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The ability of *Escherichia coli* isolated from different clinical cases to form biofilm and the detection of *CsgA* and *Yja*A genes

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ABSTRACT

One hundred forty five samples were collected from various clinical sources at several hospitals in Baghdad city. After collection, the cultures were diagnosed, microscopic examinations and biochemical examinations. By Vitek-2 system, (50 isolates) were obtained from Escherichia coli with percentage 50(34%) distributed as follows: urinary tract infection (72%), wounds (14%), respiratory infection (2%), sputum (10%), vaginal swab (2%). After that detect of biofilm, antibiotic screening and molecular detection. The ability of E. coli to form a biofilm is one of the utmost important virulence factors and plays a role in causing infection and increasing antibiotic resistance to E. coli by using the microtiter plate method. The results indicated that (100%) of bacterial isolates were produced biofilm in different percentage, where the percentage of biofilm was weak in 11 isolates rate (91.6%), and in one isolate was medium rate (8.3%). The highest resistance to antibiotics (Cefazidime, Colistin and Ceftriaxone) with a percentage of 100% for 12 isolates. Molecular detection was chosen for the availability of the two genes responsible for the formation of biofilms (CsgA), (YjaA) for 12 isolates in E. coli, according to their ability to produce a biofilm, and thus the results appeared

that (100%) of 12 isolates carrying (CsgA) and (YjaA) genes.

Keywords: E. coli, CsgA gene, YjaA gene, Biofilm.

1. INTRODUCTION

Escherichia coli is a gram-negative selective anaerobic bacteria, a normal intestinal flora that colonizes the mucous layer (Khudhir, 2014; Abdulridha and Ibrahim, 2018). Originally called "coliform bacteria" initially, it was isolated from children's feces in 1885 by Theodore Escheriched (De Sousa, 2006). These bacteria are non-spore and have a rod shape (Alrekaby and Alwendawi, 2014). It is a common cause of many diseases (Mustafa and Mohammed, 2014) and is considered contaminated to the hospital (Ibrahim *et al*., 2014). It is found in the digestive tract of humans and animals, some species of these bacteria are known to be pathogenic, such as *E. coli* O157:H7. Therefore, *E.coli* bacteria cause many serious clinical symptoms such as fever, bloody diarrhea, hemolytic syndrome and uremia, which can lead to death in both children and the elderly (Khudhir, 2021; Al-Rudha *et al.*, 2016). *E. coli* bacteria possess hemolycin-producing virulence and adhesion (Alice and Al-Aubydi, 2009), as well as siderophore, Toxin, fimbriae and lipopolysaccharides biofilm (Bien *et al*., 2012).In addition to cholycin, aerobactin and cytotoxic necrosis factor with hydrophobic cell surface, some specific

serotypes of O and K antigens have resistance to phagocytosis and bactericidal action compared to natural serum (Ebraheem and Alwendawi, 2015). *E. coli* is the main pathogen causing urinary tract infections (UTI), meningitis in children, middle ear infections and wounds (Kibret and Abera, 2011; Roof and Fayidh., 2022). Urinary tract infections are called Uropathogenic *E.coli* (UPEC), which cause about 90% of urinary tract infections, as they can easily travel from the area to the urinary tract and bladder, which are about 14 times more common in females than in males due to short urethra in females (Al-Saadi, 2019; Lupo *et al*., 2021). The temperature at which *E.coli* bacteria grow is (7-50) °C, and the optimum temperature for them is (37) °C, and they have only one circular chromosome, and some contain a circular plasmid. The genus *Escherichia* includes other species such as: *E. hermanii*, *E. albertii*, *E. vulneris*, *E. blattae* and *E.fergusonii*) (Olowe *et al*., 2017).

Since the biofilm gives bacteria up to 1,000-fold antibiotic resistance and suppresses the host's immune system, this biofilm-related infection is difficult to treat (Ballén *et al.*, 2022). Antibiotics play an important role in the treatment of bacterial infections, but the wrong use of antibiotics leads to the appearance of high resistance to multiple drugs in different species of bacteria, especially gram-negative bacteria, because they produce broad-spectrum βeta-lactamase (ESβLS) (Sah and Hemalatha, 2015; Roof and Fayidh, 2021). The *yjaA* gene plays an essential role in the cell to respond to hydrogen peroxide and acid stress (Mohammad *et al*., 2018) and *CsgA* is the main component of curlin (Bleem *et al*., 2023). Therefore, genomics is not only an amazing diversity among bacteria, but there is also a great diversity even among *E. coli* strains and *E. coli* was first observed through phenotypic characterization (Welch *et al*., 2002; Dynamur, *et al*., 2021; Ruiz and Silhavy, 2022). Because of their pathogenic ability, antibiotic resistance, and possession of biofilms, the aim of this study is to diagnose biofilm genes (*CsgA*, *YjaA*) in *E. coli* isolates and antibiotic resistance.

2. METHOD

2.1. Bacterial sample collection

One hundred forty five samples were collected from different clinical sources, of different ages and both sexes, from several hospitals in the city of Baghdad, including: Baghdad Teaching Hospital, Educational Laboratories, Shaheed Ghazi Al-Hariri Hospital for Specialized Surgeries in the Medical City, Al-Imamin Al-Kazemin (PBUH) Teaching Hospital, Central Children's Teaching Hospital, and Al-Yarmouk Hospital . The study was conducted for the period from (6/11/2022) to (22/12/2022) at different clinical sources.

2.2. Isolation and identification of bacteria

The collected samples were cultured on blood agars, MacConkey agars and Eosin agars. Biochemical tests were also conducted (citrate Utilization, indole Prodution, to identify *E.coli* from other Enterbacteriacae bacteria (Mahdi and Abdullah., 2019), as well as Methyl red - Vogues Prosquer, Catalase, Glucose, Fermentation, Hemolysin and oxidase test,

The final diagnosis of the isolates was made by Vitek - 2 system compact

E.coli isolates were confirmed and recognized by the vitek-2 system (Mohamad, 2019; Hamady and Ibrahim, 2020) after that it was obtained (50) isolates of *E. coli* from different clinical sources.

2.3. Detection of biofilm formation

The ability of *E. coli* to form a biofilm was revealed using the microtiter plate method (96), as demonstrated by Bakhtiari and Javadmakoy (2017) and Fusco *et al.* (2017), the bacterial suspension was prepared using heart and brain infusion broth (BHI) with (3%) sucrose and compared with McFarland's standard solution. 100 µl of bacterial suspension was taken in 900 µl of heart and brain infusion medium, and compared with McFarland's solution. 200 µl of heart-brain infusion medium without bacterial suspension was added in the first column (negative control) with three replicates, and then placed in the first three holes of the second column by 200 µl of medium with the bacterial suspension (three replicates for each isolate). The method was then followed for all isolates until the last column after 24 hours. At a temperature of 37 °C, the contents of the holes were empty and gently rinsed three times with 200 µL of Normal Saline solution and left to dry at room temperature, then (200) µL of methanol alcohol was added for 15 minutes, after that it was poured and left to dry at room temperature, then 200 µL of crystal violet stain was added at a concentration of (1%) for 25 minutes, after that the stain was poured and washed with sterile distilled water. It was left to dry at room temperature, then 200 µL of 99% ethanol was added for 15 minutes, finally the optical density was read by an ELISA device at a wavelength of 630 nm.

2.4. Antibiotic resistance test

Disk spreading technology

Twelve isolates for susceptibility testing by using the disc diffusion technique to test the resistance and sensitivity of bacterial isolates to the following antibiotics: Amikacin, Meropenem, Levofloxacin, Imipenem, Gentamicin, Colistin, Cefotaxime, Ceftazidime and Ceftriaxone). After pure bacterial cultures of active *E. coli* isolates at the age of (24 hours) were prepared, the bacterial suspension was prepared and (3-5) bacterial colonies were transferred to tubes containing Normal Saline, with a good shake through a Vortex mixer, and then they were compared with the density of the MacFarland's solution, the standard turbidity of the standard. Its cell number is approximately (10^8×1.5 cells /mL), and then used Muller-Hinton Agar to complete this test, where some of the bacterial suspension was spread on Muller's medium using sterile cotton swabs by immersing the cotton swab inside the tubes that contain the suspension of bacteria, then it is lifted and constrained well on the inner wall of the tube to get rid of the excess bacterial suspension, after that it is planned on the surface of Mulleranton by the method of brushing the mat, then the dishes are left (10 minutes) to dry at the laboratory temperature. Tablets are placed antibiotics were placed at equal distances, the four discs were placed in each plate, and they were incubated at a temperature of 37 °C for 24 hours. After that, the inhibition zone that appears around each disc was measured in millimeters, and the result was compared using the international standard tables (CLSI, 2022).

DNA extraction

Genomic DNA was extracted from the preserved bacterial isolates (12 *E. coli* isolates) according to the company's protocol and using a DNA kit (ZYMO RESEARCH).

The primers used in the interaction

The stock solution of primers was ready as per the guidelines of the manufacturer (company Canada), as referenced in Table (1) by utilizing sterile distilled deionized water to get a concentration of 100 pmol/µl. The solution of every initiator was available independently at 10 pmol/µl by adding 10µl from each stock solution to 90 µl of distilled water and mixed well with Vortex mixture, then keeping with stock solutions at 20°C and

mixing the initiator solution after eliminating it from ice by utilizing the Vortex carburetor prior.

Table 1. The sequence and source of the gene primers utilized in the study

Gene name and primer		Primer sequence(5`—3`)	Product size (base pair)	References
csgA	F	GCAATCGTATTCTCCGGTAG	418	Olowe *et al.* (2019)
	R	GATGAGCGGTCGCGTTGTTA		
yjaA	F	TGAAGTGTCAGGAGACGCTG	211	Olowe *et al.* (2019)
	R	ATGGAGAATGCGTTCCTCAAC		

The contents of the PCR tubes were mixed well utilizing the Vortex then sited in a PCR thermal cycler, as shown in Table (2).

Table 2. Optimal conditions of PCR reaction for determination of *CsgA* and *YjaA* genes.

No.	Phase	Tm (°C)	Time	No. of cycle
1	Initial Denaturation	94°C	5 min.	1 cycle
2	Denaturation -2	94°C	30 sec.	35 cycle
3	Annealing	60°C	30 sec.	
4	Extension-1	72°C	1 min.	
5	Extension -2	72°C	7 min.	1 cycle

Agarose gel electrophoresis

It was ready according to Sambrook *et al*. (1989) method as follows; Dissolve (1.5%) grams of agarose gel in (100 ml) of TBE Buffer buffer solution at a concentration of 1x and microwave at a temperature of (60°C) for a period of 15 minutes, then let the gel cool to a temperature (45-50)°C and then added 5 microliters of red stain and mix well with the gel. Pour the agarose gel into the tray after fixing the combs, then leaved to solidify at room temperature for 30 minutes, after that the combs were prudently detached from the gel to obtain the pits.

3. RESULT AND DISSCUSION

3.1. Isolation identification:

50 (34%) isolates of *E.coli* were obtained from different clinical cases (urinary tract infections (72%), wounds (14%), respiratory tract infections(2%), sputum (10%), swab from the vagina (2%), then 12 isolates were taken for biofilm, antibiotic and molecular diagnosis (Table 3).

Table 3. Number of *E.coli* isolates for each source.

Isolate source	Number of isolate	Percentage
Urinary tract infections	4	33.3%
Wounds	3	25%
Sputum	3	25%
Respiratory tract swab	1	8.3%
Vagina swab	1	8.3%
Total	12	

3.2. Microscopic examination

It was used on all (50) isolates after staining them with a gram stain, so the result was negative rods. Other tests were also conducted on *E. coli* to confirm them on the 12 isolates so the results of the biochemical tests were negative on the 12 isolate in the test oxidase, urea, Vogues-proskauer, citrate, and gram stain. The biochemical tests were positive tests on 12 isolate tests are catalase, indole and methyl red. The positive and negative results are consistent with the results of Al-Badry (2020).

The ability of *E.coli isolates to form a biofilm:*

The results of biofilm detection for 12 isolates are presented by (100%) of the bacterial isolates formed the biofilm in different degrees. After comparing it to the negative control, the results showed one isolate with a percentage of (8.3%) was medium and 11 with a percentage of (91.6%)isolates were weak, as shown in Figure (5). The consequences of this study were agree with Poursina *et al*. (2018) in Iran, as well as in line with Boroumand *et al*. (2019), but dis agreed with Ahmed (2021) study because it showed the high mean percentage and the weak percentage very low in this study. Also, the current study is not consistent with Noori and Mohamad (2023) study, but the current study agrees with Al-Saadi (2019) because the results showed weak biofilm formation is the highest.

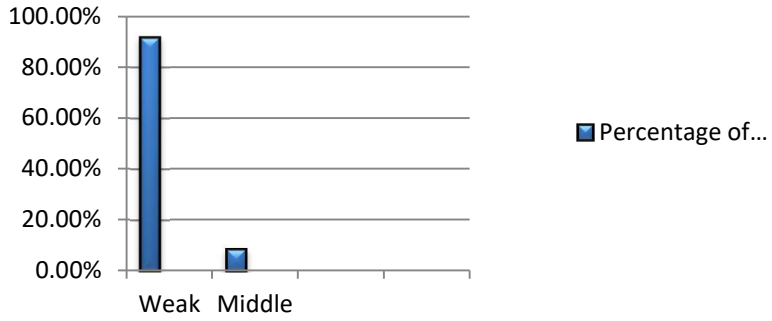

Figure 1. Percentage of *E.coli* isolates on biofilm formation for 11 isolates are presented by (91.6%) isolates were weak and one isolate presented (8.3%) was a medium formation biofilm.

3.3. Antibiotic results

Susceptibility testing of 12 *E.coli* isolates against 8 antibiotics was studied using the Disk Diffusion method with Agar Mueller Hinton medium. These antibiotics include: Levofloxacin, Meropenem, Ceftazidime, Colistin, Imipenem, Gentamicin. The consequences were verified by calculating the diameters of inhibition in millimeters (mm) surrounding the using antibiotic disc, and compared to the standard inhibition areas found in the Clinical and Laboratory Standards Institute (CLSI). The results of the recent study displayed resistance of the tested isolates to antibiotics (100%) each of ceftazidime, colistin and ceftriaxone, (25%) to imipenem, (0%) to meropenem, while gentamicin, amikacin and levofloxacin recorded (8%, 50%, 58%) respectively as shown in the Figure (2).

In the current study, *E. coli* isolates displayed a resistance percentage of (50%) to Amikacin antibiotic and sensitivity (8%). This antibiotic belongs to the group of aminoglycosides. The results of Briongos-Figuero *et al*. (2012) showed (3.33%) and this less resistance than the current study. Whereas, the reason for the resistance of Gram-negative bacteria is belong to the modified enzymes of the aminoglycosidic group have encoded plasmids or a change in the target site, whereas it represents the small ribosomal unit (30S), on which the antibiotic acts (Katzung, 2001). Ceftriaxone is one of the cephalosporin antibiotics, and it is from the third generation. The results of the current study were 100% and this disagreed with Abujnah *et al*. (2015) study. These results are consistent Raoof (2021) study which was found that the percentage of resistance (6.7%), as well as Mawlood *et al*. (2018) study was agreement with the current study. The cause for the high resistance of *E.coli* isolates to cephalosporins and penicillinases is attributed to the ability of bacteria produce enzymes (β-Lactamase) (Ibrahimagic *et a*l., 2017). The results of the current study displayed a high rate of resistance to (Colistin) (100%), and this agree with the results of Al Azad *et al*. (2019) who recorded the resistance rate (100%), while Armin *et al*. (2023) showed the resistance percentage (59.2%) and the sensitivity percentage (40.8%) and this did not agree with the results of the recent study, as well as the results of a study in Bangladesh that did not agree with the results of the present study, which recorded the sensitivity percentage (73.53%) to Colistin antibiotic (Makhol *et al*., 2011). Colistin is a polycationic antibiotic and it treats gastrointestinal infections caused by Gram-negative Enterobacteriaceae. The reason for its high resistance is chromosomal mutations by resistant plasmids. In the current study, *E.coli* isolates displayed a percentage of resistance to Ceftazidime antibiotic 100%. This antibiotic belongs to the group of the third generation of cephalosporins. The

result of current study is close to Suresh *et al*. (2016) in India by the percentage of resistance to Ceftazidime antibiotic 98%. Also, results were shown to the results of Al-Badry (2020) in Iraq by the percentage of resistance the isolates to this antibiotic reached (64%). The results of the current study disagree with Maleki *et al*. (2017) who recoded the percentage of (6.8%). As for the results of the study, the Gentamicin antibiotic, which belongs to the group of aminoglycosides, has a resistance percentage of (8%) and a sensitivity of (92%, 68%) and this study showed the weak percentage of resistance. This is due to the fact that *E.coli* bacteria have very weak virulence factors, so that they cannot resist the killing effect of antibiotics. The results of the present study displayed resistance to (Imipenem) antibiotic by (25%) and sensitization by (75%). These results are agree with Badran *et al*. (2016) in Jordan, by the resistance percentage (22.5%), and the results of the recent study did not agree with the results of Al-Badry (2020), by the percentage of resistance results (4%), and these results are completely different from the results of the current study. The results of the current study were close to the sensitivity percentage of (75%) to Polse *et al*. (2016) study conducted in Zakho, by recorded all isolates sensitive to (Imipenem), which is from the group of (Carbapenemes).

The results of the recent study showed that Levofloxacin had a resistance percentage (58%) and a sensitivity percentage (25%). This antibiotic is a broad-spectrum from the third generation of the fluroquinolone group (Islam *et al*., 2022). According to Armin *et al*. (2023), the resistance percentage was (83.3%) and sensitivity (7.16%), as well as the result of Stone and Hackel (2022) recorded the resistance percentage (74%) and it is higher than the results of the current study (58%). The rises in resistance to fluoroquinolones have been associated with the use of levofloxacin. Meropenem, which is from the group of carbapenems and it is produced by carbapenemase enzymes, and considered one of the most essential mechanisms of resistance, because it encodes for mainly gene that is located on the plasmid, and it is an inhibitor of most βeta-lactams such as Cavulanic acid and these enzymes belong to the Enterobacteriaceae family. The results of the current study showed a resistance percentage (0%) and a sensitivity percentage (58%). The current study agrees with Raoof (2021) study which recorded the sensitivity percentage (100%), and it is considered a relatively modern antibiotic.

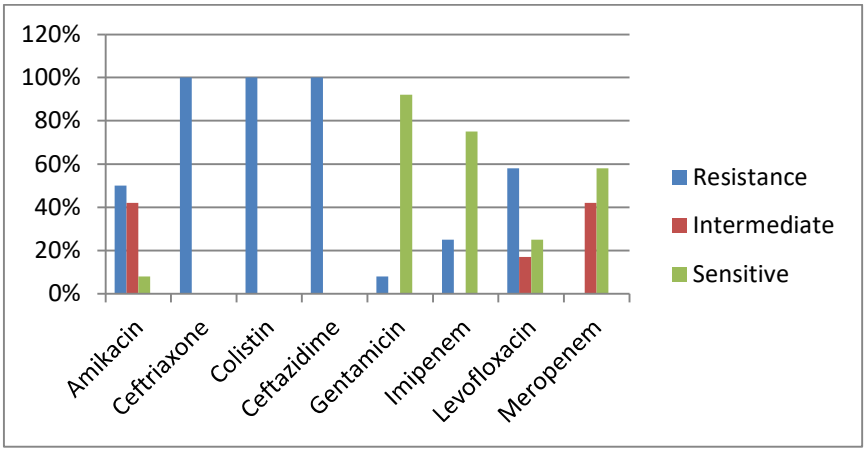

Figure 2. Percentage of *E. coli* resistance to antibiotics.

3.4. *Detection of the CsgA gene*

Analysis of PCR amplification products by gel electrophoresis revealed that the bands are 418 bp in size, 12 isolates of *E, coli* bacteria possess this bands (100%) as shown in Figure (2). These results of the recent study are agreed with Bakhtiari and Javadmakoei (2017) study which show similar results. In the current study, the results were (94.3%), as well as the results of Olowe *et al*. (2019) showed close to the results of the current study by (81.1%). Whereas *CsgA* is the major subunit of the twisted amyloid fibrils, and acts as a protein on the surface to form extracellular amyloid polymers, whereas (*CsgB*) found in the cell and aggregate upon nucleoprotein interaction, and this proving (*CsgA*) role in biofilm formation (Hammer *et al*., 2007).

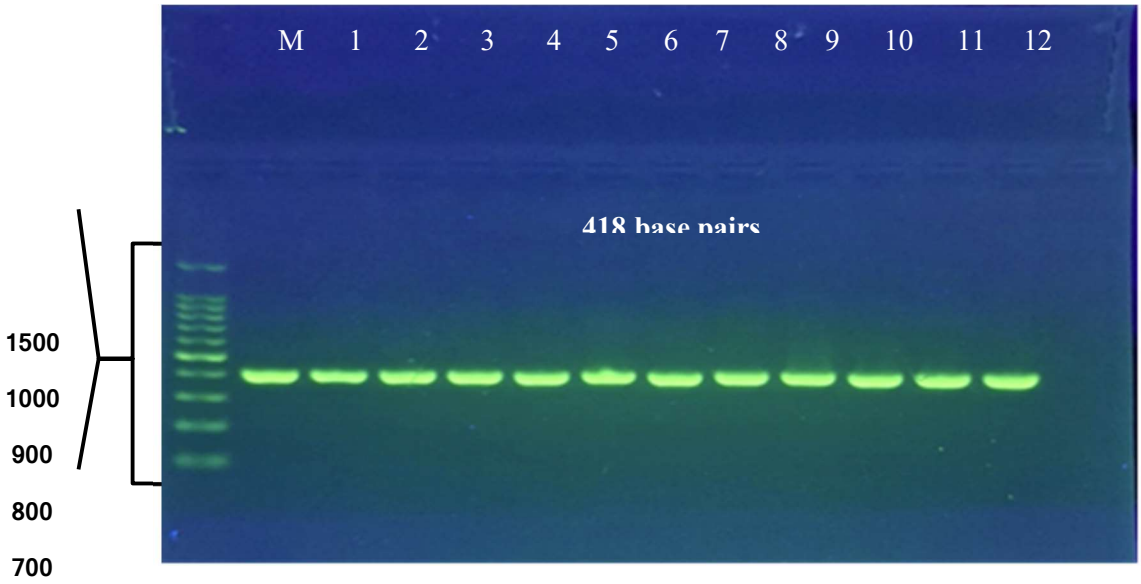

Figure 3. *CsgA* gene results for 12 isolates and PCR product of *E. coli* on (2%) agarose gel electrophoresis, (M) DNA ladder, (1500) bp of *E. coli* isolates (Olowe *et al.,* 2019).

Detection of YjaA gene

Analysis of the PCR amplification products in gel, electrophoresis found that the bands are of (211 bp) size. All isolates of *E. coli* have this gene (100%) as shown in Figure 3. The current study is consistent with Olowe *et al.* (2019) study. The results of the current study are also consistent with the results of the study in Iraq (Jremich and Al-Taei, 2020) and the current results are also consistent with the results of Hussain and Saleh (2019) study where the results appeared 100%. The *yjaA* gene was first identified in the recent whole genome sequencing of *E. coli* K-12 (Clermont *et al*., 2013; Jremich and Al-Taei, 2020). The results of a study Al-Hashmay *et al*. (2021) are consistent with the results of the current study, where the percentage appeared to be 100%. The results of a study in Iran (Ranjbar *et al*., 2020) are also consistent with the results of the current study, where the results showed 100% of the appearance of the *YjaA* gene.

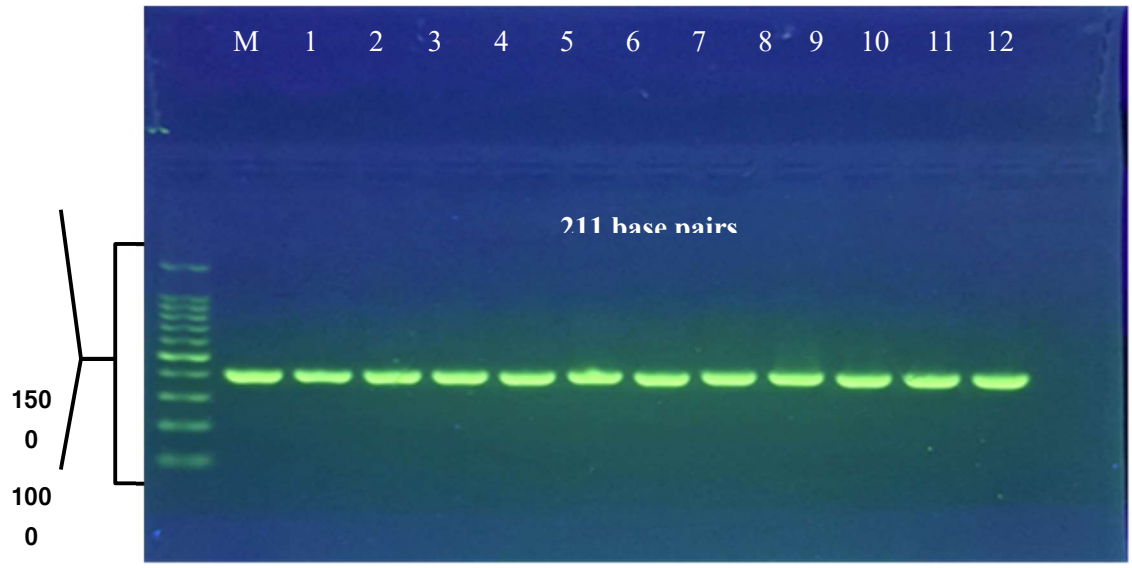

Figure 4. *YjaA* gene results for 12 isolates and PCR product of *E.coli* on (2%) agarose gel electrophoresis. (M) DNA ladder, 1500 bp from *E. coli isolates* for (Olowe *et al*., 2019).

4. CONCLUSION

The result of the biofilm showed 11 isolates with a rate of (91.6%), weak biofilm, and one isolate with medium biofilm, with a rate of (8.3%).the antibiotics that gave the highest resistance were (Cefazidime, Colistin and Ceftriaxone) at a rate of 100%. and the result of the *CsgA* gene for *E.coli* had 12 at a rate of (100%), and the result of the *YjaA* gene for *E. coli* had 12 at a rate of (100%).

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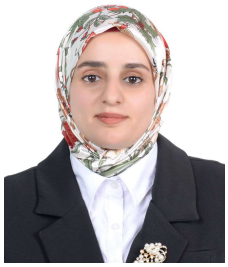

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