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Phenotypic and genotypic study of biofilm formation in multi-drug resistance *Enterobacter* species in Al-Najaf province, Iraq

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ABSTRACT

The objective of this work was to isolate and identify *Enterobacter* spp. responsible for various clinical infections in individuals, and to assess their ability to form biofilms. This study also aimed to detect bacterial taxa with antibiotic resistance. Recovered *Enterobacter* spp showed resistance to many antibiotics out of 17 examined antibiotics based on antibiotic susceptibility test. This study revealed that all isolated *Enterobacter* spp. under investigation could produce strong biofilms when assessed by using the tissue culture plate technique, whereas the majority of bacteria were able to do so using the Congo red agar method. Our results revealed that bacterial isolates that formed biofilms exhibited greater antibiotic resistance compared to bacterial isolates that did not develop biofilms. Analysis of biofilm-associated genes revealed that among the 21 isolates of *Enterobacter* spp., 47.6% exhibited the *fliC-d* gene, whereas 42.9% were characterised by the *csgD* gene.

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Introduction

Enterobacter species, particularly *Enterobacter cloacae*, play a significant role as nosocomial pathogens and have been found to be accountable for around 13.20 percent of urinary tract infections among patients in Iraq as indicated by a prior investigation (Al-Saadi *et al*. 2017). The bacteria exhibit the capability to flourish on both living and non-living surfaces and boundaries in order to form biofilms, which are intricately structured communities of microorganisms (Jahid & Ha 2014). Bacteria's ability to thrive in various settings, both natural and man-made like food processing facilities, primarily hinges on their capacity to adhere to surfaces (Giaouris & Simões 2018).

The rise of antibiotic resistance presents a pressing global challenge, particularly in connection to diseases instigated by *Enterobacteriaceae* bacteria. Resistance can manifest through two primary mechanisms: extrinsic processes involve the incorporation of resistance genes into the bacterial genome due to external influences such as the rampant, inappropriate, and negligent administration of antibiotics, while intrinsic pathways entail the pre-existence of resistance genes within the bacterial genome (Salgueiro Fins, 2020). New variants of bacteria with innate resistance traits can acquire resistance mechanisms through genetic inheritance or by harboring mobile genetic elements like integrons, transposons, and plasmids that carry diverse factors impeding the efficacy of multiple drugs (Henriques *et al*., 2013). The current study aims to determine the association between genotypic and phenotypic biofilm formation *Enterobacter* spp isolates.

Materials and Methods

Sampling

A total number of 160 clinical samples were collected from out- and inpatients who attending hospitals in AL-Najaf in Al-Sadder Medical City, Al-Hakeem General

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Hospital and Al-Zahra'a Hospital for Childbirth and Children in Al-Najaf province during the period from January 2023 to December 2024. The patients included both sexes and the age range from 1-70 years.

Bacterial isolation and identification

All collected specimens were transferred directly to the lab for isolation. Samples were inoculated into sterilized brain heart infusion broth and incubated at 37°C for 24 hours. Later on, samples were inoculated on MacConkey (MAC) agar and inoculated at 37°C for 24 hours. For identification, biochemical test and Gram stain procedure according to Macfaddin (2000) was conducted. A Vitek-2 compact system was employed for the confirmation of identification. Bacterial isolates were kept on deep Nutrient agar slant (Himedia) at -20 ◦C with periodic subculture and nutrient broth (Himedia) with 20% glycerol (Funke and Funke-Kissling, 2005).

Antibiogram test

This research employed 17 types of commonly used antibiotic including Piperacillin 100µg, Cefotaxime 30µg, Imipenem 10µg, Nalidix acid 30µg, Ceftriaxone 30µg, Ceftazidime 30µg, Meropenem 10µg, Ampicillin 10µg, Azetreonam 30µg, Levofloxacin 5µg, Trimethoprim (sulphamethoxazole 25µg, Erythromycin 15µg, Tetracycline 30µg, Nitlmicin 30µg, HLG (high level Gentamicin) 120µg, Cefepime 30µg, and Azithromycin 15µg resepctively.

The antibiotic sensitivity report was performed according to the Kirby-Bauer disc diffusion method on Mueller-Hinton agar. Briefly, the investigated isolates were allowed to multiplication overnight at 37ºC in BHI broth referred to 0.5 McFarland turbidity standard equal to $1.5X10^8$ CFU/ml, the MH agar plates were fully spreading with 0.1 ml of growth suspension and then fixed antibiotics disks on the surface. The applied plates were incubated for a duration of 10-15 minutes, followed by incubation for a period of 24 hours at a temperature of 37ºC, in adherence to standard cultural conditions. The antibiotics that were fixed underwent classification as either sensitive (S), Intermediate (I), or resistant (R) based on the measurement of diameters of the halo zone surrounding the individual disk in millimeters (mm). The findings obtained were subsequently juxtaposed with a definitive reference list of CLSI *et al*. (2023).

Biofilm Formation

Tissue Culture Plate Method (TCPM)

TCPM served as the established method for assessing biofilm formation. An amount equivalent to a loopful of recently cultured isolates was introduced into 10 ml of trypticase soy broth supplemented with 1% glucose. The inoculated broth was subsequently placed in the incubator at a temperature of 37°C for a duration of 24 hours. The bacterial suspensions underwent an additional 1:100 dilution with fresh medium. Distinct wells within a sterile polystyrene tissue culture plate, which consisted of 96 flat bottom wells each containing 200 µl of the prepared bacterial suspension, were utilized. Similarly, Control organisms were introduced into the tissue culture plate, while sterile broth exclusively was employed to ensure sterility and to detect non-specific binding. Following an incubation period at 37°C for 24 hours, the plate was delicately tapped to eliminate the contents of the wells, and then washed with 200 µl of phosphate buffer saline. This washing procedure was repeated four times to eliminate any free bacteria in the wells. Subsequently, sodium acetate (2%) was introduced to the wells and left for 30 minutes to fix the biofilms formed by bacteria attaching to the wells. The fixed biofilms were stained with crystal violet (0.1%). After 30 minutes, the wells were thoroughly rinsed with deionized water to eliminate any excess staining. Upon drying, a micro-ELISA reader (at 570 nm wavelength) was utilized to determine the optical densities (OD) of the stained bacterial biofilms. The experiment was conducted in triplicate, and an average of three OD values was obtained. The optical density values served as indicators of bacterial adherence to the wells and biofilm formation. These OD values were then computed, and the biofilm production was categorized as strong, moderate, or non/weak in accordance with findings from previous research (Panda *et al*., 2016).

Congo Red Agar Method

Congo red agar is a specifically formulated medium consisting of brain heart infusion (BHI) broth (37 g/l), supplemented with sucrose (50 g/l), agar No1 (10 g/l), and Congo red (0.8 g/l). A concentrated aqueous solution of the Congo red stain was prepared and subsequently autoclaved at 121ºC for a duration of 15 minutes. Subsequently, this solution was introduced to the autoclaved BHI agar along with sucrose at a temperature of 55ºC. The prepared CRA plates were then subjected to inoculation with the isolated pathogens and were aerobically incubated at a temperature of 37°C for a period of 24 hours. The presence of black dry crystalline colonies on the CRA plates was indicative of biofilm production, while the colonies of non-biofilm

producers remained either pink or red in color. This method was employed for ensuring sterility and for differentiating non-specific binding (Ruchi *et al.,* 2015).

Extraction and Isolation of DNA

The Genomic DNA Extraction Kit from (Geneaid) was utilized to extract DNA. The concentration of the DNA was assessed through spectrophotometric analysis, which involved measuring its optical density at 260 nm (the Extinction coefficient of dsDNA being 50 µg/ml at 260 nm). The purity of the DNA solution can be ascertained by the ratio of OD 260-280 falling within the range of 1.8±0.2 for pure DNA. The thermocycler employs a specific PCR program. Subsequently, the PCR products and the ladder marker are separated through electrophoresis on a 1.2% agarose gel (Sambrook & Russell 2000).

Polymerase Chain Reaction (PCR) Technique

In the present investigation, monoplex PCR methodology was utilized for the identification of various genes responsible for encoding virulence factors within isolates of *Enterobacter* spp. The application of monoplex PCR was specifically for the purpose of detection *csg*, *filC* genes. The PCR mixture was prepared with a total volume of 30 µl, comprising 15 µL of PCR premix, along with 2µl of each primer and 5µl of extracted DNA. The remaining volume was adjusted with 6µl of sterile deionized distilled water, followed by vertexing. The negative control included all components except the template DNA, which was substituted with distilled water. Subsequently, the PCR reaction tubes were briefly centrifuged to ensure thorough mixing and settling of contents at the tube bottom before being transferred to a thermocycler PCR instrument for DNA amplification. as detailed in tables 1 and 2.

Results and Discussion

Overall, during the study period that 160 different clinical specimens were collected from the biggest Hospitals in Al-Najaf city -Iraq. The findings indicated that *Enterobacter* spp was 21\160 (13.13%).

The Antibiotic Susceptibility Test

The susceptibility of *Enterobacter* spp. to commonly used antibiotics for the treatment of bacterial infections was assessed through the implementation of the Kirby-Bauer disk diffusion method according to CLSI (2023) guidelines. The study encompassed 17 antibiotics derived from six distinct antimicrobial classifications.

The overall resistance rate to β-lactams/β-lactamase inhibitor combination antibiotics, including Ampicillin, was observed in 85.71% of isolates. The resistance of bacterial isolates to the third-generation Cefotaxime, with 100% representation by Cefepime, was noted in 85.71% of isolates. Similarly, resistance to the fourth-generation Tetracycline was 85.71%, and 71.42% of the evaluated isolates exhibited resistance to Ceftriaxone. Additionally, 42.85% of the isolates demonstrated resistance to SXT. Levofloxacin resistance was found in 14.28% of bacterial isolates, while 4.76% showed resistance to Gentamicin. Erythromycin resistance was recorded in 100% bacterial isolates. Resistance to Imipenem was observed in 85.71%, and resistance to Meropenem antibiotics appeared in 23.80%. Furthermore, resistance to Ceftazidime antibiotics was observed at 100%, with Piperacillin resistance recorded in 85.71% of bacterial isolates. Resistance to Aztreonam was noted at 76.19%, and resistance to Azithromycin was observed at 52.38%. The resistance of bacterial isolates to Nitlmicin was found in 33.33%, while resistance to Nalidix acid appeared at 23.80% (Table 3).

Table 1: The primer used in this study

Primer Type	DNA sequences (5-3)	Product size bp	References
fliC-d	F: ACTCAGGCTTCCCGTAACGC R: GGCTAGTATTGTCCTTATCGG	763	Azou & Pollard, (2010)
csgA	F: GAAARYTGGCCGCATATCAATG R: ACGCCTGAGGTTATCGTTTGCC	276	Kim SM *et al* (2012)

Table 2: Programs of PCR Thermocycling conditions of primers

Gene name	Temperature (°C)/ Time					Cycle number
	Initial	Cycling Conditions			Final	
	Denaturation	Denaturation	Annealing	Extension	Extension	
fliC-d	95/5min	95/30sec	55/5min	72/1min	72/5min	35
csgA	95/5min	95/1min	57/5min	72/1min	72/5min	30

Table 3: The Antibiotic Susceptibility Rates among *Enterobacter spp*

Antibiotic	Resistant no (%)	Intermediate no (%)	Sensitive no (%)
Piperacillin	18 (85.7)	1(4.7)	2 (9.5)
Cifteraxone	15 (71.4)	4 (19.04)	2 (9.5)
Cefotaxime	21 (100)	0 (0)	0(0)
Nalidic acid	5 (23.8)	2 (9.5)	14 (66.7)
Cefepim	18 (85.7)	0 (0)	3 (14.3)
Gentamicin	1 (4.8)	3 (14.3)	17 (80.9)
Ceftazidime	21 (100)	0 (0)	0 (0)
Meropenem	5 (23.8)	0 (0)	16 (76.2)
Impenem	18 (85.7)	1 (4.8)	2 (9.5)
Tetracyclin	18 (85.7)	1 (4.8)	2 (9.5)
Erythromycin	21 (100)	0 (0)	0 (0)
Aztreonam	16 (76.2)	1 (4.8)	4 (19.5)
Azithromycin	11 (52.4)	0 (0)	10 (47.6)
Trimethprim (sulphamethoxazol)	9 (42.8)	1 (4.8)	11 (52.4)
Nitlmicin	7 (33.3)	1 (4.8)	13 (61.9)
Levofloxacin	3 (14.3)	2 (9.5)	16 (76.2)
Ampicillin	18 (85.7)	2 (9.5)	1 (4.8)

R= signifies resistance, I= denotes an intermediate level, and S= indicates sensitivity.

Previous research has shown that *Enterobacter* species were resistant to a wide range of antibiotics, such as β-lactam antibiotics, macrolides, cephalosporins, fluoroquinolones, aminoglycosides, carbapenems, tetracyclines, and polymyxins. Additionally, the presence of chromosomally encoded multidrug resistance efflux pumps, β-lactamases, and antibiotic-modifying enzymes contributes to the intrinsic antibiotic resistance of *Enterobacter* spp. Antibiotic resistance is horizontally transferred by integrons, transposons, integron-like elements, plasmids, and insertion element common region (ISCR) elements, resulting in the acquisition of drug resistance mechanisms (Sanchez *et al.* 2009).

The fact that just 3 of the pathogen isolates (14.28%) had mild resistance to levofloxacin, as opposed to 16 (76.19%), suggests that this antibiotic is more effective. Last but not least, the event shown in Table (1) declared that Erythromycin and Ceftazidium medications show remarkable results against various *Enterobacter* isolated species. Of them, 21 (100%) were susceptible to the medicine, while 0 (0%), were resistant.

Azevedo *et al*. (2018) was found the resistance profile in the studied isolates corroborate with a study conducted by Cabral *et al*. (2017) which examined a Brazilian set of isolates from the *E. aerogenes* and *E. cloacae* complex and discovered that amikacin, gentamicin, and tobramycin had the lowest resistance rates. However, the current study has detected 71.4% resistance to trimethoprim-sulfamethoxazole, whereas they reported low levels of resistance. Fortunately, imipenem sensitivity was observed in all isolates except for isolate EA06, as published reports indicate that imipenem is still one of the most potent antibiotics for treating *E. cloacae* infections.

While Rodulfo, (2016) was found the *Enterobacter* spp. strain showed resistance to the following: ampicillin (AMP), amoxicillin-clavulanate (AMC), cefalotin (CF), piperacillin (PIP), piperacillin-tazobactam (TZP), cefuroxime (CXM), cefoxitin (FOX), cefotaxime (CTX),

ceftriaxone (CRO), aztreonam (ATM), ceftazidime (CAZ), cefepime (FEP), imipenem (IPM), meropenem (MEM), ertapenem (ETP), trimethoprim-sulphamethoxazole (SXT), amikacin (AK), tobramycin (NN), netilmicin (NET), ampicillin-sulbactam (SAM), ciprofloxacin (CIP), chloramphenicol (C).

Dimitrova, (2019) the majority of the ESBL isolates in this investigation exhibited multidrug resistance phenotypes (MDR), with the highest resistance rates to tobramycin (98%), gentamicin (91.6%), ciprofloxacin (81%) and trimethoprime/sulphometoxazole (59.6%), according to the results of antimicrobial susceptibility tests.

Most Gram-negative bacteria (GNB), including Enterobacteriaceae, have long been blamed for being the most common MDR carriers (WHO 2017).

Enterobacter cloacae (7/274), *Enterobacter hormaechei* (1/274), and *Enterobacter kobei* (1/274) were the three species belonging to the *Enterobacter* genera that had the second highest incidence (3.28%, 9/274). These species were also the most widely distributed, having been isolated from five different types of animal-derived food. Notably, both *E. hormaechei* and *E. kobei* were completely resistant to AMC, CTR, CXM, and VA (Edris *et al*. 2023).

Biofilm formation

The results appear that CRA examine was a good method for detection of the ability of slime and biofilm production (figure 1) and agreed with researcher Arciola *et al*., (2006) it is recommended by experts that the CRA experiment is a reliable approach for assessing biofilm production. The slime layer functions to encapsulate the bacterial cell, creating delicate, viable membranes referred to as biofilm. This serves as a protective barrier that hinders the entry of antibiotics into the bacterial cell, thereby providing resistance (Al-Khafaji 2018, Jawade *et al.* 2024).

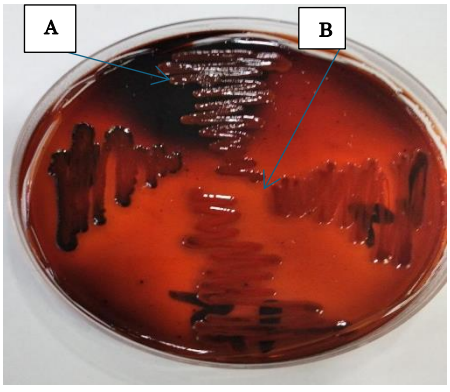

Fig 1. Colony of *Enterobacter* spp on Congo Red Agar A: dark colonies of biofilm producer, B: red colonies of non-biofilm producer**.**

The outcome demonstrated that 9 (42.85%) of the colonies were identified as black biofilm producers, whereas 12 (57.14%) of the isolated colonies were categorized as non-biofilm producers.

Furthermore, the study demonstrated the capacity of the majority of bacteria to form biofilms using the Tissue Culture Plate Method (table 2).

Phenotypic detection of biofilm formation through the Microtiter Plate Method (MTP) is discussed in the study by Christensen *et al*., (1985). The study utilized *Enterobacter* spp isolates to assess their capacity for biofilm formation, revealing that all 40 isolates (100%) exhibited robust biofilm production. However, no isolates (0%) were weak to form the biofilm, as shown in table 4 and figure 2.

Fig 2. Phenotypic detection of biofilm formation of *Enterobacter* spp by TCP method.

Table 4: The Percentage of Biofilm Formation by *Enterobacter* spp

Type of biofilm formation	*Enterobacter* spp out of 21
Strong (> 0.240 ± 0.022)	21(100%)
Moderate (0.120-0.240 ± 0.020)	0 (0%)
Weak/Non (< 0.12 0 ± 0.012)	0 (0%)
Total	21 (100%)

The findings of this investigation revealed that all clinical isolates of *Enterobacter* spp can strongly produce biofilm. Within the biofilm structure, bacteria are effectively shielded from a variety of stressors, such as immune responses and antimicrobial agents. The formation of biofilm by bacteria has been linked to heightened levels of antibiotic resistance and the development of persistent recurrent infections. Within the framework of biofilm, which consists of

extracellular polymeric substances, a multitude of microorganisms may exist either individually or in aggregated micro-communities. An important aspect of virulence is the capacity of a microorganism to create biofilm, as it creates a protective environment that enables survival and resistance to antibiotics for the particular species (Dincer *et al*., 2020). Many Gram-negative bacteria are classified as biofilms that confer resistance to environmental stress and bactericides in microbial classes (Garde *et al*., 2015). Within the biofilm, there exist bacterial microorganisms known as sessile bacteria, which undergo a stationary or dormant phase of growth, exhibiting phenotypic characteristics that set them apart from planktonic bacteria (Muhammad *et al*. 2020).

Molecular detection of Biofilm

We used the uniplex-PCR approach to amplify genes using specific primers mentioned in Table (1) for 21 Enterobacter spp. isolates, identifying the genes csgD and fliC-d, as shown in Figures 3 and 4. The results revealed that the presence of the csgD gene conferred a higher frequency and percentage (9/21, 42.9%), while the fliC-d gene showed rates of percentage (10/21, 47.6%).

Several surface structures of bacteria, including curli, flagella, pili, and exopolysaccharides, play roles in different aspects of the development of biofilms (Kalantar et al., 2008). High regulation controls the expression of csgD. E. cloacae isolates were efficient. Biofilm-forming significantly correlated with the csgA and csgD gene mRNA expression rates. The curli protein fimbriae showed up as tangled fibers, and the strain that was high in curli formed mature biofilms (Kim et al., 2012). The expression of curli fimbriae in E. cloacae is a key part of biofilm formation, which is the first step in a bacterium becoming pathogenic (Rasheed et al., 2021).

The results of the current study showed that 5 (23.8%) of E. cloacae isolates were carrying csgA in the genome of bacteria. Curli fibers are also considered a main agent for autoaggregation, as studies have shown that autoaggregation increases with Curli production at lower temperatures (Goulter et al., 2010). Curli belongs to the class of amyloid fibers, and their expression is regulated by two operons, csgBA and csgDEFG, that encode for the protein's structural subunits and accessory proteins that regulate or mediate transport to the extracellular matrix. The CsgA subunit is secreted to the exterior of the cell and is then assembled into fiber by CsgB, who is anchored to the bacteria's outer membrane.

Wang et al. (2021) found in their study that 59% of isolates carry the csg gene, while Al-Mulla and Al-Muhanna (2023) found 2 (20%) of isolates carry csgD.

The study of Kim et al. (2012) documented that the expression of curli fimbriae plays a crucial role in the process of biofilm formation in E. cloacae. This was demonstrated by a previous investigation, which identified the presence of csgA and csgD genes in a majority (78.6%) of the 14 isolates; also, Zogaj et al. (2003) and Abdulla et al. (2024) suggested that biofilm formation is associated with the cellulose and curli fimbriae expression (Oleiwis et al. 2021).

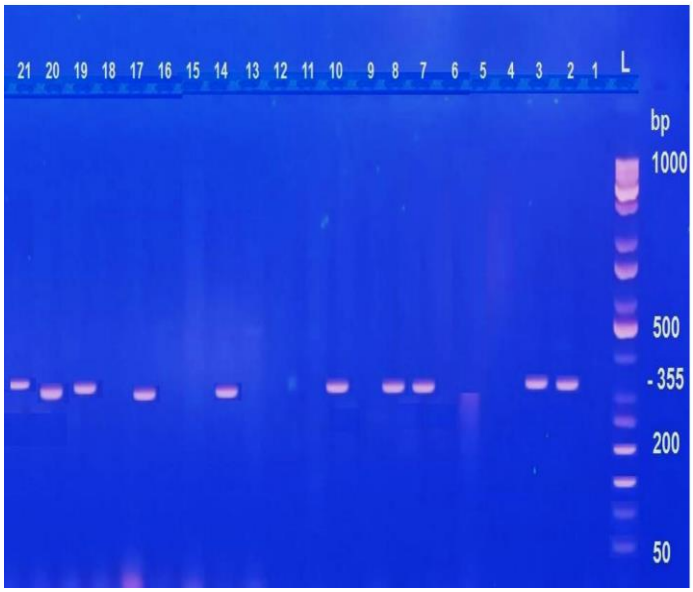

Fig 3. Agarose Gel Electrophoresis of *Enterobacter* spp utilizing primer *csgD* gene with product 355 bp. The DNA molecular size marker (50–1500 bp ladder) lane (L) was electrophoresed for 1.30 hours at 70 volts.

The flic-d gene encodes a universal subunit of flagella that makes Enterobacter spp. flic copies and sequences them in bacteria that can move. The FLiC loci contain the majority of the 53 H-serotypes. Using specific primers (Figure 4), we increased the amount of flic-d found in 10 out of 21 Enterobacter species that were taken from urinary tract infections. Despite repeatedly tuning the reaction conditions, certain isolated bands still amplified non-specific bands.

Previously, many studies have noted that amplification of flic producing different amplicon size reflection the H-type of flagella belong it (Kadhim & Abdulhasan 2023). One possible explanation for the lack of fliC amplification in all isolates is that some H-antigen genes, such as flnaA, fllA, fmlA, or flkA, are located at loci other than fliC (Majeed & Motaweq 2024).

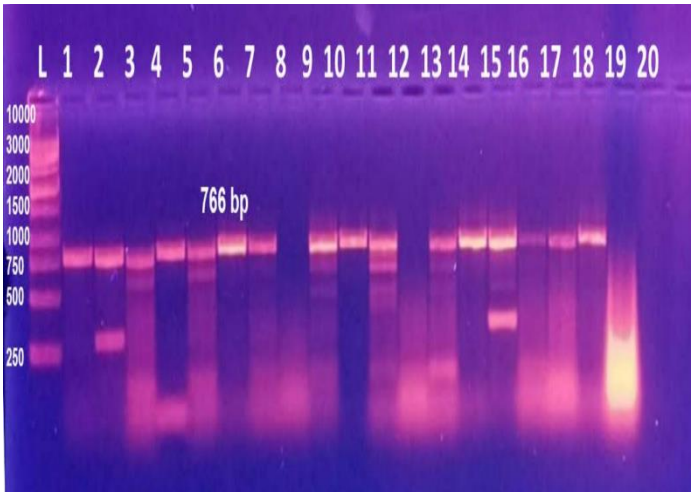

Fig 4. Agarose gel electrophoresis of *Enterobacter* spp PCR amplification products amplified for one hour at 80 vol using *fliC-d* gene primers, yielding a product of 763 bp. The DNA molecular size marker (250–10000 bp ladder) lane (L).

Conclusion

The Enterobacteriaceae family comprises a diverse array of Gram-negative bacteria, primarily residing in the intestinal tracts of humans and animals. Urinary tract infections (UTIs) are the most prevalent hospital-acquired infections caused by Enterobacteriaceae, but lower respiratory tract and bloodstream infections are the most fatal. Virulence factors in Enterobacteriaceae encompass several adhesins, hemolysin synthesis, serum resistance, and biofilm development. These features, particularly the capacity to establish biofilms in the human intestine, may facilitate gut colonization and significantly influence the functionality of the intestinal microbiome and its interactions with the host.

This study shows that all of the csgD bacteria are associated with strong biofilm formation in Enterobacter spp., which may be multidrug resistant. Furthermore, in order to determine the precise etiology of the infection, molecular identification and characterization are necessary.

Ethical of Specimen

All the participants provided informed consent for inclusion in the study and were assured that all the information provided would be used solely for the purposes of this study and treated confidentially.

Conflict of interest

The authors declare that they have no conflict of interest.

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