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MOLECULAR CHARACTERIZATION AND ANTIBIOTIC SUSCEPTIBILITY ANALYSIS OF ESCHERICHIA, KLEBSIELLA, AND SHIGELLA SPECIES

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Abstract:
Bacterial pathogens are the primary cause of waterborne diseases among people and antimicrobial resistance of these pathogens has become a challenge and serious cause of mortalities in many countries. The current study aimed to characterize bacterial pathogens (E. coli, Shigella and Klebsiella) at molecular level from drinking water and their antimicrobial resistance against different antibiotics. 215 water samples were collected from different locations of Kasur, Faisalabad and Gujrat with the help of GPS essentials. All the samples were plated, cultured and specific bacterial isolates were obtained. For molecular characterization, the genomic DNA of bacterial isolates were amplified using 16S rRNA universal primers. Antibacterial resistance was assessed by using disc diffusion method. The results revealed that the streptomycin and azithromycin (80-95%), amoxicillin (50-100%), ampicillin, penicillin, neomycin and gentamycin (70-30%) showed higher resistance against E. coli, Shigella and Klebsiella species. Other antibiotics like cefotaxime, cephalothin, clarithromycin and gatifloxacin (5-20%) resistance against bacterial species. Finding of this study revealed that higher resistance of bacteria against antibiotics limits the effects of antibiotics and there is pressing need of advancement in treatment and eradication of these bacterial species from drinking water.

KEYWORDS: Antibiotic resistance, bacterial pathogens, molecular characterization, potable water

Introduction

Drinking water quality is a global concern having great influence on human health. People suffering from immune system deficiencies and kidney disorders, heart and urinary diseases are recommended with mineral water therefore it is curtail to make it sure that drinking water is safe to consume because microorganisms present in water can harm the consumers health. Likewise, packaged water shared among persons is cause of transfer of pathogens [1].

Shigellosis is a gastrointestinal (GI) disorder triggered by Shigella species it is predictable as severe health issue globally. It is most commonly occurred in under developed countries because of inadequate waste management system, reduced sanitary condition and unhealthy drinking water. Among the industrialized countries, it mostly occurred by traveling to undeveloped countries and use of contaminated food stuff. Worldwide, the mortality and diseases caused by shigellosis were highest

in kids under five years of age. Kiyoshi Shiga in 1897 reveal 1st report on isolation and characterization of Shigella [2].

Genus Shigella is categorized in family Enterobacteriaceae, a gram negative bacilli, non-motile, non-spore forming and 0.5-0.7µm in size. It is facultative anaerobic pathogenic bacteria that is closely related to E. coli. It is distinguished from E. coli on basis of pathogenesis serology, and physiology. Shigella spp. typically ferment sugars deprived of the production of lactose and gas, it is urease and oxidase negative bacteria [3].

Genus Shigella is classified into four species i.e. Shigella dysenteriae having serogroup A, Shigella flexneri including serogroup B, Shigella boydii that is serogroup C and Shigella sonnei comprises serogroup D. Regarding biochemical classification as well as serological characteristics, all these species can sub divided into numerous serotypes, like Shigella dysenteriae have fifteen serotypes, Shigella flexneri having fourteen serotypes and subserotypes, Shigella boydii consisted of 20 serotypes and Shigella sonnei having a single serotype [4].

All these species are pathogenic agents of Shigellosis and also recognized as bacillary dysentery. Its indications might be ranging from minor watery diarrhea to major inflammatory dysentery with mucoid as well as bloody stools. Other medical indicators includes abdominal cramp, nausea, fever, malaise, convulsions and vomiting. Some other problems of shigellosis included septicemia, joint pains, dehydration, hypoglycemia, neurological complications as well as hemolytic uremia [2]. The route of transmission is via fecal-oral route and due to direct interaction with some infected persons. Shigella species are potentially transmissible as only ten to hundred organisms can cause disease. Moreover, this bacteria is highly resistant for stomach acid as it can easily passes via gastric acid barrier present in stomach [5].

Escherichia coli are the bacteria that generally originate in the lower gastrointestinal tract (GIT) of human and other warm-blooded animals [6]. Most of the strains of E. coli are non-pathogenic whereas a remarkable number of strains have developed virulence factors that can cause diseases [7]. E. coli contaminate drinking water resources when it is extensively defecated by humans [8]. Open defecation, usage of manure in agriculture practices, poor drainage system are major causes of impurities and contamination water sources with pathogenic microbes like E. coli [9]. E. coli present in water serve as a bacteriological indicator of poor water quality and fecal contamination [10]. Pathogenic E. coli occurred in water consumed for drinking, recreational purposes and irrigation draw a potential health risk for humans and aquatic animals. E. coli isolated from environmental water samples is multidrug resistant and has importance for public health. Multidrug resistance at this point can be defined as acquirement of non-susceptibility to minimum number (at least) of anti-microbial agent among three or more anti-microbial classes [11].

Several pathogenic bacteria can cause infectious gastroenteritis that can lead to acute diarrhea [12]. Moreover, the diarrheal disease outbreak among toddlers is surely caused by pathogenic E. coli [13]. The pathogenic E. coli is capable of causing diarrhea is distinguished as; ETEC that is enterotoxigenic E. coli, EPEC i.e. enteropathogenic E. coli, EAggEC termed as enteroaggregative E. coli, VTEC is verotoxigenic E. coli, DAEC diffusely adherent E. coli last one is EIEC that as enteroinvasive E. coli [14].

Klebsiella pneumoniae is a prominent pathogenic microbe occurred in hospital samples. These bacteria are causative agents for foodborne infections such as pneumonia, septicemia, urinary tract infections, liver abscess, diarrhea, and bloodstream infection [15, 16]. K. pneumonia is also pathogenic for humans as well as various animals. In Egypt, it was described to be the cause of 35% mortality in broilers [17]. Street foods can be easily infected by K. pneumoniae, which causes health problem in consumers.

It was stated that ninety nine strains 9.9% of K. pneumoniae were identified in 998 food samples that are tested and the percentage of infection in the cooked food stuff was account as 7.5% [18]. Moreover, [19], and [15] also reported its occurrence in ready to eat food, meat, vegetables and aquatic food items. The present study is planned for identification and molecular characterization of E. coli, Shigella spp., Klebsiella spp. isolated from drinking water of Kasur using 16S rRNA gene.

1. Materials and Methods

2.1 Water Sample collection

Total 73 drinking water samples were collected from four tehsils of district Faisalabad. Systematic random sampling was carried out depending of area and population size each tehsil. 20 samples were collected from Jaranwala comprises of 1,811 Sq. km, 21 samples collected from Tehsil Faisalabad having area of 168 Sq.km, 17 samples were collected from tehsil Tandlianwala with area 1,284 Sq. km, 15 samples from Sammundri having area of 754 Sq. km. Water samples were collected in autoclaved falcon tubes (100 ml) and brought to laboratory for microbiological analysis. Falcon tubes were closed tightly to avoid the environmental microbial contaminants. All the samples were brought to Microbiology Laboratory of University of Veterinary and Animal Science in ice chest having ice packs and analyzed within 24 hours [14].

2.2 Culturing of Bacterial Isolates

In order to isolate E. coli, Shigella spp., and Klebsiella, 100 micro liter of each water sample was pipetted and evenly spread using a sterile glass spreader onto MacConkey, Salmonella Shigella (SS), and Klebsiella ChromoSelect selective agar, respectively. The plates were then incubated at 37°C for 24 hours.

For E. coli, pinkish red colored round colonies were observed on MacConkey agar plates that are morphologically identified. On SS agar, colorless round and convex colonies presumptively indicated as Shigella. No bacterial growth was observed on Klebsiella ChromoSelect Selective agar which indicated that Klebsiella spp. was not present in water samples. Colony forming units (cfu) were ranged from 30 to 300 after incubation.

2.3 DNA Extraction

A single bacterial colony was suspended in 1 milliliter of lysis buffer and 15 microliters of proteinase K (200 micrograms/milliliter). The solution was then vortexed and incubated at 56°C for 30 minutes, then at 95°C for 10 minutes. To separate the DNA, an equivalent proportion of ice-cold isopropanol was added. Additionally, the pellet of DNA was twice cleaned with 70% ethanol, dried, and then reconstituted in 50 µl of TE buffer [20]. Confirmation and concentration of DNA was checked through Agarose Gel electrophoresis and (NanoDrop 2000 and 2000c Thermo Scientific™).

2.4 Amplification of target gene

16S rRNA universal primers were used to amplify genomic DNA of bacterial isolates. The primers are shown in Table 1. Polymerase Chain Reaction (PCR) was done by following the manual instructions of Qiagen PCR kit. 1 min of denaturation at 94°C, followed by 25 cycles of 96°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final expansion under 72°C for 10 min. PCR product was analyzed on 1% (w/v) agarose gels. A 100 kb marker was used as a DNA marker using TBE as a buffer. Finally, the PCR product was purified using a QIA quick PCR purification kit (Qiagen) and eluted in 50 µl Tris–HCl before sequence. Purified products were sent for sequencing analysis to Nova gene Bioengineering Company.

Table 1: Primer Sequence for the amplification of 16S rRNA for different species

Bacterial spp.	Forward primer	Reverse primer	Reference
Escherichia spp.	5'- AGAGTTTGATCCTGGCTCAG -3'	5'- CTTGTGCGGGCCCCCGTCAATTC-3'	[21]
Klebsiella spp.	5`– CGCTGGCGGCAGGCTTAACA–3	5`– CCAGCCGCAGGTTCCCCT–3`	[22]
Shigella spp.	5'- TGAATACCAAGTCTCAAGAGTG-3'	5'- GTCCACGCTGTAAACGAT-3'	[23]

2.5 Antibiotics susceptibility test

Antibacterial sensitivity test will be done by using disc diffusion method according to instructions manual of CLSI, 2020 (Table 2).

Table 2: Classes and types of antibiotics selected for antibiotic susceptibility test

Class of Antibiotics	Types of Antibiotics	Mechanism of Action	Reference
Aminoglycosides	Streptomycin, Neomycin, Gentamycin	Inhibit protein synthesis	[24]
Beta Lactam	Penicillin, Ampicillin, Amoxicillin	Inhibit bacterial cell wall biosynthesis	
Cephalosporins	Cefotaxime, Cephalothin, Cephalexin	Inhibit bacterial cell wall biosynthesis	
Macrolides	Azithromycin, Erythromycin, Clarithromycin	Inhibit protein synthesis	
Sulfonamides	Sulphanilamide, Sulfisoxazole, Sulfadiazine	Inhibit growth and multiplication	
Quinolones	Ciprofloxacin, Gatifloxacin, Levofloxacin	Interferes bacterial DNA replication and transcription	

2.6 Statistical analysis

The zone of inhibition was mentioned as mean±SD. The whole genome sequence of pathogenic strains was edited in BIOEDIT software and compared using BLAST against the public database available in gene bank (http://www.ncbi.nlm.nih.gov/). The variations and resemblance of nucleotides was used to identify the strains. For phylogenetic analysis, sequences will be aligned using ClustalW & MEGA (version 7) will be used to perform phylogenetic tree.

2. Results

A total of 215 samples of potable water were collected from different Tehsils of District Kasur, Faisalabad and Gujrat. E. coli was mostly occurring pathogen than numerous isolates of Shigella spp. were obtained and Klebsiella species were least prevalent in water samples. To check the antimicrobial resistance of these bacterial species 18 different antibiotics were used.

Antibiotics of different classes' presents varying resistance values against E. coli, Shigella and Klebsiella species. Amoxicillin (100%), streptomycin (95%) and erythromycin (87%) showed high resistance against E. coli, whereas ampicillin showed 72%, cefotaxime (70%), ciprofloxacin and gentamycin showed 60% resistance for E. coli. Cephalothin showed 51% resistance. Penicillin, azythromycin, and neomycin showed moderate resistance for E. coli i.e. 35%, 29% and 23%, respectively. Cephalexin 5%, clarithromycin 4%, sulphanilamine 4%, sulphisoxazole 5%, sulphadiazine 5%, gatifloxazine 2% and levofloxacin 2% all these showed minimum resistance for E. coli isolated from drinking water samples (Figure 1).

As far as Shigella specie is concerned cephalexin showed (95%), streptomycin and azithromycin (90%), erythromycin (85%), cephotaxime (80%) and sulphisoxazole (77%) all the prior mentioned antibiotics represented high resistance. Whereas ampicillin (35%), penicillin (32%), cephalothin (30), neomycin (20%) and gentamycin (19%) presented moderate resistance for Shigella specie. Very less resistance was observed in clarithromycin, sulphadiazin, gatifloxazin, ciprofloxacin and levofloxacin that varies in values 0% to 10% (Figure 2).

Regarding Klebsiella spp., streptomycin and erythromycin represented (80%) high resistance, whereas sulphisoxazole (75%), cephotaxime (61%), amoxicillin (50%) presented moderate resistance. Penicillin (29%), ampicillin (23%), cephalothin and azithromycin (25%), neomycin and gentamycin presented (15%) resistance for Klebsiella. Sulphanilamide (10%) and clarithromycin represented (2%) for Klebsiella species sulphadiazine, ciprofloxacin, gatifloxacin, and levofloxacin showed 0% resistance against Klebsiella species (Figure 3).

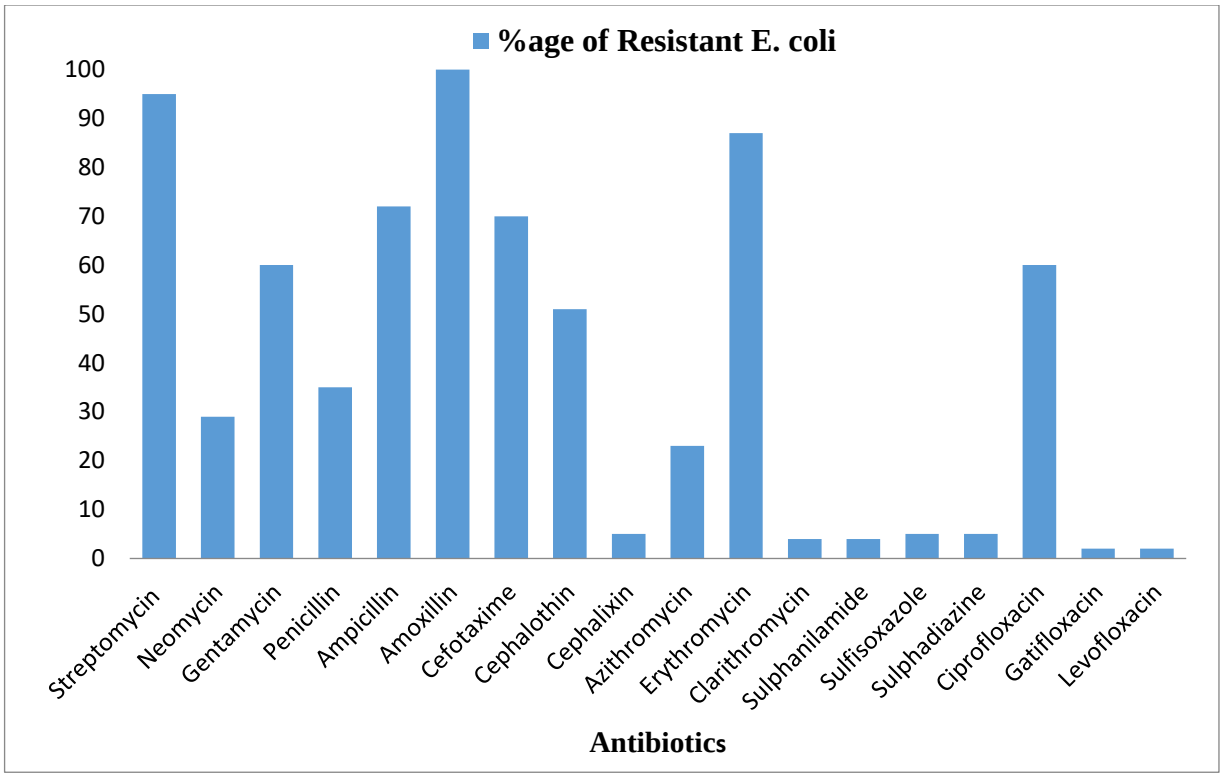

Figure 1: Antimicrobial resistance of E. coli against different antibiotics

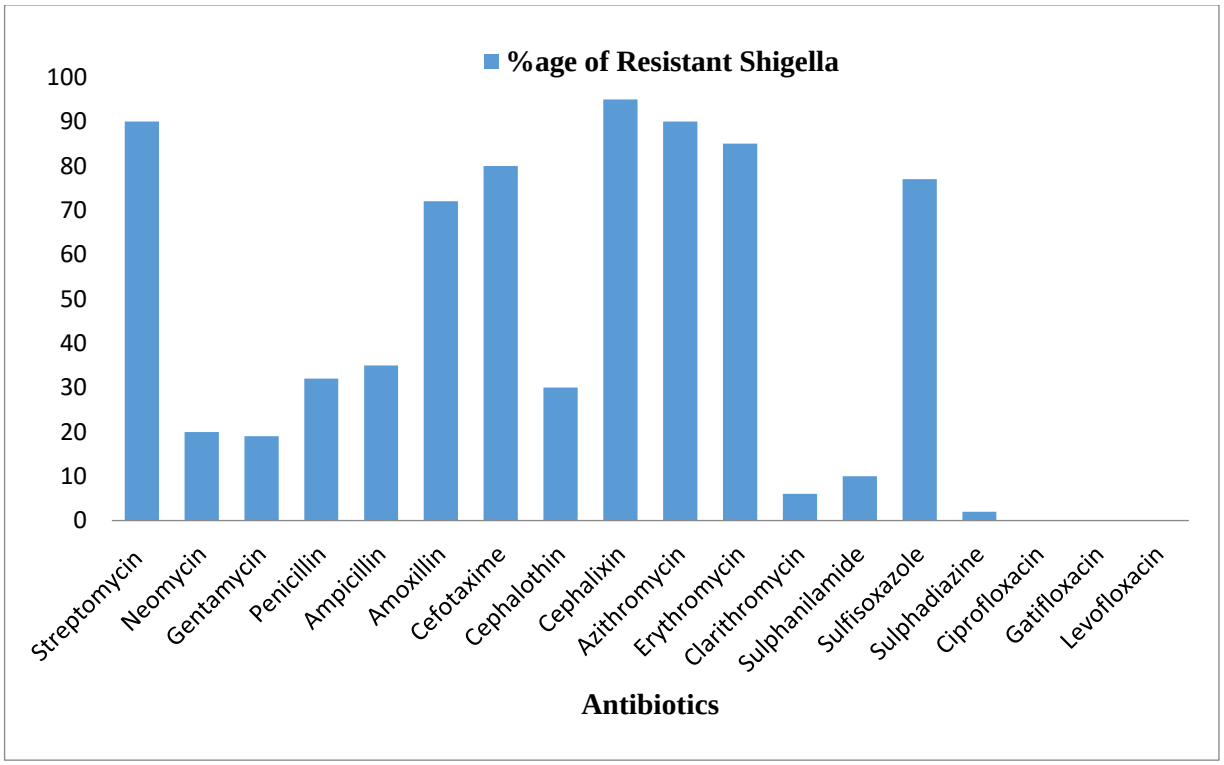

Figure 2: Antimicrobial resistance of Shigella against different antibiotics

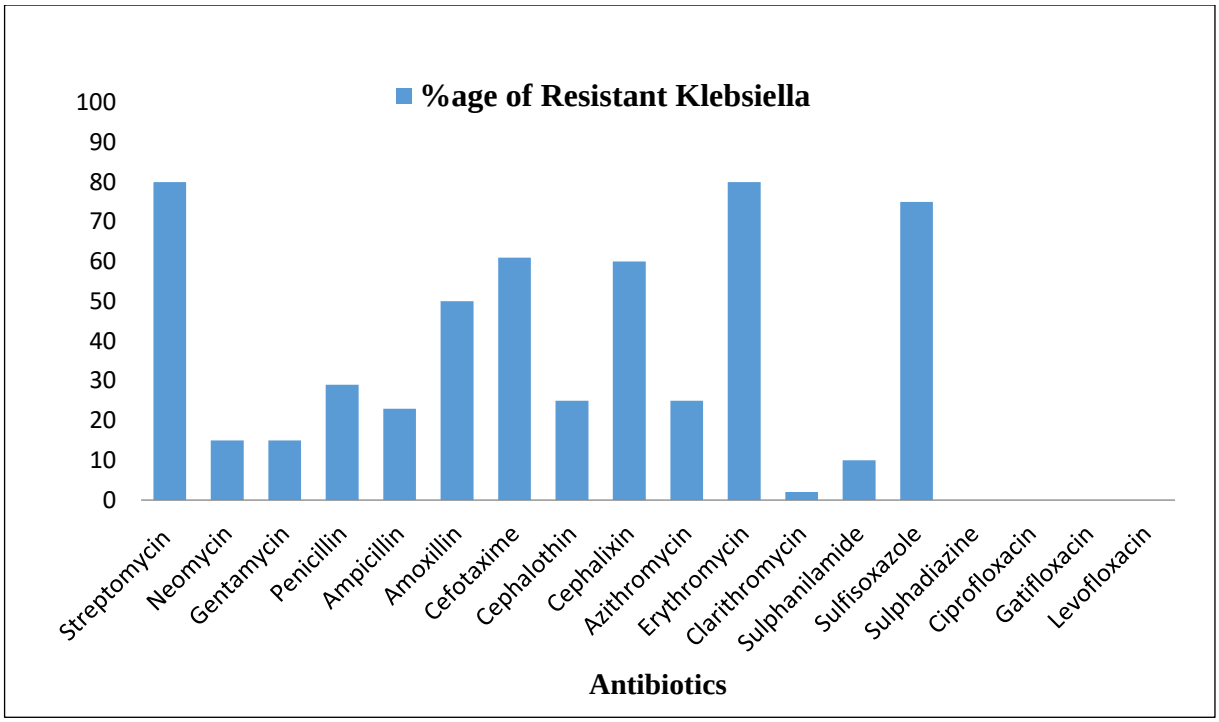

Figure 3: Antimicrobial resistance of Klebsiella against different antibiotics

Figure 4 showed the analysis of target gene 16S rRNA which were extracted from genome of E.coli. Arrows shows the band of 16S rRNA that amplified by PCR, M:(2000bp) DNA ladder; Lane 1-3,16S rRNA gene.

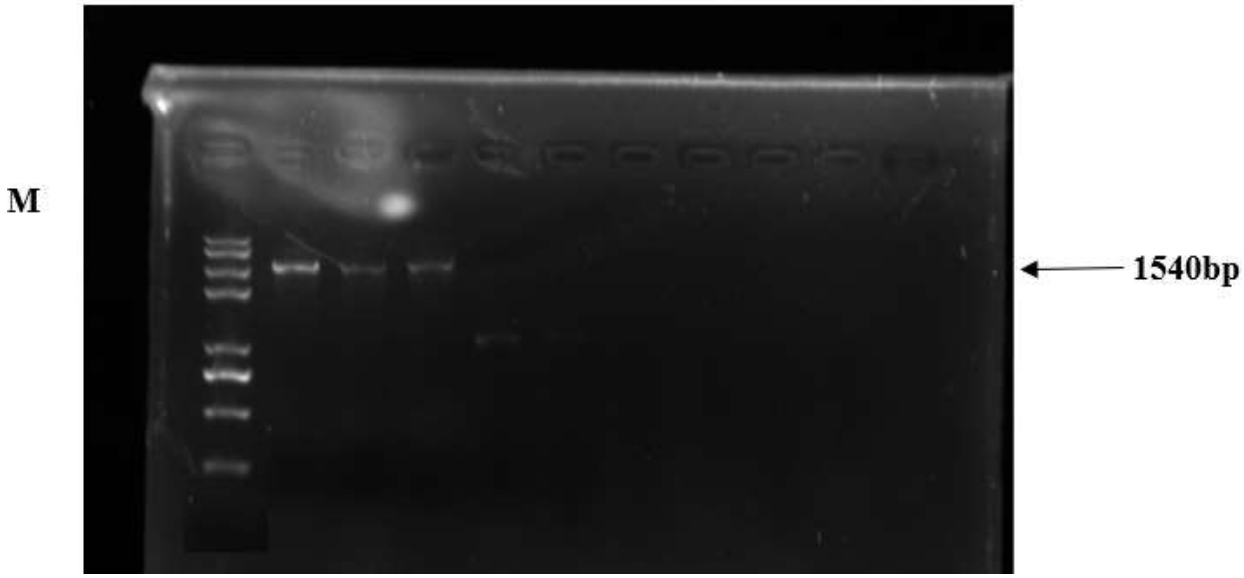

Figure 4: Confirmation of 16S rRNA gene of E.coli by PCR amplification

Confirmation of 16S gene (1623bp) in Shigella and Klebsiella species was confirmed through PCR amplification. The DNA marker Trans 2K was used for size reference (Figure 5). Specifically, Lane 1 and Lane 2 demonstrate the presence of the 16S gene in Shigella species, while Lane 4 and Lane 5 indicate the presence of the gene in Klebsiella species. Lane 3 serves as the negative control in the experiment (Figure 6).

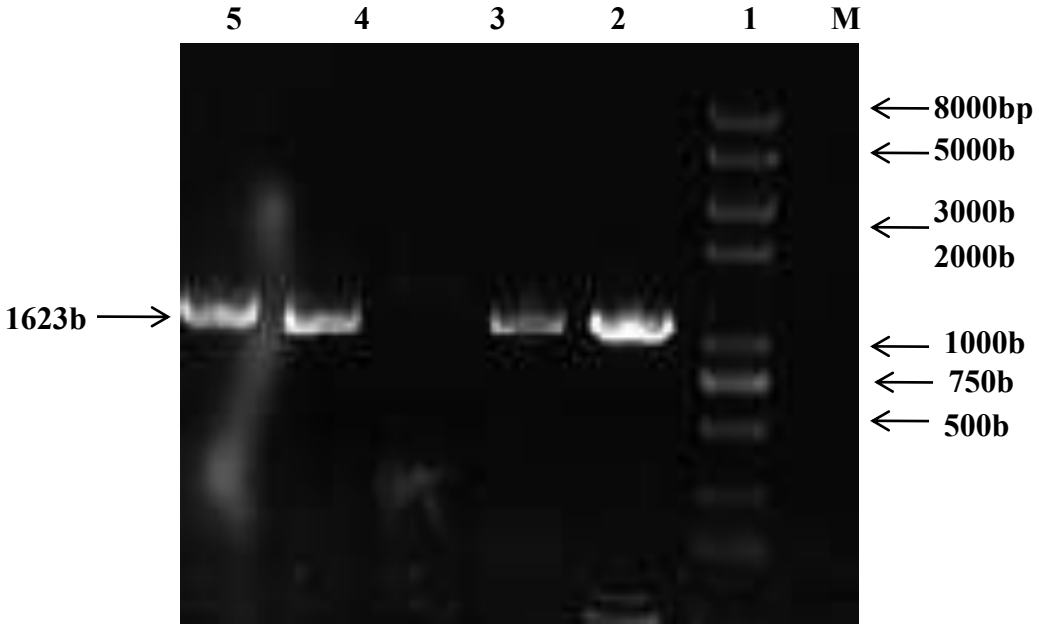

Figure 5: Confirmation of 16S rRNA gene of Shegiella and Klebsiella by PCR amplification

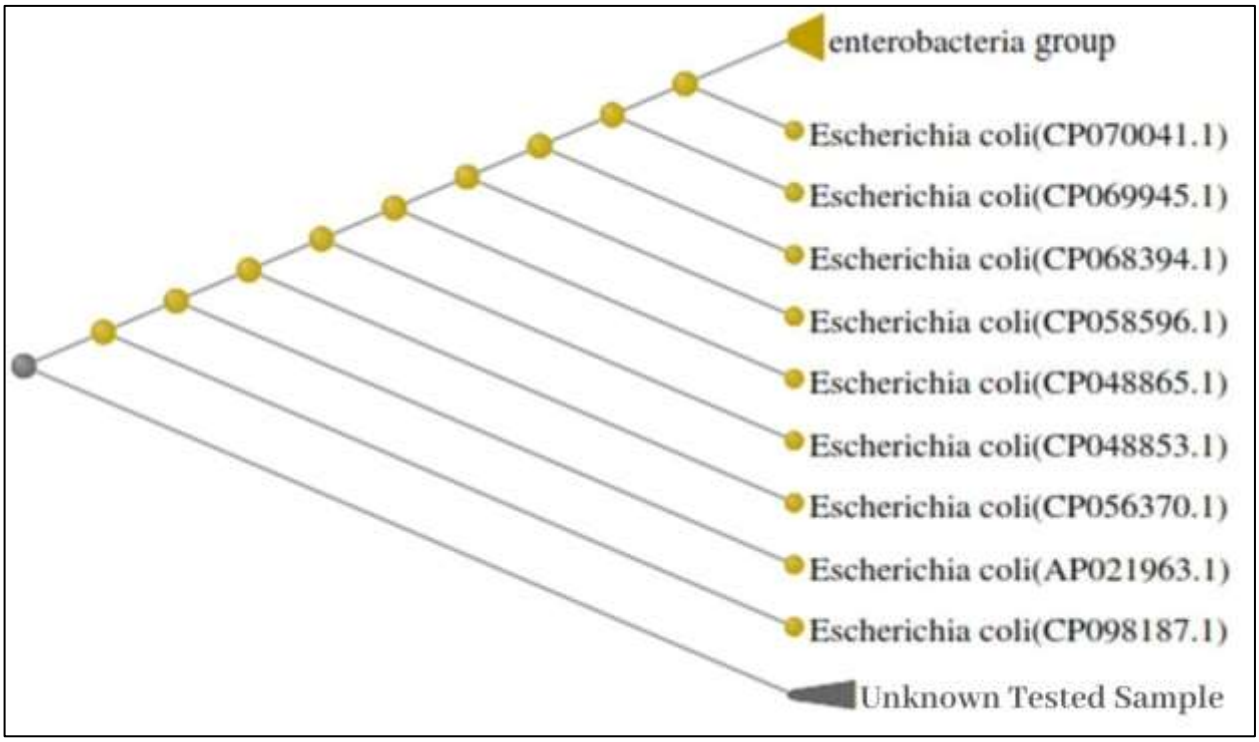

Figure 6: E. coli sequence was used for the construction of phylogenetic tree on the basis of 16S rRNA gene

3. Discussion

Antimicrobials are the agent containing inhibitory or killing properties for microorganism. In a more detaining term the antibiotics refers to natural sources of antimicrobial representatives. Antibiotics were mainly reflected as low molecular weight organic metabolites that are used against response for microorganisms against others which inhabit same habitat and strive for same nutrients [25].

The result shows the antibiotic resistance percentages of three bacterial species (E. coli, Shigella, and Klebsiella) to various antibiotics. The diminishing efficacy of medicines in treating bacterial infections, resulting in higher morbidity and mortality, makes antibiotic resistance a major healthcare concern.

E. coli exhibits high resistance to some antibiotics, with amoxicillin being 100% ineffective against it. This is particularly alarming as urinary tract infections and other illnesses are often caused by E. coli. The E. coli isolates' antimicrobial susceptibility profile to different antimicrobial drugs was also reported by [26]. The most common antibiotics for which resistance was found were ampicillin (93.6%), ceftizoxime (9.2%), cefotaxime (12.9%), sulfamethoxazole/trimethoprim (41.3%), levofloxacin (14.7%), and ciprofloxacin (16.5%). Multi-drug resistance (MDR) phenotype, which is resistance to three or more antimicrobial agent classes, was present in 34.9% of the isolates. A significant number (62.96%) of E. coli isolates showed MDR to antibiotics, which raised serious public health concerns [27]. While 11.11% of highly resistant bacteria were found to be highly resistant to six antibiotics, 25.92% of isolates only exhibited resistance to three antibiotics. Ampicillin (88.89%), ciprofloxacin (37.04%), ceftazidime (25.23%), gentamicin (18.52%), cefotaxime (18.52%) and ceftriaxone (40.74%) were all shown to be resistant.

Shigella also shows high resistance to certain antibiotics, including cephalothin (resistance rate of 80%) and azithromycin (resistance rate of 90%). Shigella is a pathogenic bacterium responsible for causing shigellosis, a diarrheal disease. Same results showed that Shigella spp. also exhibited resistance to third-generation cephalosporins [28], as evidenced by the fact that 27 (48.2%) and 33 (58.9%) of isolates were incompatible with ceftriaxone and cefotaxime, respectively. S. sonnei strains were extremely resistant to these drugs, with 16 (88.9%) of its isolates being cefotaxime resistant and 13 (72.2%) being ceftriaxone resistant, despite third generation cephalosporin resistance being reported in all serogroups.

S. flexneri strains were mostly resistant to quinolones and chloramphenicol, although overall testing revealed that S. sonnei was more resistant to antibiotics than other species. The antibiotic ciprofloxacin was effective against all Shigella isolates. Each antibiotic had a varying impact on water-borne enteric bacterial isolates [29]. Antibiotics had the greatest impact on all isolates (92.63%), completely killing all Shigella strains. Ciprofloxacin was also sensitive to all Shigella isolates.

Klebsiella displays varying degrees of resistance to different antibiotics, with cephalexin being the most effective (resistance rate of 60%) among those listed. Streptomycin, neomycin, and erythromycin are antibiotics that show significant resistance in all three bacterial species. On the other hand, some antibiotics, like sulfisoxazole and sulfadiazine, have low resistance rates, which indicate they might still be effective against these bacterial species. Klebsiella pneumoniae showed highest antimicrobial resistance to ceftazidime (33.33%), ciprofloxacin (42.11%), trimethoprim-sulfamethoxazole (36.84%), and amoxicillin (36.84%) [30].

In conclusion, the research highlights the need of using antibiotics sparingly as well as the ongoing need for new antibiotic research and development to tackle resistance among bacteria. The data shows the importance of prudent antibiotic use and the need for continued research and development of new antibiotics to combat bacterial resistance. The implementation of efficient infection control measures is emphasized as a crucial step in halting the development of bacteria resistant to antibiotics in healthcare centers and in public.

4. Conclusion

Findings of this study revealed that higher resistance of bacteria against antibiotics limits the effects of antibiotics and there is pressing need of advancement in treatment and eradication of these bacterial species from drinking water.

Conflict of Interest

No potential conflict of interest was reported by the authors.

Authorship credit

Sana Arshad: Concept and design of study:
Syed Mohsin Bukhari: Acquisition of data
Arshad Javid: Analysis and interpretation of data
Shahid Mehmood: Drafting the article or revising it critically for intellectual content

Ali Ahmed Sheikh: Help in study design and Execution

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