK
Ceftriaxone	CRO	30µg	Mast/ UK
Cefixime	CFM	5µg	Mast/ UK
Ceftazidime	CAZ	30µg	Mast/ UK
Chloramphenicol	CAM	30µg	Mast/ UK
Ciprofloxacin	CIP	5µg	Mast/ UK
Gentamicin	GEN	30µg	Mast/ UK
Erythromycin	ERY	15µg	Mast/ UK
Nalidixic acid	NA	30µg	Mast/ UK

3.1.5. Primers

In this investigation, primer3 plus online and the NCBI-Genbank database were used to build PCR primers. According to Table (3-6), the Korean company Macrogen provided these primers:

Table (3-6): PCR primers for 16S RNA gene.

Primers		Sequence (5'-3')	Product Size	Genbank
16S RNA gene Shigella sp.	F	CGCAGGCGGTTTGTTAAGTC	515bp	MW600518.1
	R	ACATTCGAGCAACACGGGG		

Table (3-7): PCR primer for *Shigella* typing with their nucleotide sequence and product size

Primer		Sequence (5'-3')	Product Size	Ref
Shigella flexneri	F	TTT ATG GCT TCT TTG TCG GC	537bp	Ojha *et al.*, 2013
	R	CTG CGT GAT CCG ACC ATG		
Shigella sonnei	F	TCT GAA TAT GCC CTC TAC GCT	430bp	Ojha *et al.*, 2013
	R	GAC AGA GCC CGA AGA ACC G		
Shigella dysenteriae	F	TCT CAA TAA TAG GGA ACA CAG C	211bp	Ojha *et al.*, 2013
	R	CAT AAA TCA CCA GCA AGG TT		
Shigella boydii	F	TCTGATGTCACTCTTTGCGAGT	248bp	Ranjbar *et al.* 2014
	R	GAATCCGGTACCCGTAAGGT		

Table (3-8): PCR primers for virulence factor genes.

Primer		Sequence (5'-3')	Product Size	Ref and gene bank code
Stx1 gene	F	CCAGAGGAAGGGCGGTTTAA	582bp	HM017965.1
	R	CATTCTGGCAACTCGCGATG		
Stx2 gene	F	GCGACGCCTGATTGTGTAAC	201bp	AJ271153.1
	R	AACTTCGCTGAATCCCCCTC		
ShET-1 gene	F	TCACGCTACCATCAAAGA	309 bp	Vargas *et al.*, 1999
	R	TATCCCCCTTTGGTGGTA		
ShET-2 gene	F	GTGAACCTGCTGCCGATATC	147bp	Vargas *et al.*, 1999
	R	ATTTGTGGATAAAAATGACG		

Table (3-9): PCR primers for extended-spectrum *β-lactamase* genes.

Primer		Sequence (5'-3')	Product Size	Genbank
bla-CTXM	F	TTGTTAGGAAGTGTGCCGCT	652bp	MW657993.
	R	TATCCCCCACAACCCAGGAA		
bla-TEM	F	AGATCAGTTGGGTGCACGAG	508bp	HQ203209.
	R	TTGTTGCCGGGAAGCTAGAG		
bla-SHV	F	CCACTATCGCCAGCAGGATC	364bp	HQ203196.
	R	GACTCGATCGTCCACCATCC		
bla-AMPC	F	AAACGACACTCTGCGCCTTA	422p	UYIS01000011.1
	R	GGATTTCACCTCATCCGGCA		

3.1.6. Molecular study kits

3.1.6.1. PCR detection kits

Table (3-10): Kits

No.	Kits	Company	Country
1	Mini gDNA Bacteria	Geneaid	Taiwan
2	Maxime PCR PreMix	iNtRON	koria

3.2. Methods

3.2.1. Patients

A total of 200 stool specimens were taken from individuals of all ages (adults, adolescents, and children), including both males and females. Ages of patients range from four days to thirty years. Stool specimens were collected from patients with diarrhea who were admitted to general hospitals, the teaching hospitals for women and children, and Al-Diwaniyah teaching hospitals between November 2020 and May 2021. A case sheet for each patient listed their name, age, gender, typical look, and symptoms.

3.2.2. Specimens collection

A total of 200 specimens of diarrheal stools were collected from patients under strict safety handling guidelines using sterile containers that were clearly labeled with the patients' information and sent straight to the lab. In some cases, samples are transported in buffered glycerol saline (BGS) or Cary-Blair medium (CB) depending on when they were collected and when they arrived at the lab. For the purpose of identifying *Shigella* species, all samples were streaked in accordance with industry standards on differentiation and selective media (3.2.3) and incubated aerobically at 37^0C for 24 hours.

3.2.3. Bacterial culture media preparation

All culture media were prepared depending on manufacturing instructions as following:

A. MacConkey agar: The medium was used for primary bacterial confirmative isolation. It was prepared by diluted 37gm in 1 liter distilled water and dissolved by microwave for 5 minutes.

B. Brain heart infusion agar: The medium was used for activation of bacterial isolation. It was prepared by diluted 40gm in 1 liter distilled water and dissolved by microwave for 5 minutes.

C. Brain heart infusion broth: The medium was used for preparation of storage stock isolates. It was prepared by diluted 37gm in 1 liter distilled water and dissolved by microwave for 5 minutes.

D. Mueller-Hinton agar: The medium was used for antimicrobial susceptibility test. It was prepared by diluted 38gm in 1 liter distilled water and dissolved by microwave for 5 minutes.

3.2.4. Bacterial biochemical tests

The biochemical identification of *Shigella sp* isolates were done according to method described by MacFaddin, (2000) that includes; the catalase test which was determined by bubble production in H_2O_2 solution, and oxidase test was determined by using Kovac´s oxidase reagent (Kovac's oxidase test determines the presence of cytochrome oxidase). The bacterial motility was determined using hanging drop method (Janda and Sharon, 2002).

3.2.4.1. Indole Test

Procedure of Indole Test

A 4 ml of sterilized tryptophan was added and incubated for 24-28 h at 37 °C. Then, a 500 µl of Kovac's reagent was added to the broth culture in order to get ring formation.

Indole Spot Reagent Procedure

1. Place several drops of indole on a filter paper.

2. Place a portion of the bioisolation onto the filter paper 3. Look into it right away.

Positive: The top layer of reagent forms a pink to red color seconds after reagent addition.

Negative: No change even after the proper reagent has been added.

3.2.4.2. Catalase Test

Protocol

The catalase test has a number of uses and procedure variants. The most widely used method in clinical bacteriology is the catalase slide method or drop method, in which a small amount of the organism is used.

3.2.4.3. Oxidase Test

An organism will pass the oxidase test if it has the cytochrome c oxidase enzyme. To assist differentiate between species, the test is use(oxidase positive) (Macfaddin, 2000). The reagent becomes colorless when reduced, and it changes to a dark-blue to maroon hue when it is oxidized. Cytochrome oxidase or indophenol oxidase are existing in bacteria that produce oxidases. Both of these facilitate the transfer of electrons between molecules that act as electron acceptors and donors(NADH). The oxidase enzyme uses the TMPD test substance as an(oxidation). The results of this procedure are either water or hydrogen peroxide,

Procedures

All disks should have four deionized water inoculating loops on it.

transfer a significant quantity of germs to the disk using a loop. Spend no more than three minutes watching the disk. A positive outcome is achieved if the injection site changes from dark blue to maroon to virtually black. If there isn't a hue shift after three minutes, the outcome won't be good. Alternately, using a sterile procedure and a single-line streak *inoculation*, live bacteria can be grown on trypticase soy agar plates> After colonies have formed on the medium (37^0C for 24-48h), 1-2 drops of reagent DMPD are given to the surface of each organism to be studied. A purple tinge will appear after 10 t0 20 seconds if the test is positive (OX+). An OX^- test result that is negative will appear as a very light pink or as no color at all.

3.2.5. Antibiotic susceptibility test

The disc diffusion method for *in vitro* antibiotic susceptibility tests was done according to Sheikh method (Sheikh *et al*., 2019). The antibiotic disc agents, concentrations, and the interpretation of zones of inhibition for *Shigella* spp. (CLSI, Humphries *et al*., 2021). The preparation of test was done by using Mueller-Hinton agar plate that inoculated by 0.5 McFarland tube dilution of bacterial culture which spread by sterile cotton swab(37ºC /24 h). Present or absence zone of inhibition around each of the disc after the period of incubation was explained the antibiotic action and the diameter of zone of inhibition produced by each antibiotic was measured to determinate the patterns of antibiotic susceptibility **(**Ericsson and Sherris, 1971).

3.2.6. Polymerase Chain reaction

The 16S ribosomal RNA gene was amplified using the PCR technique for confirmational molecular detection. Other key virulence factors and

antibiotic resistance genes were also discovered. All of the PCR primers

used in the technique were created for this investigation in the manner described below:

3.2.6.1. Bacterial DNA Extraction

Bacterial genomic DNA was extracted from XDR *Shigella* isolates according to company instructions by using (Presto™ Mini gDNA Bacteria Kit) (Chien *et al*., 1976):

1-Sample Preparation

Two ml of bacterial cells that have been cultivated for 18 hours and transferred to microcentrifuge tube, centrifugation (10,000 rpm /1 min), and the floatable was then discarded.

2-Steps of Cell Lysis

A.The tube was filled with 180 l of GT (guanidium thiocyanate) buffer, the cell pellet was suspended by a vortex, 20 µl (Proteinase K) was added, and incubated at 60 ^{0}C /10 min. The mixture tubes were turned over every three minutes during the incubation periods.

B-Each tube received 200 µl of the GB buffer, which was then vortexed (10 sec) at 60 °C /10 min, inverted every three minutes.

3-Steps in DNA binding

A. A 200 ml of absolute ethanol were added, quickly mixed by vortex, and any precipitates that formed were broken up with pipetting.

B. All mixture (including any precipitate) were transferred to a GD column, which was positioned in a 2 ml collection tube, then, the mixture was spin for one minute at 10000 rmp. The flow-through-containg 2 ml collection tubes were discarded, and a fresh 2 ml collection tube was used to hold the GD column.

3.2.6.2. Estimation of Extracted of Whole DNA

The extracted of whole DNA was evaluated as follows using a Nanodrop, which was used to measure DNA content (ng/µL) and assess RNA purity at absorbance (260/280 nm):

1 Proper program (DNA) was selected.

2. The measurement pedestals were repeatedly cleaned with a dry wipe. 2 l of free nuclease water was then carefully pipetted and placed on the lower measurement pedestals to blank the apparatus.

3. A 1 l DNA sample was measured after the Nanodrop sampling arm was lowered.

3.2.6.3. PCR mix preparation

PCR reactions were created using the PCR master mix that created in accordance with the provided company instructions. The standard maxime PCR PreMix kit tubes, which also contained every other component required for the PCR reaction, such as those in the table above, were then mixed with the PCR master mix components. The

entire set of PCR tubes was then put into an Exispin vortex centrifuge and spun at 3000 rpm for three minutes (BioRad-USA).

3.2.6.4. PCR Thermo cycler Conditions

Using a conventional PCR thermocycler, the following conditions methodology was used for each gene as in Table (3-11):

Table (3-11): PCR thermo cycler conditions protocol

No.	Gene name	PCR Amplicon (bp)	Initial denaturation Temp./time	Denaturation Temp./time	Annealing Temp./time	Extension Temp./time	Cycle	Final extension Temp./time	Hold Temp./time
1	*Stx*1	582	95°C/ 4 min	95°C/ 30 sec	59.3°C/ 30 sec.	72°C/ 60sec.	33	72°C/ 5min	4°C/forever
2	*Stx*2	201	95°C/ 4 min	95°C/ 30 sec	59.3°C/ 30 sec.	72°C/ 60sec.	33	72°C/ 5min	4°C/forever
3	*ShET*-1	309	95°C/ 4 min	95°C/ 30 sec	59.3°C/ 30 sec.	72°C/ 60sec.	33	72°C/ 5min	4°C/forever
4	*ShET*-2	147	95°C/ 4 min	95°C/ 30 sec	58.3°C/ 30 sec.	72°C/ 50sec.	33	72°C/ 5min	4°C/forever
5	*blaCTX-M*	652	95°C/ 4 min	95°C/ 30 sec	58.4°C/ 30 sec.	72°C/ 20sec.	33	72°C/ 5min	4°C/forever
6	*blaTEM*	508	95°C/ 4 min	95°C/ 30 sec	58.3°C/ 30 sec.	72°C/ 40sec.	33	72°C/ 5min	4°C/forever
7	*blaSHV*	364	95°C/ 4 min	95°C/ 30 sec	57.2°C/ 30 sec.	72°C/ 60sec.	33	72°C/ 5min	4°C/forever
8	*blaAMPC*	422	95°C/ 4 min	95°C/ 30 sec	58.4°C/ 30 sec.	72°C/ 40sec.	33	72°C/ 5min	4°C/forever

3.2.6.5. Analysis of PCR product

PCR results were examined using the agarose gel electrophoresis method in the manner described below:

1- Agarose gel (1.5%) was made by dissolving 1.5g of the agarose in 100ml of 0.5X TBE (Tris/Borate/EDTA) for 5 minutes, heat in the microwave, and then allowed to cool at 50°C.

2- The agarose gel solution was then stained with 3 ul of ethidium bromide dye.

3- Then, the comb was removed after the agarose gel solution was hardened in the tray for 15 min at room temperature. The comb was replaced in the proper area and the solution was poured into the tray. PCR results were examined using the agarose gel electrophoresis method in the manner described below.

4- A 0.5X TBE buffer was added to tray. Each well received five tenths of a liter of PCR output, with the first well receiving five liters of DNA marker ladder (100-1500 bp).

5- UV transilluminators were used to see the PCR results (Suchman, 2016).

3.2.7. DNA sequencing

DNA sequencing method was performed for study the confirmative genetic identification and genetic variation (substitution Mutations)

analysis based on 16S ribosomal RNA gene pathogenic *Shigella* spp. isolates. The DNA sequencing study was performed using (Mega 6.0), partial metE and metF gene-based ClustalW alignment analysis, multiple sequence alignment analysis, and the Maximum Composite Likelihood technique using phylogenetic tree UPGMA method. the identification of homologous sequences and analysis of mutations using NCBI BLAST. The genes were finally submitted to the NCBI-Genbank database in order to get Genbank accession numbers.

3.3. Statistical analysis

The statistical analysis was done using Chi-square ($P \leq 0.01$) and LSD in one way ANOVA method at ($P \leq 0.05$) as well as histograms analysis were performed by using SPSS,2010 VERSION Statistics software (Daniel, 2009).

4. Results and Discussion

4.1. Description of study specimens

4.1.1. Demographic characteristics according to the gender and age

Out of 200 patients, only 20 (10%) were diagnosed as shigellosis (Table 4-1). The samples were divided into eighty-five (43%)-males and One hundred and fifteen (57%)-females. The age of patients was divided into three categories. Table 4.1 showed that the less than 11 years was 107 (53.5%), 11-20 years was 48 (24%), and 21-30 years was 45 (22.5%).

Table (4-1): Distribution of patients according to gender and age.

Characteristic	Category	No. (%)	X^2	P-value
Gender	Male	85(42.5)	9*	0.003
	female	115(57.5)		
	Total	200(100)		
Age	Less than 11	107(53.5)	55.005*	0
	11-20	48(24)		
	21-30	45(22.5)		
	total	200(100)		
Total	Positive	20(10%)	8.5*	0.002
	Negative	180(90%)		
	Total Samples	200(100%)		

* Significant difference at $p \leq 0.05$

However, females were infected at a higher rate than males in the total population samples. Age groups were further divided into three subgroups ranging from 4 to 30 years, with approximately 53.5% of children infected, approximately 24% in the 11-20 age group, and approximately 22.5% in the early teens and adults

(21–30 years). In contrast to *S. flexneri* serotype variant X (prevalent in urban areas), which equally affects men and women, Jain *et al*. (2020) discovered that *S. flexneri* serotype 2 affected women much more than males (predominantly in rural areas). Males were more resistant to disease, and antimicrobial treatments were more successful because men's metabolic systems were biologically faster and more potent than women's. *Shigella* spp. persisted in the body for longer than usual due to women's more sensitive temperaments, making them less resistant (Ranjbar and Farahani, 2019). Since children's guts are still developing, they receive low dosages of antibiotics to avoid acquiring resistance to the bacteria, which is beneficial to their gut health.

Additionally, broad-spectrum antibiotics eliminate a large number of important intestinal microbes. Similar trends were discovered in adolescents, although they are more likely to be explained by changes in food intake and sanitation than by heredity (Ngoshe *et al*., 2017). The typical method for examining the cause of dysentery involves doing precise bio-chemical checks to determine the type and class of bacteria through culture, characterized by a tedious and long time. For the detection of shigellosis, DNA-dependent molecular approaches, particularly the PCR method, are now often utilized in scientific and research facilities (Farfán *et al*., 2010; Alipour *et al*., 2012).

4.2. Detection of *Shigella* isolates using traditional methods

4.2.1. Identification of *Shigella* isolates on different media

Shigella are small, rod-shaped organisms. A 20 *Shigella* isolates were identified from the total samples. As shown in figure (4-1), when *Shigella* isolates were cultured colonies of non-lactose fermenters that are transparent and pale developed on MacConkey agar. *Salmonella-Shigella* agar performed worse than Hekton

enteric and xylose-lysine-desoxycholate agar, and required more work to do so (Pollock and Dahlgren, 1974). Samples must be plated on MacConkey with one of the following agars: xylose-lysine-deoxycholate, Hektoen enteric, or deoxycholate citrate in order to best detect *Shigella* in feces.

Shigella does not create H2S, hence its colonies on Hektoen agar were bluish-green rather than the salmon-colored *Salmonella* colonies' black centers. *Shigella* is not a lactose or xylose fermenter and exhibits a high level of biochemical inertness (Dekker and Frank, 2015). *Shigella* most commonly does not create gas, with the exception of some *S. flexneri* strains, which are outliers and may ferment lactose (Nataro *et al*., 2007). Kligler iron or triple sugar iron agar were used to further describe the appropriate colonies. *Shigella* lysine decarboxylase tests usually come out negative. The biochemical properties of Groups A, B, and C are comparable, although *S. sonnei* has ornithine decarboxylase activity and beta-galactosidase activity, according to Koneman *et al*. (1997).

4.2.2. Identification of *Shigella* isolates using biochemical test

The results of the current study of the chemical tests (Indole, catalase and oxidase) showed that all isolates of *Shigella* were positive for Indole and Catalase. As for the negative, it was for examining the oxidases in the types identified in table (4-2).

Table (4-2): Biochemical tests conducted on the isolated pathogenic bacteria (n=20).

Shigella species	Indole	Catalase	Oxidase
S. flexneri	+	+	-
S. Sonnei	+	+	-
S. dysenteriae	+	+	-
S. boydii	+	+	-

This result is in line with the Bergey's Manual of Determinative Bacteriology (Holt *et al*., 2012). Regarding the negative biochemical tests, they included urease, lysine decarboxylase, ornithine decarboxylase, and some isolates produce a red ring as a positive result in the indole assay (Saima *et al*., 2018). Other study found that *Shigella* spp. have positive result in Methyl Red, Voges Proskaure, Indole, and Triple Sugar Iron (Chhanda *et al*., 2019).

4.3. Antimicrobial susceptibility test

Shigella species differed in the presence of resistance genes, and there were statistically significant differences among them as shown in Table 4-3. The bacteria were highly resistant to ampicillin and nalidixic acid (=60%), cefixicin (59.5%), ceftazidime (55%), and ceftriaxone (55%), were all *Shigella* spp. Resistant (43.6 %). *Shigella* spp. isolates were 76.6 % susceptible to ciprofloxacin and 44 % susceptible to ceftriaxone, respectively, when susceptibility to antibiotics was examined. A 100 % of the samples examined were insensitive to AMP, 45% to NAL, 55% to ceftriaxone, CIP (15%), CHL and cefixime (60%), ceftazidime (65%), and gentamicin (40%).

Regarding to *S. flexneri* 100% of isolates were resistance to ampicillin and erythromycin, while 66.66% of isolates were sensitive to eftriaxone, ciprofloxacin, gentamicin and 33.33% of isolates were resistance to ceftriaxone, ciprofloxacin and nalidixic acid as shown in table (4-4). Regarding to *S. Sonnei* 100% of isolates were resistance to ampicillin, and cefixime, while 100% of isolates were intermediate to gentamicin and 75.00 % of isolates were sensitive to ceftriaxone as shown in table (4-5).

Table (4-3): Antimicrobial susceptibility test (number and percentage).

Antibiotic	Sensitive isolates		Intermediate isolates		Resistance isolates	
	No.	%	No.	%	No.	%
Ampicillin	0	0	0	0	20	100
Ceftriaxone	6	30	3	15	11	55
Erythromycin	0	0	2	10	18	90
Chloramphenicol	4	20	4	20	12	60
Ceftazidime	3	15	4	20	13	65
Ciprofloxacin	12	60	5	25	3	15
Gentamicin	3	15	9	45	8	40
Cefixime	5	25	3	15	12	60
Nalidixic acid	2	10	9	45	9	45
*X*2	61.72*					
P-value	0					

Table (4-4): Antimicrobial susceptibility test (number and Percentage) of *S. flexneri.*

Antibiotic agent	Sensitive isolates		Intermediate isolates		Resistance isolates	
	n	%	*n*	%	*n*	%
Ampicillin	0	0.00	0	0.00	3	100
Ceftriaxone	2	66.66	0	00.00	1	33.33
Erythromycin	0	0	0	00.00	3	100
Chloramphenicol	0	00.00	3	100	0	0
Ceftazidime	0	00.00	0	00.00	2	66.66
Ciprofloxacin	2	66.66	0	00.00	1	33.33
Gentamicin	2	66.66	0	00.00	1	33.33
Cefixime	0	00.00	1	33.33	2	66.66
Nalidixic acid	0	00.00	2	66.66	1	33.33

Regarding to *S. sonnei* 100% of isolates were resistance to ampicillin, 69.23 % of isolates were resistance to ceftriaxone and ceftazidime while 53.85% of isolates were sensitive to ciprofloxacin as shown in table (4-6).

Table (4-5): Antimicrobial susceptibility test (number and Percentage) of *S. Sonnei.*

Antibiotic	Sensitive isolates		Intermediate isolates		Resistance isolates	
	n	%	*n*	%	*n*	%
Ampicillin	0	0.00	0	0.00	4	100
Ceftriaxone	3	75.00	1	25.00	0	0
Erythromycin	0	00.00	1	25.00	3	75.00
Chloramphenicol	0	00.00	2	50,00	2	50.00
Ceftazidime	1	25.00	1	25.00	2	50.00
Ciprofloxacin	0	00.00	2	50.00	2	50.00
Gentamicin	0	00.00	4	100	0	00.00
Cefixime	0	00.00	0	00.00	4	100
Nalidixic acid	0	00.00	1	25.00	3	75.00

Table (4-6): Antimicrobial susceptibility test (number and Percentage) of *S. dysenteriae.*

Antibiotic	Sensitive isolates		Intermediate isolates		Resistance isolates	
	n	%	*n*	%	*n*	%
Ampicillin	0	0.00	0	0.00	13	100
Ceftriaxone	2	15.38	2	15.38	9	69.23
Erythromycin	0	0.00	1	7.69	12	92.31
Chloramphenicol	2	15.38	1	7.69	10	76.92
Ceftazidime	2	15.38	2	15.38	9	69.23
Ciprofloxacin	7	53.85	2	15.38	4	30.77
Gentamicin	1	7.69	4	30.77	8	61.54
Cefixime	5	38.46	2	15.38	6	46.15
Nalidixic acid	1	7.69	6	46.15	6	46.15

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion method, as recommended by the clinical and Laboratory Standards Institute (CLSI, 2022). All isolates were tested for antibiotic susceptibility using ceftriaxone (30 g), ampicillin (10 g), chloramphenicol (30 g), nalidixic acid (30 g),

trimethoprim-sulfamethoxazole (SXT, 25 g), ceftazidime (30 g), ciprofloxacin (5 g), cefixime (5 g), and gentamicin (10 g) as (Mast Diagnostics Ltd., Merseyside, UK) (Ali *et al*., 2020).

The susceptibility of *Shigella* to antibiotics varies according to dosage and formulation. Shigellosis is a disease that mostly affects children in areas of developing countries where it is endemic. However, Specialists advise the need to treat infected people to prevent transmission and control of infection (Ngoshe *et al*., 2017). Although *Shigella* species-related infectious diarrhoea is frequently self-limiting, as a result, empiric antibiotic therapy is frequently used in, as a result, antibiotic treatment is frequently used in children carrying symptoms before the result of stool culture. Most patients will notice an improvement in their symptoms even before the results are available (Alemu *et al*., 2019). Resistance to nalidixic acid, tetracycline, and trimethoprim-sulfamethoxazole exists in *S. sonnei* strains. Nalidixic acid-resistant bacteria displayed decreased susceptibility to fluoroquinolones but not complete resistance (Sheikh *et al*., 2019). In a drug susceptibility test, *S. dysenteriae* showed resistance to quinolone antibacterial drugs. Streptomycin and spectinomycin are two aminoglycoside antibiotics that bind to ribosomal subunits and limit protein synthesis at intracellular sites (Rahman and Sarker, 2021). A significant ratio of immovability to AMP, TM, and TAC was noted in numerous earlier investigations conducted in Iran and other nations (Jomezadeh *et al*., 2014). *Shigella* species and cefotaxime resistance were found to be significantly correlated (p 0.05). *S. flexneri* strains were primarily resistant to quinolones and chloramphenicol, although overall study revealed that *S. sonnei* had stronger antibiotic resistance than other species. To ciprofloxacin, all *Shigella* isolates were sensitive (Sambe-Ba *et al*., 2013; Hosseini Nave *et al*., 2016). Ciprofloxacin is beneficial against shigellosis. Multi drug resistant (MDR)

Shigella developed due to the medication's overuse and abuse in the treatment of diarrhoea and urinary tract infections (Hussen *et al*., 2019). Another study from the University of Kolkata discovered that quinolones were 90% resistant to the antibiotics tested (Halimeh *et al*., 2020).

According to antibiotic susceptibility statistics, ciprofloxacin resistance increased from 57.1% to 100% over five years, while ampicillin resistance ranged between 35.70% and 81.250%. Additionally, nalidixic acid and cotrimoxazole were completely resistant to pressure (Pakbin *et al*., 2021). Sheikh *et al*. (2019) discovered identical resistance patterns in 2015-2016, with the same prevalence of indicated resistance (33.3%).

Antibiotic resistance changes as a result of plasmid-borne gene transfer. In their analysis, 43.7% of samples were insensitive to furazolidone, and 33.3% were insensitive to gentamicin (Sati *et al*., 2019). According to antibiotic susceptibility statistics, ciprofloxacin resistance increased from 57.1 to 100%, while ampicillin resistance ranged between 35.7% and 81.25%. Between 2014 and 2016, nalidixic acid and cotrimoxazole displayed 100% resistance (Anandan *et al*., 2017). Brander *et al*. (2017) discovered a similar level of resistant reluctance. About a quarter of the samples were resistant to one of the third generation cephalosporins (Chung *et al*., 2016). *S. sonnei* strains were 20% resistant to cefotaxime or ceftriaxone, whilst *S. flexneri* strains were 11.76% resistant. *Shigella* spp. resistance to cephalosporins was reported to be between 2% and 5.2% in Southeast Asian research (Chung *et al.*, 2016).

According to a large multicenter study conducted in eight Asian nations (Drprabhurajeshwar *et al*., 2015), resistance to ceftriaxone increased by 5% in *Shigella* strains between 2001 and 2004. As a referral center, they accept patients

that have been treated partially or completely, which may show the accurate occurrence of *Shigella* spp. (Jain *et al*., 2020).

4.4. Molecular methods for further identification of *Shigella*

DNA was extracted from 20 bacterial isolates after the initial diagnosis based on biochemical and morphological tests of *Shigella* spp. for PCR technique to detect the genes of 16S rRNA.

The whole DNA was extracted using genomic DNA kit, then, of DNA concentration and purity were measured, DNA concentration were 20.9 – 49.5 ng/µl and purity of the DNA were 1.48 – 1.98. It was found that only 20 (10%) positive samples out of 200 samples of *Shigella* spp. isolates were highly detectable in 16s ribosomal RNA, demonstrating the presence of *Shigella* spp. isolates in the sample amplified by PCR and run in gel. The 16S rRNA gene is 1550 kb in length and contains both conserved and variable segments due to the prevalence of interspecific polymorphisms in the 16S rRNA gene.

The number of people infected was lower than expected based on the symptoms; it's possible that one of them had been infected previously and it was a mild infection that was passed on to other age groups, or that some people had shown resistance to the *Shigella* species.

The results of the current study showed after collecting 200 samples from patients with clinical signs and abdominal pain, where 20 (10%) positive samples out of 200 samples were diagnosed by PCR, (Figure 4.1).

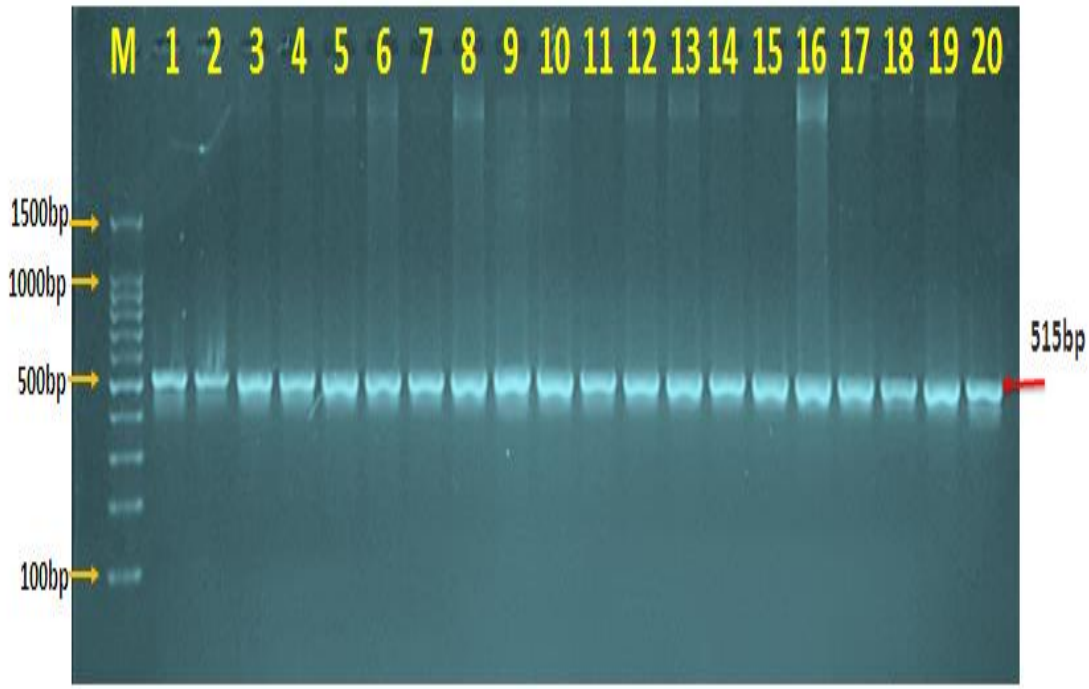

Figure (4.1): Agarose gel electrophoresis image showed the PCR product analysis of 16S ribosomal RNA detection gene in *Shigella* spp. isolates. Where Marker ladder (1500-100bp), Lane (1-20) showed positive *Shigella* sp. 16S ribosomal RNA gene at 515bp PCR product size.

The results illustrated, upon examination by PCR method of *Shigella* species, showed important differences among types (Table 4.7), *S. flexneri* (15%, 3 ⁄ 20), *S. Sonnei* (20%, 4 ⁄ 20), *S. dysenteriae* (65%, 13 ⁄ 20), and *S. boydii* (0%, 0 ⁄ 20) (Figures 4-2, 4-3, 4-4 and 4-5).

Figure 4-2 shows the presence of *S. flexneri* isolates from the samples collected. *S. flexneri* was vaguely present in the PCR amplified products at a 537 base pair of PCR product size, which is noticeable easily. The majority of isolates (38.46%) were collected from children aged one to ten years. This was statistically significant compared to the other age categories ($P \leq 0.05$).

Figure 4-3 shows the presence of *S. sonnei* isolates from the samples collected as *S. sonnei* was vaguely present in the PCR amplified products at a 430 base pair of PCR product size.

Table (4-7): Identification of *Shigella* species according to the PCR.

Shigella species	Positive samples	%
S. flexneri	3	15
S. Sonnei	4	20
S. dysenteriae	13	65
S. boydiii	0	0
Total	20	100
X^2	25.06*	
P-value	0	

Figure 4-4 shows the presence of *S. dysenteriae* isolate from the samples collected. *S. dysenteriae* was vaguely present in the PCR amplified products at a 211 base pair of PCR product size. The Figure shows the positive results of isolates found from the samples.

The findings create a new phylogenetic tree that separates *Salmonella* from *Shigella* and *E. coli* while yet showing close relationships between the three bacteria. In order to distinguish between bacterial species that are closely related to one another, the *gyr*B gene may prove to be helpful. 16S rRNA is used to identify the genus of unknown bacteria for easy amplification . However, examination of the more variable *gyr*B sequence region allows for the species-level identification of unidentified bacteria. According to reports, there are drawbacks to employing

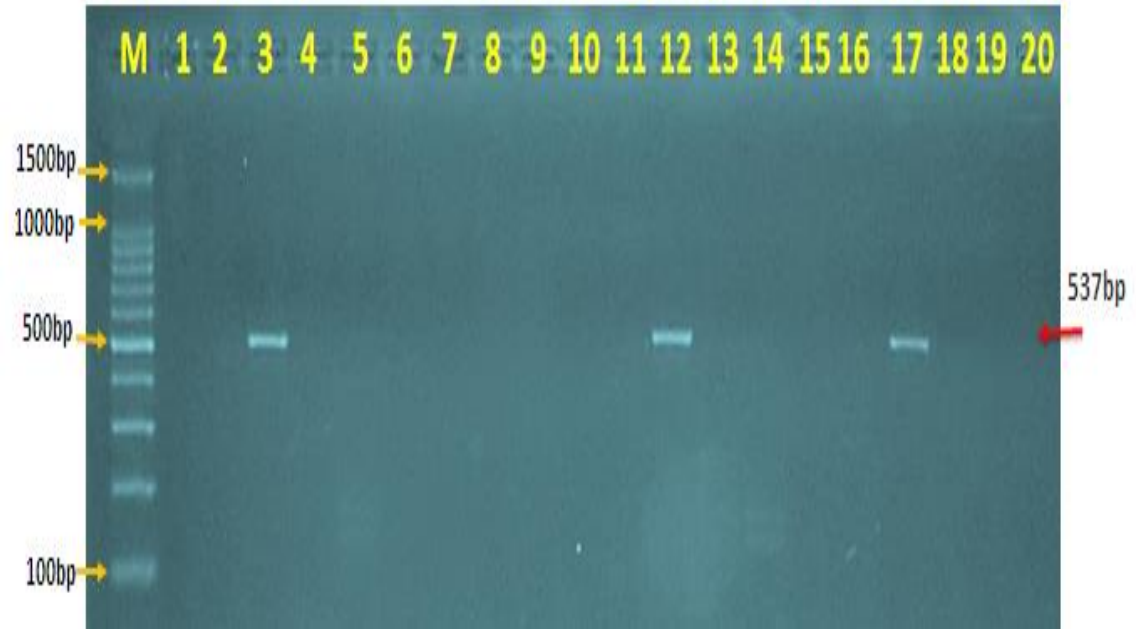

Figure (4.2): Agarose gel electrophoresis image showed the PCR product analysis of *S. flexneri* isolates. Where marker ladder (1500-100bp) and Lane (1-20) showed some positive *S. flexneri* isolates at 537bp PCR product size. Lane 21, negative control.

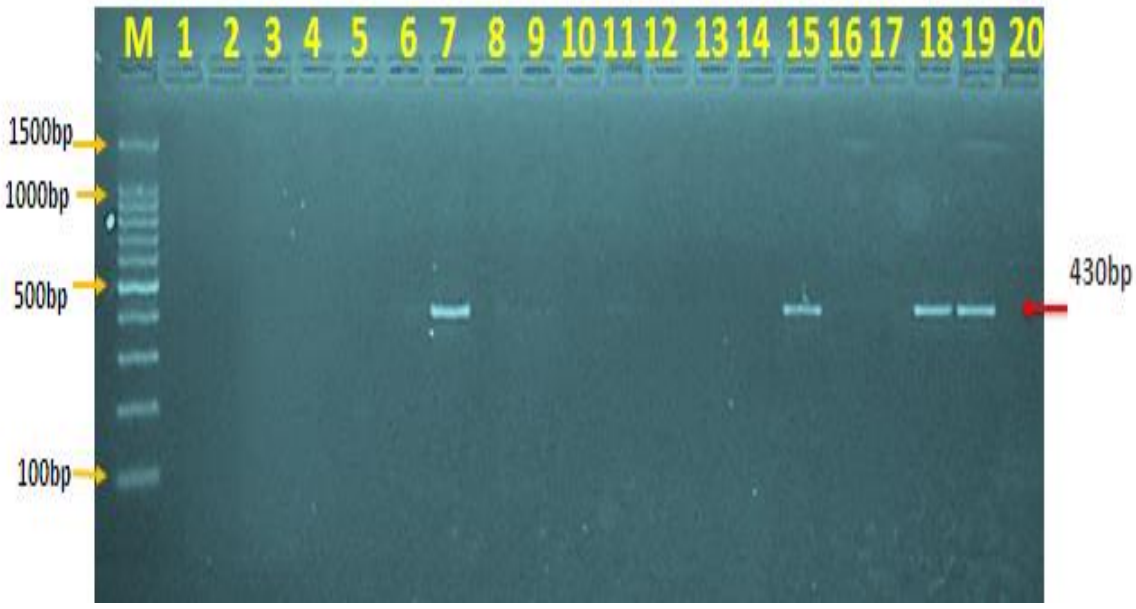

Figure (4.3): Agarose gel electrophoresis image showed the PCR product analysis of *Shigella sonnei* isolates where marker ladder (1500-100bp) and Lane (1-20) showed some positive *Shigella sonnei* isolates at 430bp PCR product size.

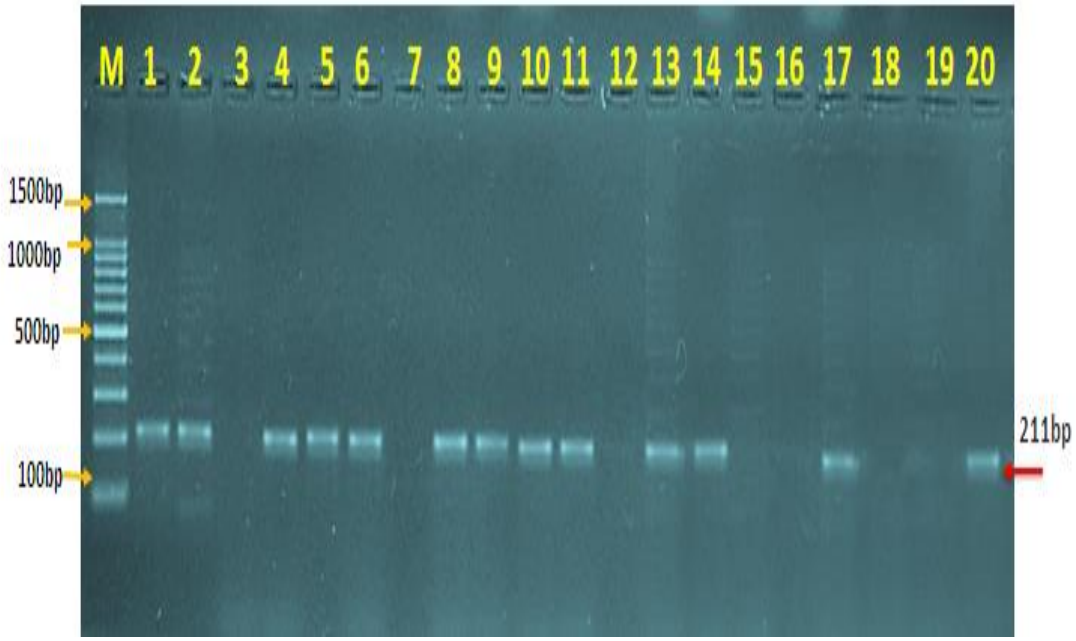

Figure (4.4): Agarose gel electrophoresis image showed the PCR product analysis of *Shigella dysenteriae* isolates. Where marker ladder (1500-100bp), Lane (1-20) showed some positive *Shigella dysenteriae* isolates at 211bp PCR product size.

Figure (4.5): Agarose gel electrophoresis image that showed the PCR product analysis of *Shigella boydii* isolates. Marker ladder (1500-100bp) and Lane (1-20) showed negative *Shigella boydii* isolates at 228bp PCR product size.

16S rRNA sequencing for bacterial identification. One of these is the inability to tell atypical *E. coli* from *Shigella* spp. *S. flexneri, S. sonnei*, and *S. boydii* share 99.8, 99.9, and 99.7% of the sequences of *E. coli*, respectively (Fukushima *et al*., 2002). *Shigella*, a non-serotypeable species, must be molecularly typed because it is the main cause of bacterial diarrhea in underdeveloped nations. This study demonstrates that the kmer technique and *gyr*B gene analysis can overcome some 16S rRNA sequence method shortcomings, such as the inability to discriminate between closely related species (Dhiviya Prabaa *et al*., 2017). Chen *et al*. (2014) did not distinguish atypical *E. coli* and *Shigella* spp. by molecular identification using 16S rRNA sequencing. 16S rRNA sequence similarity between different pathogenic strains of Shigella bacteria using the reference 16S rRNA sequence available from (NCBI) (Devanga Ragupathi *et al*., 2017).

Shigella species are classified into three serogroups and one serotype: Serogroup A contains 15 serotypes of *S. dysenteriae*, Serogroup B contains 9 serotypes of *S. flexneri*, Serogroup C contains 19 serotypes of *S. boydii*, and serogroup D contains one serotype of *S. sonnei* (Knirel *et al.*, 2015). In another study in six Asian countries, it was found that the *flexneri* type is the most common, while the *dysenteriae* type is the least common (Von Seidlein *et al*., 2006). *S. sonnei* is found abundantly in various parts of the world and is considered as one of the most abundant species found after *S. flexneri* which is reported to be found in (49.2%) of the cases of shigellosis (Sheikh *et al*., 2019). From stool samples, 56 (9%) *Shigella* strains were identified. *S. flexneri* 31 was the most prevalent species, tracked by *S. sonnei* (33%) and *S. boydii* (13%). In the current investigation, *S. dysenterae* was not found. The multiplex PCR technique was used to confirm the serologically determined *Shigella* spp. identities of all the isolates (Hosseini Nave *et al.*, 2016). As the diarrhea progresses, bloody stools become

more common. Anemia is uncommon unless the infection is worsened by the development of HUS or thrombotic thrombocytopenic purpura, which typically manifests as left shift leukocytosis (Ullah *et al*., 2019).

4.5. Distribution of *Shigella* spp. isolates according to the gender and age by PCR

Based on the PCR results, as shown in Table 4-8, the ratios of males and females were close, and there was no difference between them. The *Shigella* spp. isolates were divided into (9/85) males (10.58 %) and (11/115) females (9.56 %). The age of the samples was divided into three categories; Less than 11yeas about 11 (10.28 %), and 11-20 years, about 4 (8.33 %), and 21-30 years, 5 (11.11 %).

This prevalence among children was higher than the one Mohammed *et al.* (2004) found in Basra, where they found that 1.4% of children with diarrhea had shigellosis. According to Ali *et al*. (2020), children aged 1 month to 10 years had the highest incidence of *Shigella*, whereas children aged 10 to 20 had the lowest incidence and those aged 60 to 70 had the fewest isolates.

Furthermore, a comparable finding by Orrett, (2008) showed that 88.8% of cases were recovered from children under the age of 10, with an 8% isolation rate for *Shigella*. In the other trial, there were a total of 241 individuals. *Shigella* was present in 13.3% (32/241) of people. *Shigella* was found to be quite prevalent (22.6%) in children aged between 12 and 23 months. The age range of 0 to 5 months did not have any *Shigella* isolates. Most *Shigella* isolates came from cases of bloody and mucoid diarrhea. Children among the age group of 12–23 months were highly affected.

Table (4-8): Distribution of *Shigella* spp. isolates according to the age and gender.

Age interval (year)	Gender	Age (year)	*Shigella* spp.	Isolate No.
Less than 11	male	6	*S. sonnei*	10
		8	*S. desyenteriae*	12
		10	*S. sonnei*	14
		10	*S. flexneri*	17
	female	4	*S. desyenteriae*	15
		5	*S. desyenteriae*	8
		6	*S. desyenteriae*	19
		7	*S. desyenteriae*	9
		8	*S. desyenteriae*	6
		9	*S. desyenteriae*	4
		10	*S. desyenteriae*	2
11-20	male	15	*S. sonnei*	1
		17	*S. flexneri*	3
		20	*S. flexneri*	7
	female	12	*S. desyenteriae*	11
21-30	male	22	*S. sonnei*	20
		30	*S. desyenteriae*	5
	female	25	*S. desyenteriae*	13
		28	*S. desyenteriae*	18
		29	*S. desyenteriae*	16

Therefore, responsible bodies should work hard on preventive measures to reduce or eradicate the problem that has occurred due to shigellosis (Kahsay and Teklemariam, 2015).

4.6. Distribution of *Shigella* spp. isolates according to clinical signs and diarrhea

The results, upon examination by PCR method as shown in table (4.11); 100% (20 ⁄ 20) of patients suffering from abdominal pain, fever (25%, 5 ⁄ 20), vomiting (20%, 4 ⁄ 20), and fatigue (5%, 1 ⁄ 20). About 55 % (11/ 20) patients suffering from mucoid bloody, soft bloody (20. %, 4/ 20), liquid bloody (15 %, 3 /20), liquid mucoid (10 %, 2/ 20) as shown in table (4-9).

The most frequent signs of *Shigella* infection are diarrhoea (often bloody), fever, and cramps. Symptoms usually appear 1-2 days after infection and last for seven days or longer. Antibiotics are rarely necessary for the majority of situations. Antibiotics should be administered if there is a serious illness or a condition that impairs immune system's effectiveness (Ahmed *et al*., 2021).

The clinical symptoms associated with specific bacterial infections, such as high body temperature, intestinal colic, nausea, vomiting, watery stools, and bloody stools, were observed frequently in diarrheal patients (Qu *et al*., 2012). The most common complaint was fever (38%), followed by diarrhea (33%), vomiting (8%) and abdominal pain (7%). Remarkably, 45% of the patients had a history of hospitalization (Sethuvel *et al*., 2019).

To promote infection, *Shigella* spp. must endure in the stomach's acidic environment and get inside the colon's epithelial layer. *Shigella* spp. multiplies inside colonic epithelial cells and invades surrounding cells, killing the infected cells in the process. Shigellosis frequently results in the bloody mucoid diarrhea that is distinctive of the condition.

The colon becomes inflamed and ulcerated, and the damaged mucoid cells are excreted (Warren *et al*., 2006; Montville and Matthews, 2005). There are four main species within the genus *Shigella*, namely: *dysenteriae,sonnei, flexneri, boydii* (Tickell *et al*., 2017).

Shigellosis typically has an incubation period of 1-4 days. Asymptomatic infections can happen, mainly in people who have already been afflicted. It starts out with headaches, fever, vomiting, and diarrhea. Patients who are immunocompetent typically experience a

Table (4-9): Distribution of *Shigella* spp. isolates according to the clinical signs and diarrhea types.

Shigella spp.	Isolate No.	Clinical signs	Diarrhea type
S. desyenteriae	2	Fever, abdominal pain	Soft bloody
	4	Fever, abdominal pain	Soft bloody
	5	Abdominal pain	Mucoid bloody
	6	Vomiting, abdominal pain	Mucoid bloody
	8	Vomiting, abdominal pain	Mucoid bloody
	9	Abdominal pain	Liquid mucoid
	11	Abdominal pain	Liquid bloody
	12	Vomiting, abdominal pain	Liquid bloody
	13	Fever, abdominal pain	Soft bloody
	15	Fatigue, abdominal pain	Liquid mucoid
	16	Abdominal pain	Mucoid bloody
	18	Abdominal pain	Liquid bloody
	19	Fever, abdominal pain	Soft bloody
S. flexneri	3	abdominal pain	Mucoid bloody
	7	abdominal pain	Liquid bloody
	17	Fever, abdominal pain	Mucoid bloody
S. sonnei	1	Abdominal pain	Mucoid bloody
	10	Vomiting, abdominal pain	Mucoid bloody
	14	Abdominal pain	Mucoid bloody
	20	Abdominal pain	Mucoid bloody

minor illness with short-lived symptoms. There can be a progression to dysentery in some people, especially immunocompromised patients, such as reactive arthritis, bacteremia, colonic obstruction, colic distension, and hemolytic uremic syndrome (Khan *et al*., 2013). Symptoms of the disease differ from one person to another, as persistent diarrhea is one of the main complications in poor countries (Black *et al*., 1982).

4.7. Detection of *Stx*1, *Stx*2, *ShET*-1 and *ShE*T-2 genes

The results of the present study, upon examination by PCR method of *Shigella* virulence factors, deferent PCR product of *Stx*1, *Stx*2, *ShET*-1 and *ShET*-2 genes of

microbe were detected as shown in Figures (4-6, 4-7, 4-8 and 4-9). The presence of the stx1 gene that is highly detectable in *Shigella* spp. isolates, proving the

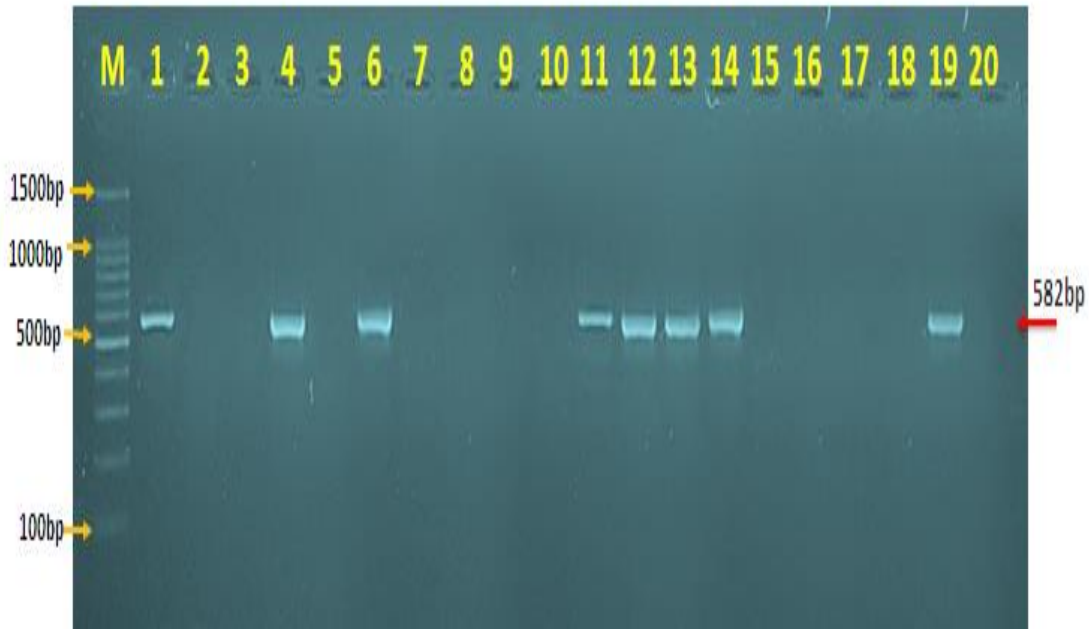

Figure (4.6): Agarose gel electrophoresis of the PCR product of the *stx*1 gene in *Shigella* spp. isolate where marker ladder (1500-100bp) and Lane (1-20) showed some positive stx1 gene at 582bp PCR product size. Lane 21, negative control.

presence of *Shigella* spp. strain in the sample that was amplified in PCR and was run in gel electrophoresis and bands were observed at 582 base pairs of PCR product size.

Non-significance differences among species and virulence factors were found; *S. flexneri Stx*1 (33.33 %,1/3), *Stx*2 (0%, 0/3), *ShET*-1 (0%, 0/3) and *ShET*-2 (0%, 0/3), while *S. sonnei Stx*1 (25 %,1/4), *Stx*2 (0.00 %, 0/4), *ShET*-1 (50.00 %, 2/4) and *ShET*-2 (0%, 0/4), *S. dysenteriae Stx*1 (46.15 %,6/13), *Stx*2 (38.46 %, 5/13), *ShET*-1 (23.07 %, 3/13) and *ShET*-2 (23.07 %, 3/13) as shown in Table (3.10).

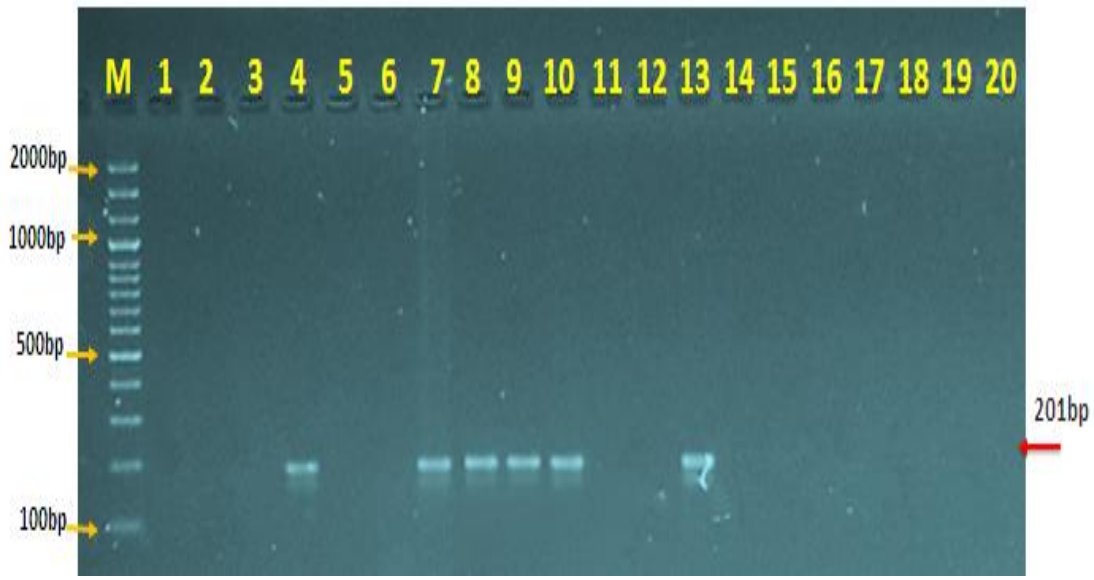

Figure (4.7): Agarose gel electrophoresis of PCR product *stx*2 gene in *Shigella* spp. isolates where marker ladder (1500-100bp) and Lane (1-20) showed some positive *stx*2 gene at 201bp PCR product size.

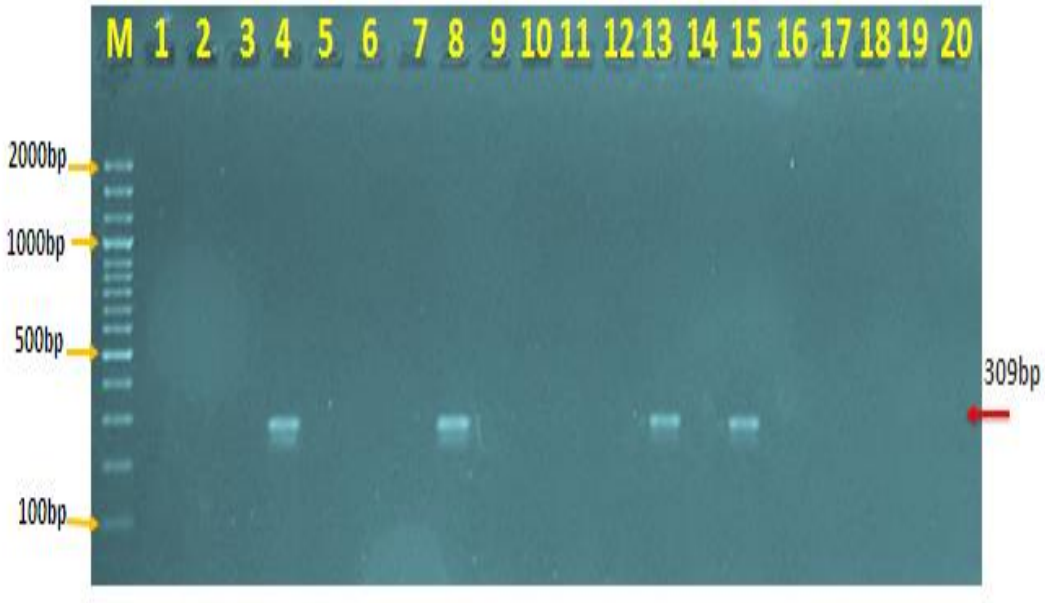

Figure (4.8): Agarose gel electrophoresis of PCR product of *ShET*-1 gene in *Shigella* spp. isolates where marker ladder (1500-100bp) and Lane (1-20) showed some positive *ShET*-1 gene at 309bp PCR product size.

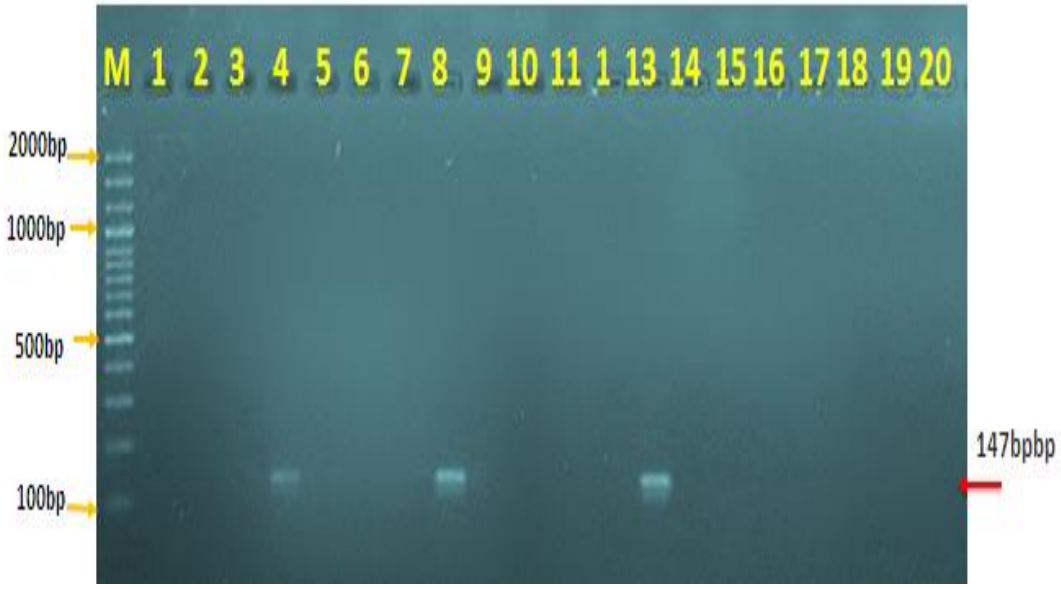

Figure (4.9): Agarose gel electrophoresis of PCR product of *ShET*-2 gene in *Shigella* spp. isolates where Marker ladder (1500-100bp) and Lane (1-20) showed some positive *ShET*-2 gene at 147bp PCR product size.

Table (4-10): Distribution of virulence genes (*Stx*1, *Stx*2, *ShET*-1 and *ShET*-2)

Shigella species	No.	Virulence factors (Frequency and %)				X^2	P-value
		*Stx*1	*Stx*2	*ShET*-1	*ShET*-2		
S. flexneri	3	1(33.33)	0(0)	0(0)	0(0)	3.27*	0.351
S. Sonnei	4	1(25)	0(0)	2(50)	0(0)	4.51*	0.211
S. dysenteriae	13	6(46.15)	5(38.46)	3(23.07)	3(23.07)	2.36*	0.501
X^2		0.636*	3.59*	2.35*	1.9*		
P-value		0.728	0.166	0.307	0.387		

*Stx*s (also known as vero toxins and previously as shiga-like toxins) are 70kDa proteins produced by bacteria that inhibit protein synthesis in sensitive cells. *Stx*s removes an adenine residue from the 28S rRNA of the 60S ribosome in order to limit protein synthesis. N-glycosidase activity is present in a component of the toxin. The pentamer's B subunits form a tetramer (Gb3) to interact with the globotriaosylceramide receptor (Zhu *et al*., 2018).

Shiga toxin (*Stx*) is a highly potent bacterial toxin. *Stx* is present in *S. dysenteriae* type 1 and various *E. coli* serogroups (*Stx*1 in *E. coli*). Certain strains of *E. coli* develop a second *Stx* and *Stx*2, which shares the same mechanism of action as *Stx*/*Stx*1 but is antigenically distinct. Due to the finding of subtypes of each toxin, *Stx*1a and *Stx*2a have become the prototype toxins for each group (Varghese and Aggarwal, 2011; Hussen *et al*., 2019). The *Stx*s are composed of a subunit and a noncovalently linked pentamer of five identical B subunits. It prevents protein synthesis in target cells by infecting the ribosome with the toxin's component. The B. pentamer is used to attach to the Gb3 globotriaosylceramide cellular receptor (Alemu *et al*., 2019). The toxin's A subunit reaches the cytoplasm only after retrogradely passing from the endosome to the Golgi apparatus and then to the endoplasmic reticulum. *Stx2a*-producing strains have been reported to be more likely to cause hemolytic uremic syndrome (HUS) in humans who have been infected with *STX*-producing *E. coli* (STEC), and this relative toxicity has been replicated in animals such as mice and baboons (Jafari *et al*., 2009). *Stx*1a and *Stx*2a exhibit varying degrees of cytotoxicity depending on the cell type, and they bind receptor analogues or mimics differently, eliciting a variety of chemokine responses. SHET-1 is one of the elements connected to the diarrhea's watery phase. SHET-2 is present in large pathogenic plasmids and is implicated in invasion (Schroeder and Hilbi, 2008). *hET*-1 and *ShET*-2 are vital for the transfer

of electrolytes and water in the colon in addition to their enterotoxic action (Zaidi and Estrada-Garca, 2014).

According to Talebreza *et al*. (2015); Ali *et al*. (2020); Ahmed *et al*. (2021); Pakbin *et al*. (2021); and Rahman and Sarker (2021), *ShET*-1's active toxin may consist of a single A subunit and several B subunits (A1-Bn). The *sen* gene produces the second enterotoxin, *Shigella* enterotoxin; invasion-associated pathogens possess a 140 MDa plasmid carrying this gene. According to Tickell *et al.* (2017), *ShET*-2 is visible in the gel following PCR amplification. Other research has shown that *S. flexneri* has a high prevalence of enterotoxin genes. *S*tx-1 and stx-2 were encoded after being found carried on the chromosomes.

Thirty *Shigella* isolates were found in an etiologic research that included 1,339 kids between the ages of 0 and 10. The most widespread type, poses about 60.0%, was *S. flexneri*. *S. sonnei* made up 22.2% of the sample, and *S. dysenteriae* and *S. boydii* made up 6.6%. *ShET-1B's* pathogenic potential was observed in relation to dehydration and *ShET*-2 in relation to intestinal injury as evidenced by the presence of bloody diarrhea. There is an association between symptoms of shigellosis and virulence genes of clinical isolates of *Shigella* spp. (da Cruz *et al*., 2014).

4.8. Distribution of extended spectrum *B*-lactamase genes according to the PCR results

The results of the current study, upon examination by PCR method of extended spectrum B-lactamase (*ESBL*s) genes, showed deferent PCR product of *Stx*1, *Stx*2, *ShET*-1 and *ShET*-2 genes as shown in Figures (4-11, 4-12, 4-13 and 4-14). The *bla-CTXM* gene that is highly detectable in *Shigella* spp. isolates, proving the presence of *Shigella* spp. strain in the sample that was amplified in PCR and was

run in gel electrophoresis and bands were observed at 652 base pairs of PCR product size as shown in figure **(**4-10**).**

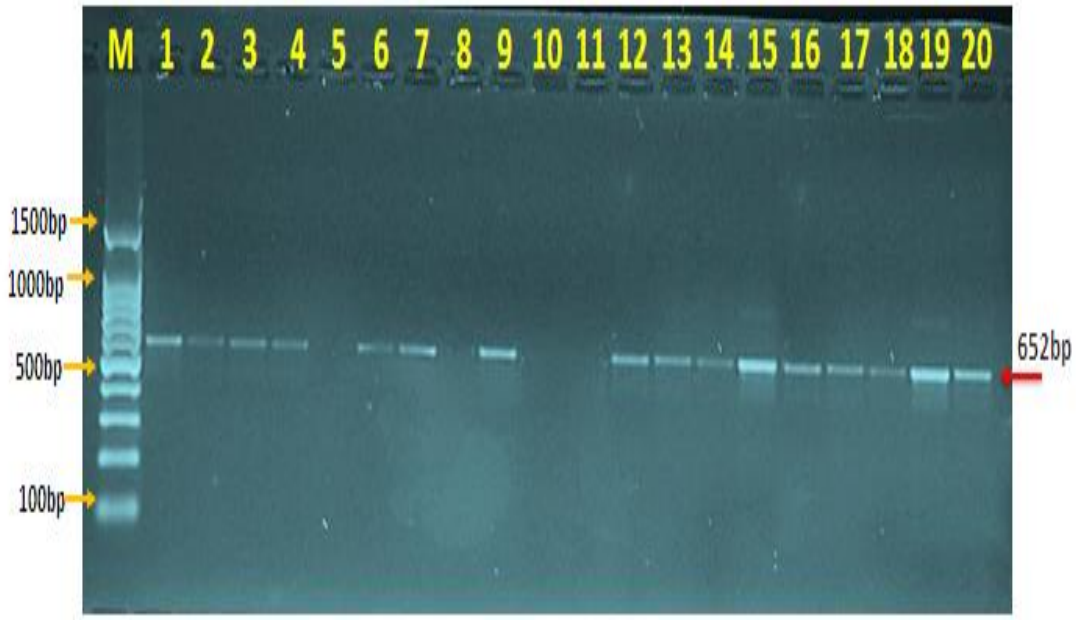

Figure (4.10): Agarose gel electrophoresis of PCR product of *bla-CTXM* gene in *Shigella* spp. isolates where marker ladder (1500-100bp) and Lane (1-20) showed some positive *bla-CTXM* gene at 652bp PCR product size.

The *bla-TEM* gene that is highly detectable in *Shigella* spp. isolates, which proves the presence of *Shigella* spp. strain in the sample that was amplified in PCR and was run in gel electrophoresis and bands were observed at 508 base pair of PCR product size as shown in figure (4-11).

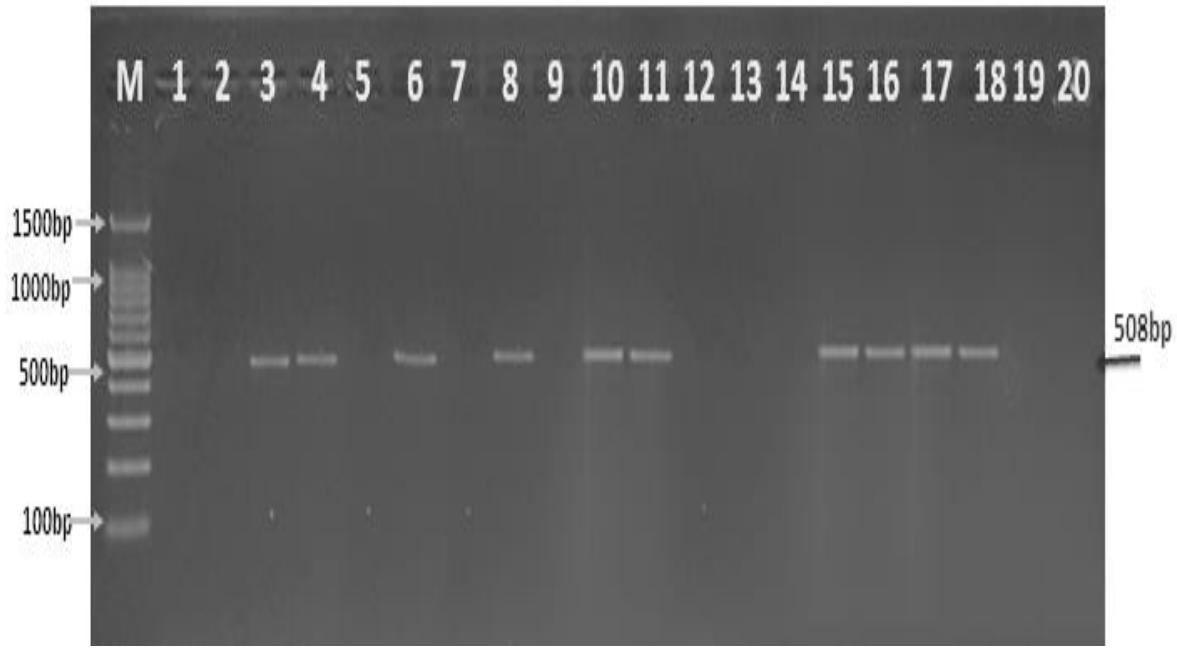

Figure (4.11): Agarose gel electrophoresis of PCR product. *bla-TEM* gene in *Shigella* spp. isolates where marker ladder (1500-100bp), and Lane (1-20) showed some positive *bla-TEM* gene at 508bp PCR product size.

The *bla-SHV* gene that is highly detectable in *Shigella* spp. isolates, proving the presence of *Shigella* spp. strain in the sample that was amplified in PCR and was run in gel electrophoresis and bands were observed at 346 base pair of PCR product size as shown in figure (4-12).

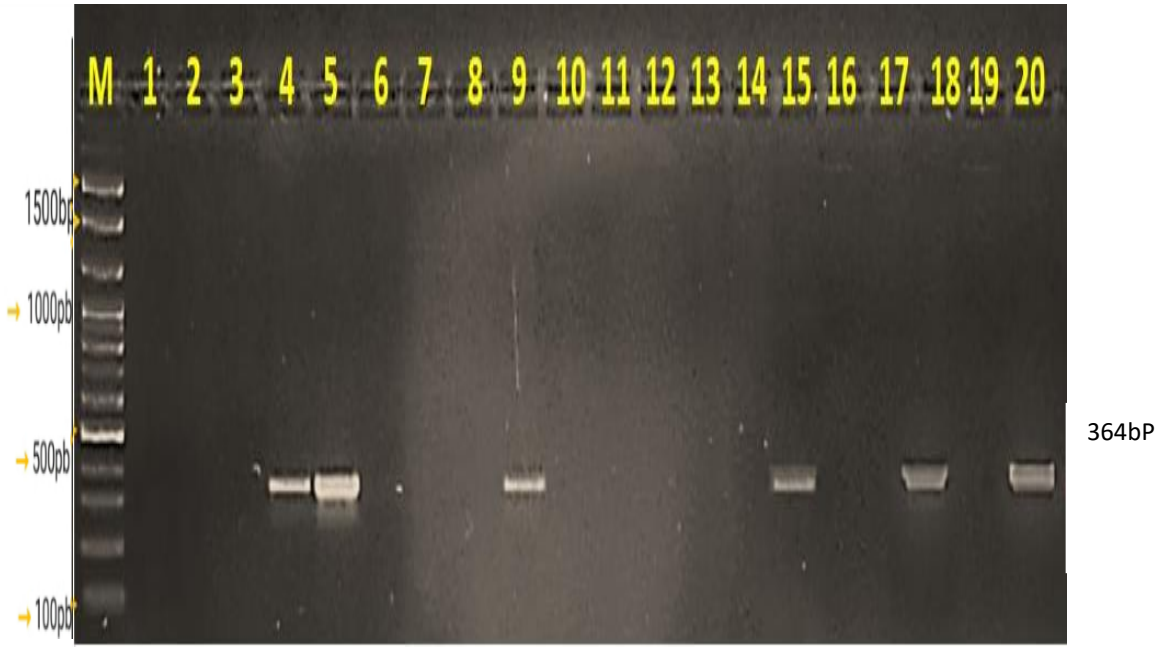

Figure (4.12): Agarose gel electrophoresis of PCR product analysis of *bla-SHV* gene in *Shigella* spp. isolates where marker ladder (1500-100bp), and Lane (1-20) showed some positive *bla-SHV* gene at 364bp PCR product size.

Table (4-11): Distribution of extended spectrum *B-lactamase* genes.

Shigella species	No.	Resistance genes (frequency and %)				X^2	P-value
		blaCTX-M	*blaTEM*	*blaSHV*	*blaAMPC*		
S. flexneri	3	3(100)	2(66.66)	1(33.33)	2(66.66)	3*	0.392
S. Sonnei	4	4(100)	2(50)	0(0)	2(50)	8**	0.046
S. dysenteriae	13	9(69.23)	7(53.84)	4(30.76)	5(38.46)	4.54*	0.208
X^2		2.69*	0.212*	1.67*	0.834*		
P-value		0.260	0.899	0.433	0.659		

Figure (4-13): Agarose gel electrophoresis of PCR product of *bla-AMPC* gene in *Shigella* spp. isolates where marker ladder (1500-100bp), and Lane (1-20) showed some positive *bla-AMPC* gene at 422bp PCR product size.

In the current study, antibiotics resistance and molecular detection of *blaCTX-M*, *blaTEM*, *blaSHV* and *blaAMPC* of antibiotics in *Shigella* sp. isolates revealed that there was a harmony between results of antibiotic resistance and positive molecular detection of these genes.

The highest resistance rate to most antibiotics was observed in the isolates of *blaCTX-M* gene, the isolates that have *blaCTX-M* gene was resistance to all antibiotics (100%), the isolates that have *blaTEM*, *blaSHV* and *blaAMPC* genes were resistance to ciprofloxacin (66.66 %), the isolates that have *blaSHV* genes was resistance to chloramphenicol (25%), ceftriaxone (27.27%) as shown in Table (4-12).

Table 4-12: *blaCTX-M*, *blaTEM*, *blaSHV* and *blaAMPC* of antibiotics of *Shigella* sp. Isolates.

Antibiotic	**No. of Resist. isolate**	***blaCTX-M***		***blaTEM***		***blaSHV***		***blaAMPC***	
		No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Ampicillin	20	20	100	10	50	7	35	9	45
Ceftriaxone	11	11	100	5	45.45	3	27.27	3	27.27
Erythromycin	18	18	100	11	61.11	6	33.33	7	38.88
Chloramphenicol	12	12	100	6	50	3	25	4	33.33
Ceftazidime	13	13	100	6	46.15	4	30.76	5	38.46
Ciprofloxacin	3	3	100	2	66.66	2	66.66	2	66.66
Gentamicin	8	8	100	5	62.5	3	37.5	4	50
Cefixime	12	12	100	6	50	4	33.33	5	41.66
Nalidixic acid	9	9	100	5	55.55	4	44.44	5	55.55
***X*2**		0*		1.66*		2.63*		3.2*	
P value		1		0.990		0.955		0.921	

* No significant difference at P≤0.05

Shigella spp. isolates were *CTX-M* positive, and all of them were also *ISEcpI* positive. Protein translation analysis compared to reference sequences established that all *CTX-M* isolates are members of the *CTX-M* gene. According to the findings, *Shigella* spp. that produce *ESBL*s are particularly resistant. As a result, it is critical to develop strategies for controlling the spread of ESBL-producing isolates (Sati *et al*., 2019). Hospitals in Iran face significant infection control management challenges due to *CTX-M* Generating *Salmonella* and *Shigella* species, which may contribute to antimicrobial drug resistance (Rahman and Sarker, 2021).

Isolates had a high prevalence of *ESBL*-encoding *blaCTXM* genes and narrow spectrum *blaOXA* 1 and *blaTEM* 1B genes were also prevalent (Abrar *et al.*, 2019).

In a close study, 66 *Sonnei* isolates were collected and *blaCTX-M*-1, *blaTEM* genes were observed in different proportions (48, 38 % respevtively) (Qian *et al*., 2018). These enzymes typically result in improper therapy, which raises the risk of infection and mortality while increasing costs and length of hospital stays. Bacterial isolates with these genes are typically resistant to different antimicrobial medicines and can be treated, leaving limited therapeutic options (Tseng *et al*., 2014). The variable region between the 540 and 1550 bp areas is used for comparative taxonomy, along with universal primers that complement the conserved sequences at the start and end of the gene (around the 1550-bp region). Even though these are the most frequently sequenced and compared lengths, sequences in databases can range in length from 500 to 1500 bp. According to Terfassa and Jida, (2018), numerous bacteria strains have had their 16S rRNA gene sequenced. GenBank contains over 90,000 16S rRNA sequences, making it the world's largest database of nucleotide sequences.

Fifty-nine (78.7%) of ESBL-producing isolates were found to be multidrug-resistant (MDR), i.e. showed intermediate or fully resistance to at least three different classes of antimicrobial agents including β-lactams, aminoglycosides, and fluoroquinolones (Peymani *et al*., 2014). In this study, all eight isolates that were resistant to ceftriaxone were also susceptible to imipenem, cefaperazonesulbactam, and piperacillintazobactam. In May 2007, the first *S. sonnei* strain to produce ESBLs and be ceftriaxone-resistant was found in the feces of a toddler with acute diarrhea (Thompson *et al*., 2016).

The emergence of strains of Shigella that produce ESBL is due to antibiotics being prescribed as experimental drugs. *Shigella* have high rates of antibiotic

resistance. Continuous monitoring of antibiotic-resistant isolates is essential to understand the mechanism of resistance (Madhavan *et al*., 2018). As a result, previously deposited strains' sequences can be compared to existing ones, as a result of the increased resistance to cefotaxime by the bacteria *sonnei* and the high quality of ESBL genes, it became necessary to monitor these genes and their prevalence. (Qian *et al*., 2018). Antibiotic treatment for *Shigella* infections in humans allows for clinical recovery, avoidance of complications, and bacterial eradication. Ciprofloxacin is particularly active in the handling of pathogen and MDR. The microbe may be associated with excessive and inappropriate use of this medication to treat diarrhoea and urinary tract infections (Christopher *et al.*, 2010). According to Brander *et al*. (2017), a high percentage of *Shigella* strains are usually resistant to ciprofloxacin. Quinolone resistance was reported to be 90% in another Kolkata experiment (Sethuvel *et al*., 2019).

When serotype resistance rates were compared, *S. sonnei* strains were more unaffected than *S. flexneri*. Our region is dominated by the MDR *S. sonnei* serotype, only identified in the United States, and other developing countries. This study utilized a total of six antibiotics, and 91.6% of the isolates were multi-resistant. In eight MDR strains, *S. sonnei* , *flexneri* were resistant to third-generation cephalosporium, CRO, and claforan (Ameya *et al*., 2018). In 2011, there was no indication of ceftriaxone resistance, but it was discovered in 2015 and 2016 (Tickell *et al*., 2017). Antibiotic resistance in members of the enterobacteriaceae family may be variable due to plasmid-gene. In their analysis, Sethuvel *et al*. (2019) discovered that 44% of samples were unaffected by furazolidone and 34% were unaffected by gentamicin.

When serotype resistance rates were paralleled, *S. sonnei* types were more sturdy than *S. flexneri*. As a result, the predominant serotype of *S. sonnei* in our region is

limited. Multidrug resistance was identified in this experiment against six medications, with 92 % of samples being MDR. MIC values were also significantly increased (MIC 64). Five isolates (three *S. sonnei* and two *S. flexneri*) were phenotypically verified to produce ESBLs. Other strains could not be resurrected. Experiments showed that resistance to ceftriaxone was associated with sensitivity to other antibiotics such as imipenem (Terfassa and Jida, 2018).

4.9. DNA Sequence and phylogenetic tree results

A DNA sequencing method was implemented to determine genetic relationships and analyze genetic variation (substitution mutations) in the 16S ribosomal RNA gene of the local *Shigella* (IQ 1 to No. 20). The phylogenetic tree genetic relationship analysis showed that the local *Shigella* spp. (IQ-No.1 to IQ-No.20) isolates were showed closed genetics related to NCBI BLAST *Shigella* spp. strain SK8 India isolates (MW600518.1) at total genetic change (0.0040-0.0010%) as shown in Figure 4-14 and Table (4-13) and detailed in Appendix 1.

The homology sequence identity between local *Shigella* spp. (IQ-No.1 to IQ-No.20) isolates and NCBI BLAST *Shigella* spp. strain SK8 India isolate showed genetic homology sequence identity ranging from (99.31-99.78%). The genetic variation (substitution Mutations) analysis in 16S ribosomal RNA gene between local *Shigella* spp. (IQ-No.1 to IQ-No.20) isolates and NCBI-Blast related NCBI BLAST *Shigella* spp. strain SK8 India isolates found (1-3) substitution mutations at total genetic variation percentage ranged (0.22-0.69%) as shown in the table and the Figure 14-4. Finally, the local Shigella spp. (IQ-No.1-IQ-No.20) isolates were submitted into NCBI Genbank and identified by accession numbers (OK036604.1 into OK036623.1). A neighbor-joining phylogenetic tree was constructed using the sequences of chromosomal genes in the study. The isolates are classified according

to their serotype and species. The network's major nodes all have bootstrap values greater than 50%, which aids in identifying the three primary *Shigella* genus clusters and subclusters. A comparison of *Shigella*'s gene map was examined. The pale green rings depict BLASTN comparisons of the strains as the outer rings represent annotations for known genes encoding proteins with known functions.

The PCR analysis performed to identify the most prevalent genetic characteristics in table 4.13 revealed strong similarities between those traits. *Shigella* is a member of the enterobacteriaceae family of bacteria that causes diarrhoea, fever, and stomach pain in those affected. *S. dysenteria, S. sonnei*, and the more recently found *S. boydii* are clinically relevant *Shigella* species. Shigellosis is more frequently associated with youngsters.

Epidemiological research has successfully traced pathogenic bacteria's origins and genetic links through molecular typing (Sheikh *et al.*, 2019).

Hospital acquired infections caused by bacteria that are multidrug resistant are becoming a significant issue for patients. To ascertain the epidemiology of nosocomial infections and assist in the development of logical pathogen control techniques, it is critical to comprehend pathogen relatedness. Pathogen typing is used to ascertain whether genetic relationships exist between isolates that are epidemiologically related. The methods of choice for determining the molecular relatedness of isolates for epidemiologic study are novel DNA-based technologies or molecular analysis. Pulsed-field gel electrophoresis (PFGE), PCR-based typing techniques, and multilocus sequence analysis are examples of DNA-based molecular technologies (Singh *et al*., 2006).

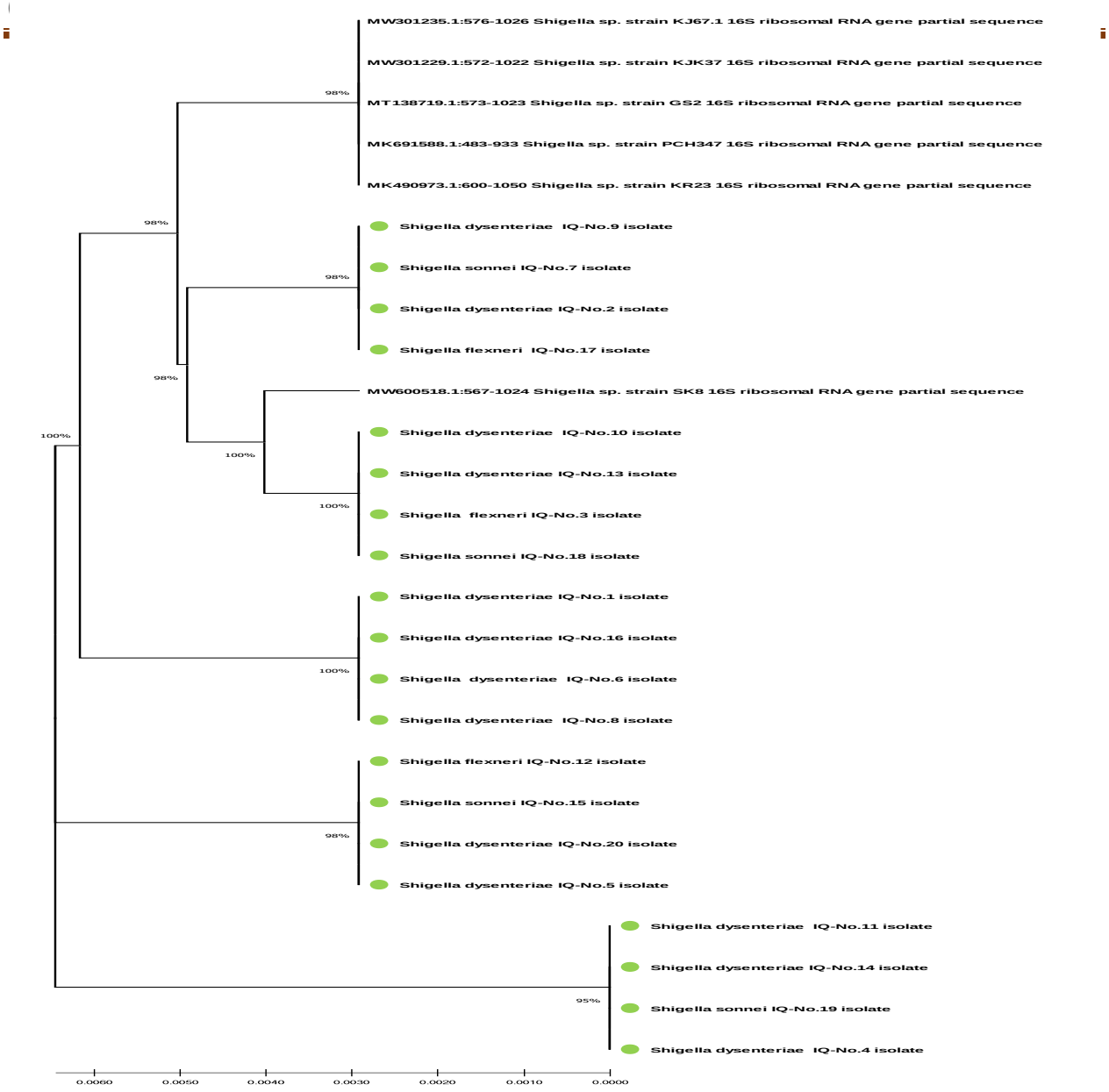

Figure (4-14): Phylogenetic tree analysis based on 16S ribosomal RNA gene partial sequence in local *Shigella* spp. (IQ-No.1 to IQ-No.20) isolates used for genetic relationship analysis. The phylogenetic tree was constructed using the evolutionary history inferred and the Neighbor-Joining method (MEGA X version). The local *Shigella* spp. (IQ-No.1 to IQ-No.20) were shown genetically related to NCBI-BLAST *Shigella* sp. strain SK8 India isolates (MW600518.1) at total genetic changes (0.0040-0.0010%).

Table (4-13): The NCBI-BLAST homology sequence identity (%) between local *Shigella* spp. (IQ-No.1 to IQ-No.20) isolates and NCBI-BLAST submitted related *Shigella* spp. isolate.

Shigella sp. isolate No.	Genbank Accession number	NCBI-BLAST Homology Sequence identity (%)			
		No. Mutation	Type of Mutation	Polymorphism Percent (%)	Identity (%)
IQ-No.1	OK036604	2	A/G, G/T	0.44%	99.56%
IQ-No.2	OK036605	1	A/T	0.22%	99.78%
IQ-No.3	OK036606	1	C/T	0.22%	99.78%
IQ-No.5	OK036608	2	T/A, C/G	0.44%	99.56%
IQ-No.6	OK036609	2	A/G, C/T	0.44%	99.56%
IQ-No.7	OK036610	1	A/T	0.22%	99.78%
IQ-No.8	OK036611	2	A/G, G/T	0.44%	99.56%
IQ-No.9	OK036612	1	A/T	0.22%	99.78%
IQ-No.10	OK036613	1	C/T	0.22%	99.78%
IQ-No.11	OK036614	3	A/T, C/G, T/C	0.69%	99.31%
IQ-No.12	OK036615	2	T/A, C/G	0.44%	99.56%
IQ-No.13	OK036616	1	C/T	0.22%	99.78%
IQ-No.14	OK036617	3	A/T, C/G, T/C	0.69%	99.31%
IQ-No.15	OK036618	2	T/A, C/G	0.44%	99.56%
IQ-No.16	OK036619	2	A/G, G/T	0.44%	99.56%
IQ-No.17	OK036620	1	A/T	0.22%	99.78%
IQ-No.18	OK036621	1	C/T	0.22%	99.78%
IQ-No.19	OK036622	2	A/T, C/G	0.69%	99.31%
IQ-No.20	OK036623	2	T/A, C/G	0.44%	99.56%

Numerous housekeeping gene segment are used in this strategy to identify microbial species based on their distinct allelic patterns (Wang *et al*., 2019). The alleles at each locus define the sequence type (ST). This study revealed two different sequence classes in *Shigella* species using BLAST homology analysis. The disease is most frequently observed in children under the age of five who reside in areas of impoverished countries where shigellosis is endemic. Many specialists feel that individuals with symptoms should be take drug to reduce the pathogenesis and the amount of fecal discharge produced by the organism, reducing the risk of transmission (Alemu *et al*., 2019; Ali *et al*., 2020; Jain *et al*., 2020; Rahman and Sarker, 2021). According to Jafari *et al*. (2009), several large outbreaks and severe sickness have been linked to *S. dysenteriae*. The serogroup *S. flexneri* appears to be the most prevalent infection in Indian investigations (Jain *et al*., 2020). In the study of Madhavan *et al*. (2018), *Shigella sonnei* was the most frequent *Shigella* serotype (62.5 per cent). There have been no outbreaks of *S. sonnei* of this magnitude observed in Bharata. *S. sonnei* is further prevalent in developed states with a more gradual development progress. In adarand, viet-nam., the dominant species has transitioned from *S. flexneri* to *S. sonnei*, most likely due to these nations' improved socioeconomic situations. Madhavan *et al*. (2018) has revealed that a similar scenario is also playing out in India's southern states. Infections with *Shigella* are typically caused by a lack of sanitation and access to safe drinking water. demonstrates that the rate of *Shigella* isolation decreased from 2011 to 2013, possibly due to enhanced public health efforts, but then increased in subsequent years. Sanitation initiatives may be to blame for this (Maharjan *et al*., 2017). Antimicrobial treatment of *Shigella* infections accelerates clinical recovery, decreases the incidence of sequelae, and prevents the spread of *Shigella* germs back into the general population (Ranjbar and Farahani, 2019).

Conclusions

1. The females and children were more susceptible to infection with shigellosis than men because of the nature of daily activities and their careless with regard to health.
2. *Shigella* spp isolates showed high levels of resistance, almost to all antibiotics understudy and *S. dysenteriae* was the main cause of shigellosis.
3. *blaCTX-M* was responsible for the resistance of the antibiotics used in this study.
4. Transmission of antibiotic-resistant bacterial isolates from countries increase with frequent travel and randomly use of antibiotics without medical advice.

Recommendations

1. Increase attention to health and hygiene, and refer to a specialist when symptoms such as bloody diarrhea appears.
2. It is need to carry out comprehensive study for detection of the type and the site of mutation in β-lactamase genes of *Shigella* spp. isolates.
3. It is important to use the antibiotic sensitivity test for checking the ability of pathogenic-bacteria toward the antibiotics in the hospitals.

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Appendix (1): Multiple sequence alignment analysis of 16S ribosomal RNA gene partial sequence in local *Shigella* spp. (IQ-No.1-IQ-No.20) and NCBI-Genbank related *Shigella* sp. related isolates. The multiple alignment analysis was constructed using NCBI BLAST alignment tool and showed the nucleotide alignment similarity as (*) and substitution mutations in 16S ribosomal RNA gene.

IQ-No.1 -
ATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.6 -
ATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.8 -
ATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.16 -
ATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.3 --
TCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.18 --
TCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.10 --
TCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.13 --
TCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.2
AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.7
AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.9
AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.17
AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.4
AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.19
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IQ-No.11
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IQ-No.14
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IQ-No.5
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IQ-No.12
AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
IQ-No.15
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IQ-No.20
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MK490973.1
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MK691588.1
AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
MW301235.1
AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
MW301229.1
AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG
****

IQ-No.1
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
IQ-No.6
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
IQ-No.8
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
IQ-No.16
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
IQ-No.3
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
IQ-No.18
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IQ-No.7
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
IQ-No.9
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IQ-No.17
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IQ-No.4
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IQ-No.19
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IQ-No.11
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IQ-No.14
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IQ-No.5
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IQ-No.12
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IQ-No.15
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
IQ-No.20
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MW600518.1
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
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MK490973.1
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
MK691588.1
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
MW301235.1
GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
MW301229.1
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****

IQ-No.1
AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA
IQ-No.6
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IQ-No.8
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IQ-No.16
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IQ-No.3
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IQ-No.17
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IQ-No.4
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IQ-No.19
AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCATACAGGA
IQ-No.11
AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCATACAGGA
IQ-No.14
AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCATACAGGA

IQ-No.5
AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA
IQ-No.12
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** ********** ******

IQ-No.1
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGGCTTGGAGGTTGTGCCCTTGAGG
IQ-No.6
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGGCTTGGAGGTTGTGCCCTTGAGG
IQ-No.8
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGGCTTGGAGGTTGTGCCCTTGAGG
IQ-No.16
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGGCTTGGAGGTTGTGCCCTTGAGG
IQ-No.3
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG
IQ-No.18
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG
IQ-No.10
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG
IQ-No.13
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG
IQ-No.2
TTAGATACCCTGGTAGTCCACGCCGTAATCGATGTCGACTTGGAGGTTGTGCCCTTGAGG
IQ-No.7
TTAGATACCCTGGTAGTCCACGCCGTAATCGATGTCGACTTGGAGGTTGTGCCCTTGAGG
IQ-No.9
TTAGATACCCTGGTAGTCCACGCCGTAATCGATGTCGACTTGGAGGTTGTGCCCTTGAGG
IQ-No.17
TTAGATACCCTGGTAGTCCACGCCGTAATCGATGTCGACTTGGAGGTTGTGCCCTTGAGG
IQ-No.4
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG
IQ-No.19
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG

IQ-No.11
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG
IQ-No.14
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG
IQ-No.5
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGATGTGCCCTTGAGG
IQ-No.12
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGATGTGCCCTTGAGG
IQ-No.15
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGATGTGCCCTTGAGG
IQ-No.20
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGATGTGCCCTTGAGG
MW600518.1
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MT138719.1
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MK490973.1
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MK691588.1
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MW301235.1
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG
MW301229.1
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG
********** ******** ******** **********

IQ-No.1
CGTGGCTTCCTGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.6
CGTGGCTTCCTGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.8
CGTGGCTTCCTGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.16
CGTGGCTTCCTGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.3
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.18
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.10
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.13
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.2
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.7
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.9
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.17
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA

IQ-No.4
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.19
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.11
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.14
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.5
CGTGGCTTCGGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.12
CGTGGCTTCGGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.15
CGTGGCTTCGGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
IQ-No.20
CGTGGCTTCGGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
MW600518.1
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA
MT138719.1
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGG-TTA
MK490973.1
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGG-TTA
MK691588.1
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGG-TTA
MW301235.1
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGG-TTA
MW301229.1
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGG-TTA
********* ** ***

IQ-No.1
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.6
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.8
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.16
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.3
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.18
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.10
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.13
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.2
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.7
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG

IQ-No.9
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.17
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.4
AAAGTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.19
AAAGTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.11
AAAGTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.14
AAAGTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.5
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.12
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.15
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
IQ-No.20
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
MW600518.1
AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG
MT138719.1 AAACTCAAATGAA-TTGACGGGGGCCCGCACAAGCGGTGG-
AGCATGTGGTTT-AATTCG
MK490973.1 AAACTCAAATGAA-TTGACGGGGGCCCGCACAAGCGGTGG-
AGCATGTGGTTT-AATTCG
MK691588.1 AAACTCAAATGAA-TTGACGGGGGCCCGCACAAGCGGTGG-
AGCATGTGGTTT-AATTCG
MW301235.1 AAACTCAAATGAA-TTGACGGGGGCCCGCACAAGCGGTGG-
AGCATGTGGTTT-AATTCG
MW301229.1 AAACTCAAATGAA-TTGACGGGGGCCCGCACAAGCGGTGG-
AGCATGTGGTTT-AATTCG
*** ********* ********** ********** ******

IQ-No.1

Score	Expect	Identities	Gaps	Strand
833 bits(451)	0.0	455/457(99%)	0/457(0%)	Plus/Plus

Query 2 ATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGG 61

Sbjct 1 60

Query 62 GGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGA 121

Sbjct 61 120

Query 122 AAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGAT 181

Sbjct 121 180

Query 182 TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGC 241

Sbjct 181 .....G..... 240

Query 242 GTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTAA 301

Sbjct 241 .....T..... 300

Query 302 AACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCGA 361

Sbjct 301 360

Query 362 TGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGAA 421

Sbjct 361 420

Query 422 TGTGCCTTTCGGGAACCGTGAGACAGGTGCTGCATGG 458

Sbjct 421 457

IQ-No.2

Score	Expect	Identities	Gaps	Strand
828 bits(448)	0.0	450/451(99%)	0/451(0%)	Plus/Plus

Query 1 AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG 60

Sbjct 1 60

Query 61 GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 120

Sbjct 61 120

Query 121 AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 180

Sbjct 121 180

Query 181 TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG 240

Sbjct 181 .....T..... 240

Query 241 CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA 300

Sbjct 241 300

Query 301 AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG 360

Sbjct 301 360

Query 361 ATGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGA 420

Sbjct 361 420

Query 421 ATGTGCCTTTCGGGAACCGTGAGACAGGTGC 451

Sbjct 421 451

IQ-No.3

Score	Expect	Identities	Gaps	Strand
837 bits(453)	0.0	455/456(99%)	0/456(0%)	Plus/Plus

Query 3 TCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGG 62

Sbjct 1 60

Query 63 GGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAA 122

Sbjct 61 120

Query 123 AGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATT 182

Sbjct 121 180

Query 183 AGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCG 242

Sbjct 181 240

Query 243 TGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTAAA 302

Sbjct 241 300

Query 303 ACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCGAT 362

Sbjct 301 360

Query 363 GCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGAAT 422

Sbjct 361 .T..... 420

Query 423 GTGCCTTTCGGGAACCGTGAGACAGGTGCTGCATGG 458

Sbjct 421 456

IQ-No.4

Score	Expect	Identities	Gaps	Strand
787 bits(426)	0.0	432/435(99%)	0/435(0%)	Plus/Plus

Query 1 AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG 60

Sbjct 1 60

Query 61 GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 120

Sbjct 61 120

Query 121 AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 180

Sbjct 121 .....T..... 180

Query 181 TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG 240

Sbjct 181 240

Query 241 CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA 300

Sbjct 241 300

Query 301 AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG 360

Sbjct 301 ...G..... 360

Query 361 ATGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGA 420

Sbjct 361 .....C..... 420

Query 421 ATGTGCCTTTCGGGA 435

Sbjct 421 435

IQ-No.5

Score	Expect	Identities	Gaps	Strand
822 bits(445)	0.0	449/451(99%)	0/451(0%)	Plus/Plus

Query 1 AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG 60
Sbjct 1 60

Query 61 GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 120
Sbjct 61 120

Query 121 AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 180
Sbjct 121 180

Query 181 TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG 240
Sbjct 181 .....A..... 240

Query 241 CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA 300
Sbjct 241 .....G..... 300

Query 301 AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG 360
Sbjct 301 360

Query 361 ATGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGA 420
Sbjct 361 420

Query 421 ATGTGCCTTTCGGGAACCGTGAGACAGGTGC 451
Sbjct 421 451

IQ-No.6

Score	Expect	Identities	Gaps	Strand
833 bits(451)	0.0	455/457(99%)	0/457(0%)	Plus/Plus

Query 2 ATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGG 61
Sbjct 1 60

Query 62 GGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGA 121

Sbjct 61 120

Query 122 AAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGAT 181

Sbjct 121 180

Query 182 TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGC 241

Sbjct 181 .....G..... 240

Query 242 GTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTAA 301

Sbjct 241 .....T..... 300

Query 302 AACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCGA 361

Sbjct 301 360

Query 362 TGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGAA 421

Sbjct 361 420

Query 422 TGTGCCTTTCGGGAACCGTGAGACAGGTGCTGCATGG 458

Sbjct 421 457

IQ-No.7

Score	Expect	Identities	Gaps	Strand
828 bits(448)	0.0	450/451(99%)	0/451(0%)	Plus/Plus

Query 1 AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG 60

Sbjct 1 60

Query 61 GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 120

Sbjct 61 120

Query 121 AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 180

Sbjct 121 180

Query 181 TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG 240

Sbjct 181 .....T..... 240

Query 241 CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA 300
Sbjct 241 300

Query 301 AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG 360
Sbjct 301 360

Query 361 ATGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGA 420
Sbjct 361 420

Query 421 ATGTGCCTTTCGGGAACCGTGAGACAGGTGC 451
Sbjct 421 451

IQ-No.8

Score	Expect	Identities	Gaps	Strand
833 bits(451)	0.0	455/457(99%)	0/457(0%)	Plus/Plus

Query 2 ATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGG 61
Sbjct 1 60

Query 62 GGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGA 121
Sbjct 61 120

Query 122 AAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGAT 181
Sbjct 121 180

Query 182 TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGC 241
Sbjct 181 .....G..... 240

Query 242 GTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTAA 301
Sbjct 241 .....T..... 300

Query 302 AACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCGA 361
Sbjct 301 360

Query 362 TGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGAA 421

Sbjct 361 420

Query 422 TGTGCCTTTCGGGAACCGTGAGACAGGTGCTGCATGG 458

Sbjct 421 457

IQ-No.9

Score	Expect	Identities	Gaps	Strand
828 bits(448)	0.0	450/451(99%)	0/451(0%)	Plus/Plus

Query 1 AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG 60

Sbjct 1 60

Query 61 GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 120

Sbjct 61 120

Query 121 AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 180

Sbjct 121 180

Query 181 TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG 240

Sbjct 181 .....T..... 240

Query 241 CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA 300

Sbjct 241 300

Query 301 AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG 360

Sbjct 301 360

Query 361 ATGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGA 420

Sbjct 361 420

Query 421 ATGTGCCTTTCGGGAACCGTGAGACAGGTGC 451

Sbjct 421 451

IQ-No.10

Score	Expect	Identities	Gaps	Strand
837 bits(453)	0.0	455/456(99%)	0/456(0%)	Plus/Plus

Query 3 TCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGG 62
Sbjct 1 60

Query 63 GGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAA 122
Sbjct 61 120

Query 123 AGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATT 182
Sbjct 121 180

Query 183 AGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCG 242
Sbjct 181 240

Query 243 TGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTAAA 302
Sbjct 241 300

Query 303 ACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCGAT 362
Sbjct 301 360

Query 363 GCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGAAT 422
Sbjct 361 .T..... 420

Query 423 GTGCCTTTCGGGAACCGTGAGACAGGTGCTGCATGG 458
Sbjct 421 456

IQ-No.11

Score	Expect	Identities	Gaps	Strand
787 bits(426)	0.0	432/435(99%)	0/435(0%)	Plus/Plus

Query 1 AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG 60
Sbjct 1 60

Query 61 GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 120

Sbjct 61 120

Query 121 AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 180

Sbjct 121 .....T..... 180

Query 181 TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG 240

Sbjct 181 240

Query 241 CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA 300

Sbjct 241 300

Query 301 AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG 360

Sbjct 301 ...G..... 360

Query 361 ATGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGA 420

Sbjct 361 .....C..... 420

Query 421 ATGTGCCTTTCGGGA 435

Sbjct 421 435

IQ-No.12

Score	Expect	Identities	Gaps	Strand
822 bits(445)	0.0	449/451(99%)	0/451(0%)	Plus/Plus

Query 1 AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG 60

Sbjct 1 60

Query 61 GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 120

Sbjct 61 120

Query 121 AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 180

Sbjct 121 180

Query 181 TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG 240

Sbjct 181 .....A..... 240

Query 241 CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA 300

Sbjct 241 .....G..... 300

Query 301 AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG 360
Sbjct 301 360

Query 361 ATGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGA 420
Sbjct 361 420

Query 421 ATGTGCCTTTCGGGAACCGTGAGACAGGTGC 451
Sbjct 421 451

IQ-No.13

Score	Expect	Identities	Gaps	Strand
837 bits(453)	0.0	455/456(99%)	0/456(0%)	Plus/Plus

Query 3 TCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGG 62
Sbjct 1 60

Query 63 GGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAA 122
Sbjct 61 120

Query 123 AGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATT 182
Sbjct 121 180

Query 183 AGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCG 242
Sbjct 181 240

Query 243 TGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTAAA 302
Sbjct 241 300

Query 303 ACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCGAT 362
Sbjct 301 360

Query 363 GCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGAAT 422
Sbjct 361 .T..... 420

Query 423 GTGCCTTTCGGGAACCGTGAGACAGGTGCTGCATGG 458

Sbjct 421 456

IQ-No.14

Score	Expect	Identities	Gaps	Strand
787 bits(426)	0.0	432/435(99%)	0/435(0%)	Plus/Plus

Query 1 AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG 60

Sbjct 1 60

Query 61 GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 120

Sbjct 61 120

Query 121 AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 180

Sbjct 121 .....T..... 180

Query 181 TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG 240

Sbjct 181 240

Query 241 CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA 300

Sbjct 241 300

Query 301 AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG 360

Sbjct 301 ...G..... 360

Query 361 ATGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGA 420

Sbjct 361 .....C..... 420

Query 421 ATGTGCCTTTCGGGA 435

Sbjct 421 435

IQ-No.15

Score	Expect	Identities	Gaps	Strand
822 bits(445)	0.0	449/451(99%)	0/451(0%)	Plus/Plus

Query 1 AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG 60
Sbjct 1 60

Query 61 GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 120
Sbjct 61 120

Query 121 AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 180
Sbjct 121 180

Query 181 TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG 240
Sbjct 181 .....A..... 240

Query 241 CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA 300
Sbjct 241 .....G..... 300

Query 301 AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG 360
Sbjct 301 360

Query 361 ATGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGA 420
Sbjct 361 420

Query 421 ATGTGCCTTTCGGGAACCGTGAGACAGGTGC 451
Sbjct 421 451

IQ-No.16

Score	Expect	Identities	Gaps	Strand
833 bits(451)	0.0	455/457(99%)	0/457(0%)	Plus/Plus

Query 2 ATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGG 61
Sbjct 1 60

Query 62 GGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGA 121
Sbjct 61 120

Query 122 AAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGAT 181

Sbjct 121 180

Query 182 TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGC 241

Sbjct 181 .....G..... 240

Query 242 GTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTAA 301

Sbjct 241 .....T..... 300

Query 302 AACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCGA 361

Sbjct 301 360

Query 362 TGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGAA 421

Sbjct 361 420

Query 422 TGTGCCTTTCGGGAACCGTGAGACAGGTGCTGCATGG 458

Sbjct 421 457

IQ-No.17

Score	Expect	Identities	Gaps	Strand
828 bits(448)	0.0	450/451(99%)	0/451(0%)	Plus/Plus

Query 1 AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG 60

Sbjct 1 60

Query 61 GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 120

Sbjct 61 120

Query 121 AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 180

Sbjct 121 180

Query 181 TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG 240

Sbjct 181 .....T..... 240

Query 241 CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA 300

Sbjct 241 300

Query 301 AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG 360
Sbjct 301 360

Query 361 ATGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGA 420
Sbjct 361 420

Query 421 ATGTGCCTTTCGGGAACCGTGAGACAGGTGC 451
Sbjct 421 451

IQ-No.18

Score	Expect	Identities	Gaps	Strand
837 bits(453)	0.0	455/456(99%)	0/456(0%)	Plus/Plus

Query 3 TCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGG 62
Sbjct 1 60

Query 63 GGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAA 122
Sbjct 61 120

Query 123 AGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATT 182
Sbjct 121 180

Query 183 AGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCG 242
Sbjct 181 240

Query 243 TGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTAAA 302
Sbjct 241 300

Query 303 ACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCGAT 362
Sbjct 301 360

Query 363 GCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGAAT 422
Sbjct 361 .T..... 420

Query 423 GTGCCTTTCGGGAACCGTGAGACAGGTGCTGCATGG 458

Sbjct 421 456

IQ-No.19

Score	Expect	Identities	Gaps	Strand
787 bits(426)	0.0	432/435(99%)	0/435(0%)	Plus/Plus

Query 1 AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG 60

Sbjct 1 60

Query 61 GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 120

Sbjct 61 120

Query 121 AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 180

Sbjct 121 .....T..... 180

Query 181 TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG 240

Sbjct 181 240

Query 241 CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA 300

Sbjct 241 300

Query 301 AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG 360

Sbjct 301 ...G..... 360

Query 361 ATGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGA 420

Sbjct 361 .....C..... 420

Query 421 ATGTGCCTTTCGGGA 435

Sbjct 421 435

IQ-No.20

Score	Expect	Identities	Gaps	Strand
822 bits(445)	0.0	449/451(99%)	0/451(0%)	Plus/Plus

Query 1 AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGG 60
Sbjct 1 60

Query 61 GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG 120
Sbjct 61 120

Query 121 AAAGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA 180
Sbjct 121 180

Query 181 TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG 240
Sbjct 181 .....A..... 240

Query 241 CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGGTTA 300
Sbjct 241 .....G..... 300

Query 301 AAACTCAAATGAAATTGACGGGGGCCCGCACAAGCGGTGGGAGCATGTGGTTTTAATTCG 360
Sbjct 301 360

Query 361 ATGCAACGCGAAGAACCTTACCCTGGTCTTGACATCCACGGGAAGTTTTCAGAGATGAGA 420
Sbjct 361 420

Query 421 ATGTGCCTTTCGGGAACCGTGAGACAGGTGC 451
Sbjct 421 451

الخلاصة

أجريت هذه الدراسة للتحري عن الصفات الجزيئية لبعض جينات الضراوة والمقاومة للمضادات الحيوية لعزلات من بكتريا الشكيلا .*Shigella* spp. جمعت 200 عينة براز من الذكور والاناث ولمختلف الأعمار (البالغين والمراهقين والأطفال). تم الحصول على عينات البراز من مرضى الإسهال الذين تم إدخالهم إلى المستشفى العام والمستشفى التعليمي للأطفال ومستشفى الديوانية التعليمي خلال الفترة من تشرين الثاني 2020 إلى مايس 2021. نظمت استمارة لكل مريض تتضمن معلومات الاسم والعمر والجنس ومظهر العينة والأعراض. من بين 200 مريض، كان هناك 20 فقط (10٪) شخصت إصابتهم بداء الشيغيلات. تضمنت العينات 85 ذكر (42.5٪) و 115 انثى (57.5٪). قسمت العينات إلى ثلاث فئات عمرية: أقل من 11 سنة حيث احتوت 107 اشخاص (53.5%)، 11-20 سنة واحتوت 48 شخص (24%)، و 21-30 سنة واحتوت 45 شخص (22.5%). وجد ان هناك فروقات معنوية عند مستوى احتمال 0.05 ($p \leq 0.05$) بين المرضى وبحسب الأعراض (اختبارX^2). وبخصوص النسبة المئوية للمرضى المصابين بداء الشيغيلات بحسب الاعراض؛ فقد لوحظ ان جميع المرضى يعانون من آلام في البطن (100٪، 20/20)، حمى (51٪، 102/ 200)، قيء (69٪، 138/ 200)، غثيان (47٪، 94/ 200) وإرهاق (16٪، 32 / 200).

عزلت بكتريا *Shigella* spp وتم تشخيصها وفقًا للصفات المظهرية والاختبارات الكيموحيوية اضافة الى استخدام تقنية الـ PCR. وجد ان هنالك فروقات معنوية عند مستوى احتمالية ($p \leq 0.05$) بين المرضى المصابين (اختبارX^2) حسب الأعراض (حالات الإسهال). كانت نسبة المرضى الذين يعانون من الغشاء المخاطي الدموي (50٪، 10/ 20)، رخو دموي (20٪، 4/ 20)، سائل دموي (20٪، 4/ 20)، والسائل مخاطي (10٪، 2/20). تم عزل عشرين عزلة من الشيغيلا من 200 عينة براز. سميت عزلات الشيغيلا IQ. No.1 الى IQ. No.20.

استخلص الحامض النووي من 20 عزلة بكتيرية بعد التشخيص الأولي بناءً على الاختبارات الكيموحيوية والشكلية لعزلات الشيغيلا بتقنية تفاعل البلمر المتسلسل (PCR) للكشف عن جين

16S rRNA. بعدها تم قياس تركيز ونقاء الحامض النووي، وكان تركيز الحامض في كل هذه العزلات (20.9 - 49.5 نانوغرام / ميكرولتر) اما النقاوة فكانت (1.48 - 1.98). قسمت العزلات إلى (85/9) للذكور (10.58٪) و (115/11) للاناث (9.56٪). تم تقسيم العينات ذات الشغيلات طبقا للعمر إلى ثلاث مجاميع: مجموعة أقل من 11 سنة وكانت فيها11 حالة موجبة من اصل 107 (10.28٪)، و مجموعة 11-20 سنة وكانت فيها 4 حالات موجبة من اصل 48 (8.33٪)، ومجموعة 21-30 سنة وكانت فيها 5 حالات موجبة من اصل 45 (11.11٪). تبين كذلك وجود اختلافات معنوية (P≤0.05) بين الأنواع، *S. flexneri* (20 ⁄ 3 ,15 %)، *S. sonnei* (20%, 4⁄20) ، *S. dysenteriae* (65% 13⁄20) و *S. boydii* (0%, 0⁄20).

بينت النتائج باستخدام تقنية PCR ان هناك اختلافات معنوية (P≤0.05) بين المجاميع (اختبار X^2). كان نصف (50%, 10⁄20) المرضى المصابين بداء الشيغيلا يعانون من آلام في البطن(3 *S. Sonnei*, 2 *S. flexneri*, 5 *S. dysenteriae*)، حمى (25%, 5⁄ 20)، قئ (20%, 4⁄20) وغثيان (5%, 1⁄20). كذلك لوحظ فرق معنوي (p≤ 0.05) حسب أعراض حالات الإسهال لدى مرضى الشيغيلات وعلى النحو التالي: جميع المرضى الذين يعانون من الغشاء المخاطي الدموي (55٪، 11 /20)، لينة الدم (20٪، 4/ 20) (فقط *S. dysenteriae*)، سائل دموي (15٪، 3 /20) (1 *S. flexneri*, 2 *S. dysenteriae*)، سائل مخاطي (10٪، 2/20) (*S. dysenteriae*).

تم اجراء اختبارات الحساسية للمضادات الحيوية للعزلات البكتيرية لـ 9 انواع من المضادات الحيوية باستخدام طريقة الانتشار القرصي وفقًا لـ CLSI-2022. أظهرت النتائج أن نسب المقاومة لهذه المضادات الحيوية كانت كالاتي: للأمبيسيلين (100٪)، للسيفترياكسون (55٪)، للاريثروميسين (55٪)، للكلورامفينيكول (60٪)، للسيفتازيديم (65٪)، للسيبروفلوكساسين (15٪)، للجنتاميسين (40٪)، للسيفيكسيم (60٪)، ولحامض الناليديكسيك (45٪). كما كان 30٪ من العينات التي تم فحصها كانت حساسة للسيفترياكسون، 10٪ لحامض الناليديكسيك، 60٪ للسيبروفلوكساسين.

كما درست بعض جينات الضراوة للشيكيلا واظهرت النتائج بانه لاتوجد فروقات معنوية (P ≤0.05) بين أنواع الشيغيلا وجينات الضراوة. فبالنسبة للـ *S. flexneri* كانت (1/3 *Stx1* 33.33٪، (0/3، 0) *Stx*2 ٪ ، (0/3، 0) *ShET*-1 ٪ ، (0/3، 0) *ShET*-2 ٪ ، بينما للـ *S. Sonnei* كانت (1/4، 25%) *Stx*1، (0/4، 0 %) *Stx*2، (0/4، 0) *ShET*-1 ٪ ، (2/4 ، 50) *ShET*-2 ٪ . وبالنسبة لنوع *S. dysenteriae* فكانت (6/13 46.15) *Stx*1 ٪، (5/13، 38.46) *Stx*2 ٪ ، (3/13، 23.07%) *ShET*-1، (3/13، 23.07) *ShET*-2 ٪ .

ولتحديد انواع بعض الجينات المسؤولة عن مقاومة *β-lactam* بالطريقة الجزيئية فاستخدم تفاعل البلمرة المتسلسل للكشف عن هذه الجينات وكانت النتائج كالتالي: فبالنسبة للنوع *S. flexneri* كانت (100 %, 3/3) ، (*blaCTX-M* (66.66%, 2/3) ، *blaTEM* ، *blaSHV* (33.33%, 1/3) ، و (66.66%, 2/3) *blaAMPC* . وبالنسبة لـ *S. sonnei* كانت النتائج (100 %, 4/4) *blaCTX-M*، (50%, 2/4) *blaTEM*، (33.33 %, 1/3) *blaSHV*، و (66.66%, 2/3) *blaAMPC*. وبخصوص النوع *S. dysenteriae* كانت النتائج *blaCTX-M* (69.23%, 9/13)، *blaTEM* (53.84%, 7/13) ، *blaSHV* (30.76%, 4/13)، و(*blaAMPC* (38.46%, 5/13).

كما لوحظ ان أعلى معدل مقاومة لمعظم المضادات الحيوية في العزلات التي تحتوي على الجين *blaCTX-M*، حيث كانت العزلات التي تحتوي على هذا الجين مقاومة لجميع المضادات الحيوية (100٪)، والعزلات التي تحتوي على جينات *blaTEM* و *blaSHV* و *blaAMPC* كانت مقاومة للسيبروفلوكساسين بنسبة (66.66٪). كما كانت العزلات التي تحتوي على جينات *blaSHV* مقاومة للكلورامفينيكول بنسبة (25٪) وللسيفترياكسون بنسبة (27.27٪).

وفيما يتعلق بالشجيرة الوراثية، فقد وجد ان جميع عزلات *Shigella* spp (IQ-No.1-IQ-No.20) قد أظهرت تقاربا وراثيا بنسبة (99.78-99.31٪) او مطابقة لمصادر العزلات المثبتة في بنك الجينات NCBI وجميعها تشبه العزلة الهندية سلالة SK8 وبنسبة تطابق وراثي (99.78-99.31%). وعند دراسة التغيرات الجزيئية للعزلات المحلية (IQ-No.1-IQ-No.20) بالاعتماد على نتائج 16S ribosomal RNA ومقارنتها مع العزلة الهندية تبين وجود ثلاث

طفرات استبدال وبنسبة تغيرات جزيئية تقدر بـ (0.22-0.69%). كما تم تقديم عزلات في هذه الدراسة (IQ-No.1-IQ-No.20) الى NCBI Genbank وتم تحديدها بأرقام الانضمام (OK036604.1 إلى OK036623.1).