World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

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Genotypic and phenotypic study of *E. coli* isolated from children suffering from severe diarrhea with some antibiotic resistant gene

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World Journal of Advanced Research and Reviews, 2022, 15(01), 683–693

Publication history: Received on 23 June 2021; revised on 26 July 2022; accepted on 28 July 2022

Article DOI: https://doi.org/10.30574/wjarr.2022.15.1.0758

Abstract

Introduction: A total of (116) diarrhea-sufficient patient samples gathered from February 15, 2019 to April 15, 2019.

Methodology: Using colonial morphology, microscopic examination, biochemical testing, and the 20 R API Enter systems, the bacteria were able to be identified. Ultimate "PCR" identification of *E. coli* ("MDH" gene).

Result: Rendering to the results obtained according to the morphological, cultural and biochemical characters From the 116 clinical specimen only 42(59.4%) isolates were belonged to *E. coli*. PCR analysis of the *MDH* gene (392bp) showed that 26/42(61.9%) were positive.

Using the Polymerase chain reaction (PCR) assay the *bla-TEM-1* gene11/42(26.19%) of *E. coli* , were carrying of $bla_{\text{TEM-1}}$ genes was as well as 30/42(71.4%) of *E. coli* using universal $bla_{\text{-CTX-M}}$ primers. Also examined were the antimicrobial susceptibility patterns of the isolates, occurred between 0.00% and 100.00%.

Conclusions: The present study revealed that of the rate bacterial infection which causes diarrhea is more causative agent and the higher infection rate among 1month – 2 years age than other age. In this study found the molecular technique method is more efficient than other methods such as (morphology, biochemical and API20E). There was a significant prevalence of ESBL-producing bacterial isolates, and the medicines ciprofloxacin and imipenem were very effective against bacteria isolates. The high prevalence of *sul-1* gene than other genes (*bla* CTX-M and *bla* TEM).

Keywords: Diarrhea; API Enteric System 20 R; Patient; *E. coli*

1. Introduction

Diarrhea diseases have been a public health problem that particularly affect in infants and young children throughout the world. It is the main factor in pediatric pneumonia. Mainly in developing countries. [1]. In Asia, Africa, and America, 20 % of children die each year, with 60 % of those deaths taking place in the first two years of life. [2].

Numerous elements can be used to categorize diarrhea, including the severity of the condition (small or large), the length of the sickness (acute or persistent), the pathophysiological mechanisms involved (osmotic and secretory), and the dependent features of the stool (watery, fatty, and bloody). [3].

One of the most effective virulence factors of Enterobacteriaceae, the capsule is crucial for pathogenicity and shields this pathogen from the host immunity surveillance. Enterobacteriaceae, which include (*E. coli, Salmonella ssp*, and

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Shigella ssp), have many virulence factors, which are molecules expressed and secreted by bacteria that play a role in pathogenicity (macrophage and serum complement). In addition to lipopolysaccharides from endotoxins (LPS) [4].

E. coli is dependent on a number of virulence factors. Along with endotoxin, P-fimbriae type 1 promotes bacterial adhesion to host mucosal surfaces, inflames the area, and aids the pathogen in evading the immune system. [5].

The diarrheagenic *E. coli* (DEC) infection strategy entails colonizing mucosal locations, dodging host defenses, growing, and causing harm to the host. Specific fimbrial antigens that are bound by certain *E. coli* strains facilitate colonization, which is the presence, growth, and multiplication of the organism in one or more body sites without obvious clinical signs or immunological response. [6].

study's objective

This study's objectives were to find several antibiotic-resistant genes and use phenotypic and genotypic techniques to diagnose *E. coli* in diarrhea patients.

2. Material and methods

The research team is made up of 116 clinical stool samples from children with diarrhea who visited the AL-Zahra Children's Teaching Hospital in Al-Najaf Province between February 15, 2019, and April 15, 2019. The samples were split into 74 male and 42 female samples.

2.1. Morphological and cultural characteristic

All stool specimens were cultured on Mac Conkey agar plates. The sediment of stool sample was cultured directly on above mentioned culture media by sterile loop. At 37°C, all plates underwent a 24-hour aerobic incubation period. To differentiate *E. coli*, one colony was selected from a primary positive culture and cultivated on Eosin methylene blue agar and CROM agar. It was identified, stained with Gram's stain based on its morphology (colonies' appearance, size, color, borders, and texture), and then examined under a light microscope. Staining was followed by biochemical assays on each isolate to complete the final identification. [7].

2.2. Antibiogram profile

2.2.1. Disk diffusion method

Antibiotic sensitivity test was according to [7] by using [8]. The experiment was carried out using certain disks that could be obtained commercially (Bio analysis, Turkey).

2.2.2. Disk Approximation Test

To all isolates' testing [9].

2.2.3. DNA amplification and detection

According to [10].

2.2.4. PCR amplification and gel electrophoresis

Table 1 The primers were used in this work

Target gene	Sequence	Bp	Reference
MDH for *E. coli*	F 5'-ACTGAAAGGCAAACAGCCAAG-3' R 5'-CGTTCTGTTCAAATGGCCTCAGG-3'	392	12
blatem-1	F 5′- CCCCTATTTGTTTATTTTTC-3′ R 5′- GACAGTTACCAATGCTTAAT-3′	962	13
bla-CTM	F 5′-AACCGTCACGCTGTTGTTAG -3′ R 5′-TTGAGGCGTGGTGAAGTAAG-3′	766	14

PCR was used to identify MDH and genes for antibiotics like (bla_{tem-1} and bla-CTM) in DNA of all isolates, these methods according to [11].

Table 2 Programs for PCR thermo cycling conditions of primers

Gene Name	Temperature (°C) / Time					Cycles Number
	Initial Denaturation	Cycling conditions			Final Extension	
		Denaturation	Annealing	Extension		
MDH	95˚ /5min	95˚ /35std	57˚ /35std	72˚ /35std	72˚C/5min	40 cycles
^{bla}TEM-1	94°C /5 min	95˚ / 1min	51°C / 1min	72°C/1min	72°C/7min	30 cycles
CTX-M	95˚ /5min	94˚ /30 std	57°C/45std	72˚C/45std	72°C/7min	35 cycles

3. Results

3.1. Description of study specimen

The present study included 116 clinical stool specimen, 69/116(59.49%) specimen were positive culture (causes bacteria), and the other 47/116(40.51%) isolates were considered negative results (Figure 1).

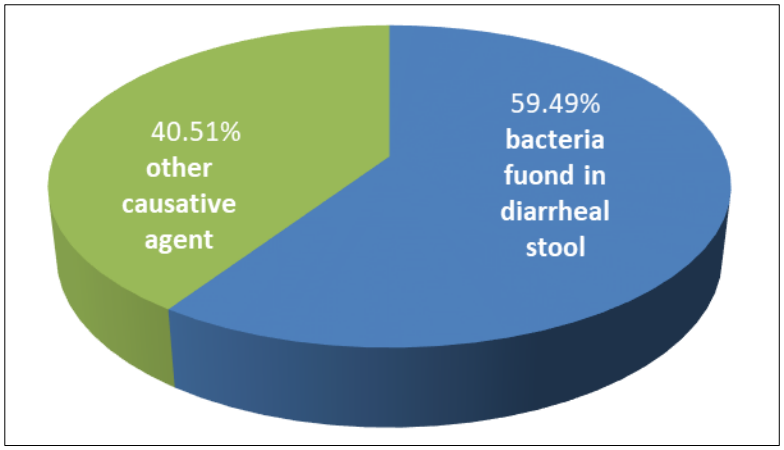


Figure 1 The occurrence of bacteria isolated from 116 children with diarrhea

3.1.1. Distribution of the diarrhea patients according to hospitalization, gender and age

Distribution of the isolates according to hospitalization, found higher frequency outpatients of 82/116(70.68%) than inpatients of 34/116(29.32%) as show in table (3).

Distribution of the isolates according to the gender, found highest frequency in male of 74/116(63.79%) than females was 42/116(36.21%) as shown in table (3).

Distribution of the isolates according to age, found that the age 1month – 2years had the highest frequency with a total 63/116(54.3%) among patients, 2 - 4 years were observed to be at the second rank in the total patients which were 26/116 (22.4%) ,while age group (4 - 6) and (6 - 8) years recorded the lowest frequency 15/116 (12.9%) patients and 12/116 (10.3%) patients, as seen in the table, accordingly (3).

Table 3 Distribution of diarrhoea patients who were hospitalised, by gender and age

Patient profile	Status	NO.(%) of sample
Hospitalization	outpatients	82(70.68%)
	inpatients	34(29.32%)
Gender	Male	74 (63.79%)
	Female	42(36.21%)
Age group (years)	1month – 2years	63 (54.3%)
	2 - 4 years	26 (22.4%)
	4 - 6 years	15 (12.9%)
	6 - 8 years	12 (10.3%)
Total		116

3.2. Bacterial isolation and identification

According to table (4)'s morphological characterization of bacteria, the *E. coli* isolates were 42/69(60.8%) which appeared small dry pink colony on Mac Concky which recorded suspected as *E. coli*, while on EMB agar had the ability to grow as circles, small in size and gave metallic shine color identified as *E. coli* and on chrom agar gave pink color (Figure 2).

Table 4 The positive results of *E. coli* isolates from Diarrhea children

bacterial species	No.	% age (%)
E. coli	42	60.8%

The biochemical tests which appeared that 42/69(60.8%) of the isolates were *E. coli* as shown table (5).

Table 5 The process of reading the results of a standard biochemical test

Test / Result	Oxidase	Catalase	urease	Citrate	VP	MR	Motility	Kliglar iron agar	H2S	Indole	Lactose fermentation Mac Conkey agar
E. coli	-	+	-	-	-	+	+	A/A/-		+	+

(-) means negative; (+) means positive; (AK) means alkaline; and (A) means acid.

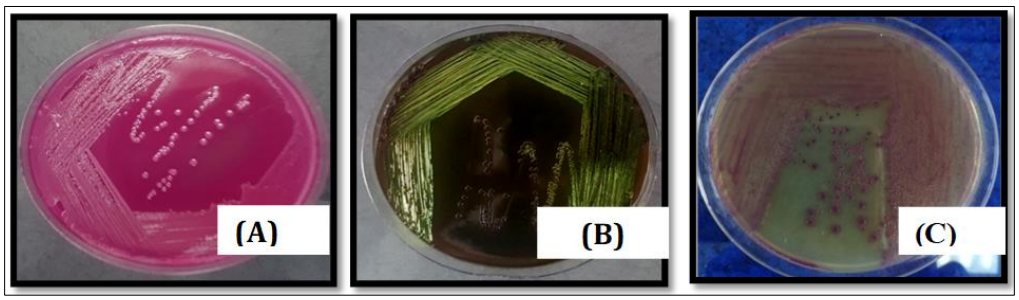


Figure 2 A- small dry pink color of *E. coli* No.(3) colony on Mac Concky agar. B- Metallic shin color of *E. coli* colony on EMB agar. C- Dry pink color of *E. coli* No.(3) colony on Chrom agar

3.2.1. Method using API Enteric System 20 R:

The API 20 R is a collection of chemical tests that are dependent upon 20 additional tests. Using the APi20R technology, the isolated *E. coli* diagnosis was verified. The manufacturer's instructions, which stated that 42 isolates of *E. coli* produce positive findings, were followed to complete this.

3.2.2. Molecular detection of bacterial isolates

E. coli confirmation using PCR amplification of *MDH* gene

The polymerase chain reaction technique of the *E. coli* clinical isolates revealed that the MDH gene gave a positive result for this gene with a product of 392 bp. The results showed that 26/42(61.9%) were *E. coli* isolates carrying *MDH* gene as shown in figure (3).

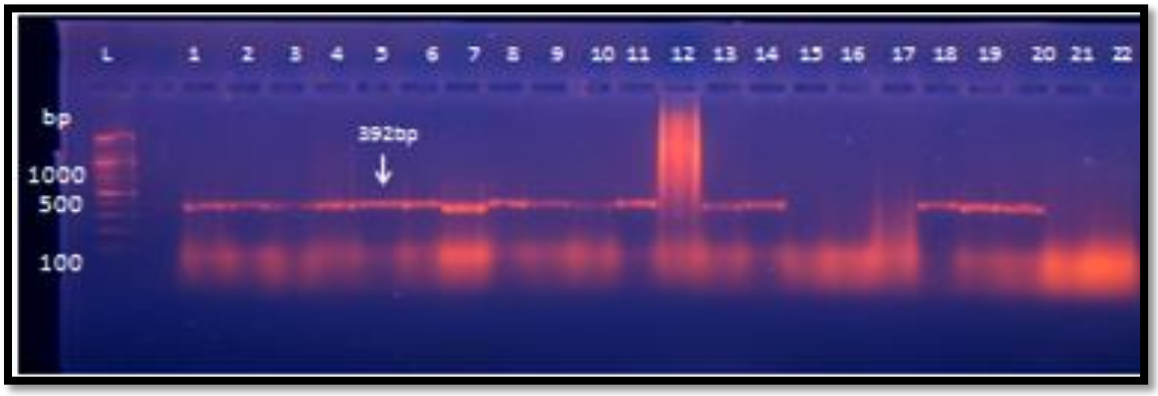


Figure 3 MDH gene primers were used to amplify *E. coli* isolates, and the result was a 392 bp product that was stained with ethidium bromide on an agarose gel

3.3. Test for Susceptibility

All isolates (42) that produced positive findings in the API Enteric System 20 R test against 18 antibiotics underwent the antibiotic susceptibility test. The outcomes are displayed in the figure (4). There were resistance rates ranging from 0.00% to 100.00%.

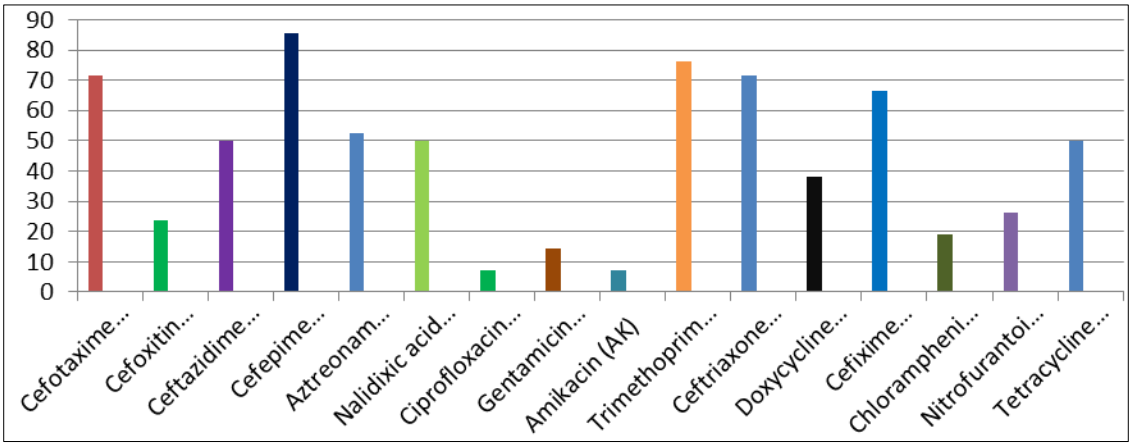


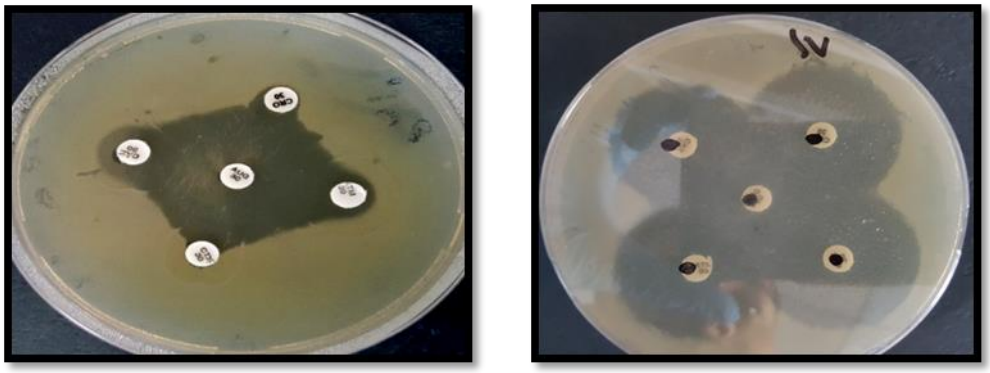
Figure 4 Antibiotics resistance of bacteria isolated from diarrheal children

3.3.1. Detection of extended-spectrum b-lactamases

The disc approximation approach confirmed all isolates (42 *E. coli*). In this procedure, an ESBL was identified by the augmentation of the inhibitory zone between two 30 mg antibiotic discs (ceftazidime, ceftriaxone, cefotaxime, and aztreonam) toward an amoxicillin-clavulanate disc (20/10 g) (Table 6), The screening test findings showed that *E. coli* 34/42 was present (80.95 %) isolates gave positive ESBLs production test, (Figure 5).

Table 6 Phenotypic characterization of ESBLs producing *E. coli* by approximation methods (n= 42)

Types of bacteria	N0. of isolates	No. (%) of positive
E. coli	42	34(80.95%)



(A) (B)

Figure 5 Approximation approaches for detecting the production of ESBL in (A) *E. coli* isolates and (D) isolates of *E. coli* that do not produce ESBL. AmC stands for Amoxi-C1av, whereas ATM and CRO stand for Ceftriaxone and CAZ for Ceftazidime. For 24 hours, the plate was incubated at 37 °C

3.3.2. Molecular study of antibiotic genes

Detection of the *bla*$_{TEM-1}$ gene

To ascertain the prevalence and varieties of extended-spectrum β-lactamases, all 57 isolates (42 *E. coli*) were examined (ESBLs). The findings showed that 11/42 (26.19%) of the *E. coli* samples had the *bla*$_{TEM-1}$ genes indicated in figure (6).

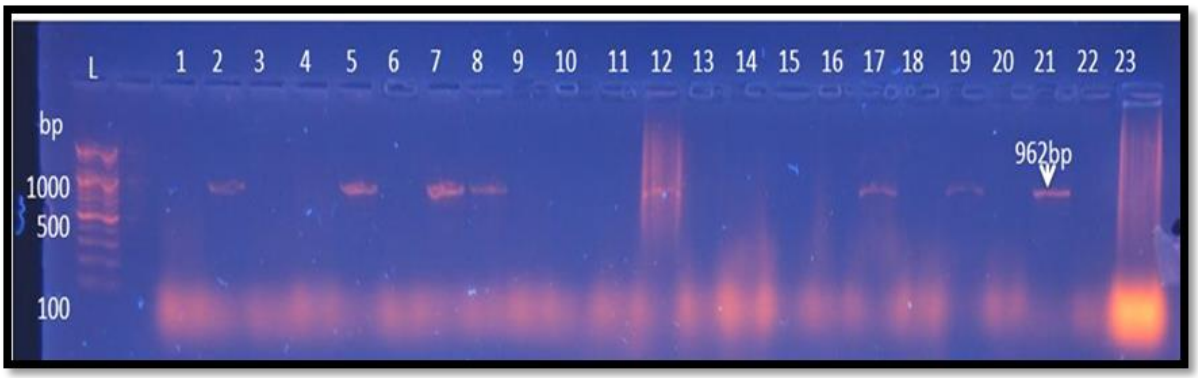


Figure 6 A 962 bp result of PCR amplification of *E. coli* isolates using the *bla*$_{TEM-1}$ gene primers is seen on an agarose gel stained with ethidium bromide (1.5 % agrose gel, 75 V, 1.20 hours)

Uncovering the Universal *bla*-$_{CTX-M}$

The goal of this study was to use universal *bla*$_{-CTX-M}$ primers to identify *bla*$_{-CTX-M}$ genes in *E. coli* isolates. The findings showed that utilizing universal *bla*$_{-CTX-M}$ primers, 30/42 (71.4 %) of the tested isolates of *E. coli* carried the *bla*$_{-CTX-M}$ gene. (7).

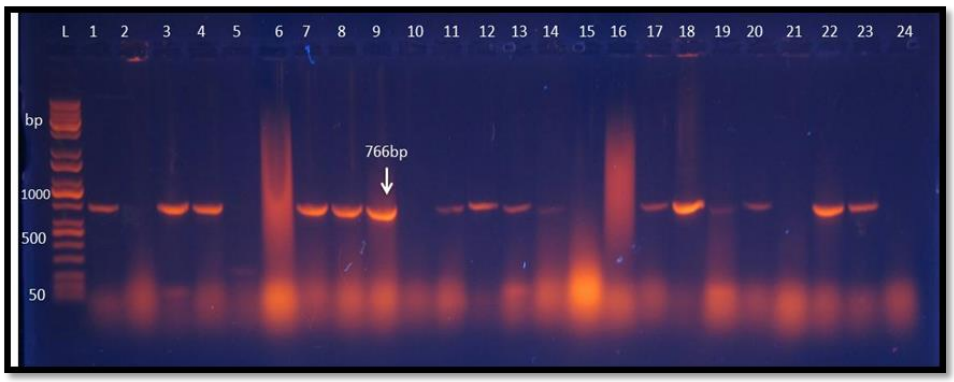


Figure 7 A 766 bp result of PCR amplification of *E. coli* isolates using the *bla-$_{CTX-M}$* gene primers is visible on an agarose gel stained with ethidium bromide (1.5 % agrose gel, 75 V, 1.20 hours)

4. Discussion

The positive results were of highly %age, which was more than half among the clinical specimens because diversity of bacteria that causes diarrhea more than other causative agent such as viruses. This observation agrees with other studies, [2] who that found most major etiology of diarrhea among children caused by bacterial infection. The finding in this study disagree with additional research in Iraq and other nations which indicating that *Rotavirus* appears as the most common cause of contagious diarrhea [15].

Regarding the characteristic of Diarrhea clinical specimens it revealed highest frequency among male compared with female, as show in table (3). This result had the same opinion with [16] who found that male was higher than females. Other studies by [17] found no significant difference between male and female.

According to morphological and biochemical reaction the result show that 92(46%) of total bacterial isolated that found *E. coli* were 42(45.65%).

This finding demonstrates the presence of enteric bacteria. Due to the presence of crystal violet and bile salts, which prevent the development of gram-positive bacteria, Conkey agar is a differential medium that is connected to enteric gram-negative bacteria. Some Enterobacteriaceae, such as *E. coli*, digest lactose while generating acidic metabolic products that lower pH and give colonies a pink colour. A selective media for *E. coli* and a differential medium for Enterobacteriaceae, which inhibits gram-positive bacteria, is EMB agar. [18].

The results of PCR of clarify that 26(61.9%) and 2(4.76%) isolates of *E. coli* producers isolates carrying *MDH* (figure 3). This research supports a study by [19], which found that 65.3 % of *E. coli* isolates were found.

Figure (4)'s findings revealed that *E. coli* was resistant to cefoxitin (23.8 %). This result dissimilar with [20] who found that resistance to Cefoxitin was (90.9%)

Figure (4)'s findings revealed that *E. coli* was resistant to ceftazidime (50 %). This outcome runs counter to [21] who found that (89.3%) isolates were resistant to Ceftazidime.

Figure (4)'s findings revealed that *E. coli* were resistant to ceftriaxone. (71.4%). This result linked with [21] who found that resistance to Ceftriaxone was (96.4%).

The results of the current investigation showed considerable resistance to third-generation cephalosporins, which is an indicative of the presence of ESBLs. ESBLs genes are typically found on big, transferable plasmids, which are easily able to propagate throughout *E. coli*. [22].

[23] Found that mechanism of resistance in *E. coli* strains may be due to expression of β-lactamases enzymes which encoded on plasmid or chromosome or as a results of cheap and facility getted without a medical prescription.

A monocyclic nucleus is covered with several side chains in monobactams like aztreonam. Gram-negative bacteria are susceptible to a variety of monobactams' activities. [24]. The study found that *E. coli* were resistant to aztreonam (52.3%). This result was near to [21] who found that resistance to aztreonam was (92.9%).

Gentamicin and other aminoglycoside resistance in Enterobacteriaceae and other Gram-negative rods is frequently caused by the expression of different modifying enzymes, such as aminoglycoside modifying enzymes (AME), acetylases, phosphorylases, and adenylases, which can reduce the effectiveness of antibiotics. Changes in bacterial membrane permeability and modified ribosomal proteins are two other resistance mechanisms. [25] .

Figure (4)'s findings revealed that *E. coli* were gentamicin-resistant (14.2 %). This outcome contradicts [21] who found (57.14%) isolates were resistant to Gentamicin, respectively. The results also support the recommendation of the aminoglycoside, Gentamicin as suitable antibiotic for treating Enterobacteriaceae and severe hospital-acquired illnesses caused by Gram-negative bacteria that are drug-resistant [26].

The frequency of antibiotic resistance of figure (4) show that *E. coli* isolates were resistance to Nalidixic acid (50%). These results were match former results obtained by [27] who found isolates of *E. coli* resistance to Nalidixic acid, respectively.

The results of figure (4) show that *E. coli* lower resistant to ciprofloxacin than the rate of [28] who found (67.7%) resistance to ciprofloxacin.

The lower resistances results of ciprofloxacin may be because of Children are not permitted to use ciprofloxacin or other quinolones due to the possibility of harm to developing joints. [29].

Tetracycline has a broad spectrum of antibacterial activity and is effective against a wide range of Gram-positive and -negative bacteria. Tetracycline primarily works by preventing tRNA from binding to the 30S rRNA's A site, leading to incorrect mRNA code reading or suppression of the protein synthesis start step. [30].

Figure (4) findings revealed that Tetracycline-resistant *E. coli* were present. (50%). This result agrees with [31] who found that resistance to Tetracycline (56%).

Figure (4) findings demonstrated that *E. coli* was resistant to trimethoprim (76.1 %). This outcome contrasts with [32] who found that resistance to Tetracycline (59%).

Despite the fact that ESBLs were discovered at least three decades ago, there is still little knowledge of their clinical importance and ability to be detected in a laboratory. The uncontrolled spread of these enzymes and occasionally unsuccessful treatments have been caused by the inability to detect them. [33].

Table (6) findings indicated that *E. coli* were thought to be ESBL producers (80.95%). Similar findings were made by [34], who discovered that ESBL-producers (85 %).

The results of PCR of clarify that 26(61.9%) isolates of *E. coli* producers isolates carrying *MDH* gene figure (3) This study is consistent with a study by [19] who detected (65.3%) of *E. coli* isolates.

The results in this study show bla_{CTX-M} β-lactamase was the most prevalent among the ESBL producing isolates; followed by bla_{TEM} β-lactamase figure (6 and 7). The result is like the study of [35] who found bla_{CTX-M} β-lactamase was the most prevalent among the ESBL producing G-ve isolates; followed by bla_{TEM} β-lactamases were the less. In India [36] reported that bla_{CTX-M} β-lactamases were more common enzymes than bla_{TEM} in clinical G-ve isolates.

The bla_{CTX-M} genes are a recent class of plasmid-mediated ESBLs; some of them are gene cassettes in integrons or were previously found in transposons.

[37]. The emergence of the bla_{CTX-M} there has been a marked shift in the epidemiology of ESBLs and A new family of plasmid-mediated ESBLs are the bla_{CTX-M} genes; some of these are gene cassettes in integrons or were previously discovered in transposons. [38].

[39] Found that bla_{CTX-M} were the most prevalent ESBL genes among the study isolates as they were the most frequently detected genes within *E. coli*.

In order to reduce the misuse of the available antimicrobials, it is important to emphasise the rational use of antimicrobials as improper use of antibiotics is the cause of the development of antibiotic resistance in isolates. Regular monitoring of antimicrobial susceptibility is also necessary for wisely identifying the patterns of resistance. To maintain the effectiveness of antibiotics and for better patient management, a national and state-level antibiotic strategy and draught plan of action should be create.

5. Conclusion

The present study revealed that of the rate bacterial infection which causes diarrhea is more causative agent and the higher infection rate among 1month – 2 years age than other age. In this study found the molecular technique method is more efficient than other methods such as (morphology, biochemical and API20E). There was a significant prevalence of ESBL-producing bacterial isolates, and the medicines ciprofloxacin and imipenem were very effective against bacteria isolates. The high prevalence of sul-1 gene than other genes (bla CTX-M and bla TEM).

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