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ORIGINAL PAPER

Spread of ESβL-producing *Escherichia coli* and the anti-virulence effect of graphene nano-sheets

Sarah Naji Aziz[1] · Suhad Abbas Abid[1] · Shaymaa Khudhr Al-Alak[2] · Israa M. S. Al Kadmy[2] · Ahmed Mahdi Rheima[3]

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Abstract

Despite the studies worldwide, the prevalence of ESβL *E. coli* in the Iraq is still unknown. Realization of the demographic characterization of ESβL *E. coli* infections will assist the prevention efforts. This study aimed to isolate clinical *E. coli*, determine their antimicrobial susceptibility, phenotypic and genotypic detection of ESβL-producing ability, detection of some virulence-related genes, estimate the impact of graphene nano-sheets as antibacterial, and study the adherence-related gene expressions in *E. coli* isolates. Graphene nano-sheets were synthesized and characterized using XRD, UV, TEM, and SEM. *E. coli* isolates were identified using 16S rRNA. Antibiotic resistance was detected, virulence genes (*blaTEM*, *blaSHV*, *BlaCTX-M-15*, *papC*, and *fimH*) were screened using PCR. The antibacterial activity of graphene nano-sheets was screened using well-diffusion assay and MIC. The gene expression of adherence genes after treatment with graphene nano-sheets was evaluated using QRT-PCR. From a total of 512 identified using 16S rRNA, 359 (69.9%) were ESβL-producing *E. coli*. The ESβL genotypes positive were 83.56% (300/359) of *E. coli* isolates with the frequencies: 85% for *blaCTX-M* gene, 26% for *blaSHV* gene, and 28% for *blaTEM* gene. Graphene nano-sheets showed effective antibacterial activity with MIC 25 µg/ml. Furthermore, graphene nano-sheets reduced the expression of *papC*, and *fimH* genes. This study has helped us to better understand the characteristics of ESβL *E. coli*, their adherence gene harboring, and the potential ability of graphene nano-sheets to reduce bacterial growth, and the expression of adherence genes. Furthermore, the current study showed further step to understand the mechanisms by which graphene nano-sheets can conflict bacterial virulence and resistance.

Keywords *Escherichia coli* · ESβL · *papC* · *fimH* · Graphene nano-sheets

Introduction

One of the major predominant normal flora members in the human is *Escherichia coli* (*E. coli*), which can cause broad spectrum of infections. *E. coli* is a major pathogenic bacteria which are responsible for urinary tract infections (UTIs), accounting for greater than 80% of these infections (Qureshi and Doi 2014). The emergence of multi-drug resistant organisms is associated with the wide-spread use of empirical antibiotics in the hospital setting that causes serious opportunistic infections (Kareem et al. 2021). Many bacteria produce β-lactamases (cephalosporinases) to confer resistance to β-lactam antibiotic (Hammoudi Halat and Ayoub Moubareck 2020). A great threat has been posed after the arrival of extended-spectrum β-lactamases (ESβLs) on the usage of majority of the antibiotics classes, particularly cephalosporins. The β-lactamases production causes resistance to the antimicrobial agents, particularly in the gram-negative bacteria. As a consequence, numerous enzymes are mutated leading to the development of ESβLs in response to increased pressure of antibiotic use (Bahr et al. 2021). The majority of the affiliates of Enterobacteriaceae have acquired mutated plasmids that are capable of producing β-lactamases, whereby conferring β-lactam antibiotics resistance (Kumar and Saikumar 2021). Amongst those

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✉ Israa M. S. Al Kadmy
israaalkadmy@gmail.com; stsf@uomustansiriyah.edu.iq

[1] Branch of Microbiology, Department of Biology, College of Science, Mustansiriyah University, POX 10244, Baghdad, Iraq

[2] Branch of Biotechnology, Department of Biology, College of Science, Mustansiriyah University, POX 10244, Baghdad, Iraq

[3] Department of Chemistry, College of Science, Wasit University, Kut, Iraq

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members of Enterobacteriaceae, an increased prevalence of ESβL producers has been found to vary between different geographical areas (Padmini et al. 2017). ESβL producers are common in hospital settings; as a result, it is a major aggravating factor for hospitalization, and the outcomes would be seriously critical (Oteo et al. 2010).

Graphene is one of the carbon allotropes that includes charcoal, graphite and carbon nanotubes. It is a two-dimensional (2D) nano-structure consisting of sp2 carbons first discovered in 2004. Graphene has unique chemical, and physical properties such as optical, electrical, mechanical strength and large surface area in addition to the catalytic activity, so it has a potential application in biomedicine such as biosensors, cancer therapy drug delivery and antimicrobial activity. Graphene nanoparticles can be synthesized in a simple method such as electrochemical as mentioned in Hussain et al. (2020). Many studies have recently proved the biocidal properties of graphene nanoparticles and nanosheets toward microorganisms, including pathogenic bacteria (Ali et al. 2019; Ghanem et al. 2020; Aziz et al. 2021). Its bactericidal effect include physical mechanism induced by the sharp edge of graphene that leading to bacterial membrane damage. The second mechanism is the chemical effect that employs the charge transfer which leading to oxidative stress and ROS generation (Kumar et al. 2019). Despite the fact that *Enterobacteriaceae* have emerged as the most questionable pathogens of critical health care interest, little is known about these organisms virulence genes which contribute to their pathogenesis (Kareem et al. 2017).

In this study, we aimed to isolate *E. coli* from clinical samples which were procured from outpatients and inpatients wards from the diagnostic and research laboratory, determine their antimicrobial susceptibility, phenotypic and genotypic detection of ESβL-producing ability, detection of some virulence-related genes, estimate the impact of graphene nano-sheets as antibacterial, and study the adherence-related gene expressions in *E. coli* isolates.

Materials and methods

Bacterial collection and analysis

1000 clinical samples were collected from different hospitals in Iraq during 2019–2021. The routine microbiological examination was carried out for bacterial isolates. To confirm the identification phenotypically, were used conventional PCR was carried out to these species by using specific primers, for 16SrRNA gene which described by (Wang et al. 2002). The primer sequences are listed in Table 1.

Synthesis of graphene nano-sheets

The electrochemical cell was used to synthesize graphene nano-sheets. (Liu et al. 2019; Hussain et al. 2020). The graphite rods (6.5×75 mm) were used in the two electrodes figurs, cathode and anode. An aqueous electrolyte of 50 ml, 0.1 M H_2SO_4 with 50 ml, 0.1 M KOH solution was used to carray out the electrochemical study. They were washed in a series of steps employing aqueous and organic cleansers. (ethanol and de-ionized water) prior to laying the graphite rods in the cell. Each cleansing stage took 180 s.The D.C voltage was utilized. The voltage between electrodes was 6 V under a current density of 2.11×10^{-3} mA/cm^2 for 8 h. After obtaining a brown precipitate, the sample was extracted, bathed with deionized water, and left to dry for further processing. Reference (Rheima et al. 2019) describes the procedure for synthesizing and characterizing graphene nanosheets.

Table 1 List of primers or PCR amplification

Gene	Sequence	Product size (bp)	Annealing temperature (°C)	References
16S rRNA	CCCCCTGGACGAAGACTGAC ACCGCTGGCAACAAAGGATA	401	50	Wang et al. (2002)
blaTEM	GAGTATTCAACATTTCCGTGTC TAATCAGAGGCACCTATCTC	800	48	Al-Kadmy et al. (2015)
blaSHV	AAGATCCACTATCGCCAGCAG ATTCAGTTCCGTTTCCCAGCGG	200	59	Al-Kadmy et al. (2015)
BlaCTX-M-15	ATGTGCAGCACCAGTAAAGT ACCGCGATATCGTTGGTGG	542	53	Namaei et al. (2017)
papC	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	328	63 °C	Hetta et al. (2021)
fimH	TGC AGA ACG GAT AAG CCG TGG GCA GTC ACC TGC CCT CCG GTA	508	63 °C	Hetta et al. (2021)

Antimicrobial susceptibility test

Antimicrobial susceptibility test toward cefoxitin, cefepime, ceftazidime, cefotaxime, ertapenem, imipenem, amikacin, gentamicin, ciprofloxacin, and chloramphenicol was carried out by diffusion disks test (Kirby Bauer method). The results were compared with Clinical Laboratory Standards Institute (Clinical and Institute 2017).

Phenotypic detection of ESβL

Screening of *E. coli* ESβL was done based on the CLSI recommendations using double-disk diffusion methods including augmentin disk (amoxicillin/clavulanic acid) placed in the midst of plate. While ceftazidime and cefotaxime disks were placed at 3 cm from a central disk. The inhibition zones of the augmentin, cefotaxime, and ceftazidime disks were compared after 24 h of incubation at 37 °C. Overlapping between cefotaxime and augmentin disk was considered as EsβL-positive producer. *E. coli* ATCC 25922 was used as the negative control strain.

Genotypic detection of ESβL

PCR amplification for *blaTEM*, *blaSHV*, and *blaCTX-M*-type ESβL genes was carried out using the boiling method to separate genomic DNA of the bacterial isolates. The amplification system was (GeneAmp PCR System 9700) from (Applied Biosystems, Foster City, CA, USA). Primers were designed for the amplification are listed in Table 1. The results obtained from PCR were analyzed using agarose gel electrophoresis.

Detection of adherence virulence genes

PCR was used to identify adherence virulence genes such as *papC* and *fimH*. Table 1 shows the primer sequences and product sizes.

Detection of antibacterial activity of grapheme nano-sheets

The antibacterial activity of the grapheme nano-sheets was screened using the agar well-diffusion test. Few colonies of *E. coli* isolates from overnight cultures were transferred to a sterile test tube containing 5 mL of normal saline and completed to equal 10^8 colony-forming unit (CFU)/mL) and cultured on Mueller Hinton agar plates. On agar plates, 5 mm wells were made and loaded with 50 µg/mL graphene nano-sheets. However, normal saline was used as a negative control. Plates were then incubated at 37 °C for 24 h and the inhibition zone diameters were measured, then incubated in 37 °C for overnight to determine the inhibition zone in mm.

Determination of the minimum inhibitory concentration (MIC) of graphene nano-sheets

The MIC was employed using the 96-well microtiter plate for graphene nano-sheets (Jasim et al. 2020). Briefly, 100 µL of Mueller Hinton broth was added to microtiter plate wells, then each well of the first vertical row was loaded with 100 µL (10 mg/mL) of graphene nano-sheets followed by mixing 1:2 serial dilutions. 100 µL from the last well was discarded. After that, all wells (except negative control row A11–H11, which was filled only with MH broth) were filled with 5 µL of diluted suspension of *E. coli* isolates and then incubated for 24 h at 37 °C. Finally 10 µL of resazurin dye was putted on the wells, then left 4 h in incubation to be ready for reading; the actions of different concentrations of nanoparticles for the bacterial growth were detected using UV–visible spectrophotometer. The assay was carried out in triplicate.

Expression of adherence-related genes in the presence of graphene nano-sheets

Real-time RT-PCR was used to evaluate the effect of graphene nano-sheets by the gene expression of adherence-related genes (*papC* and *fimH* genes). TRIzol reagent (Sigma-Aldrich, Switzerland) was used to extract RNA from bacteria treated with graphene nano-sheets, and untreated bacteria served as controls. Following that, cDNA was synthesized using the Superscript III kit (Invitrogen Inc., USA) according to the manufacturer's instructions. PCR reactions were achieved in a 7500 Sequence Detection System using ePower SYBR Green PCR Master Mix (Applied Biosystems, USA) (CFX 96 bio-rad, USA). The fold change in the expression of the target genes was calculated using the $2^{-\Delta\Delta CT}$ method and 16S rRNA gene served as the normalizing gene (Livak and Schmittgen 2001). The level of expression of each gene in cells treated with graphene nano-sheets was compared to the expression of untreated bacteria as a control.

Data analysis

The statistical analysis for this study by (SPSS) version 21.0 was used to analyze the data. The *t*-test and Chi-square tests were used to examine continuous and categorical variables, respectively. The Fisher exact test was used to calculate the difference in drug resistance levels between ESβL-producing and non-ESβL-producing isolates. The data were analyzed at a rate of 95%. (p 0.05 indicates a confidence interval).

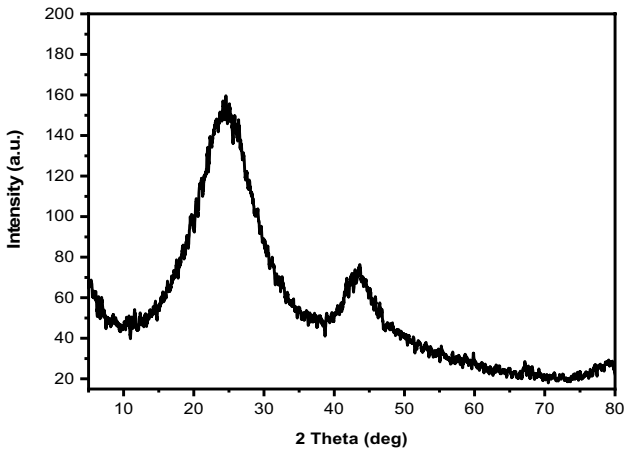

Fig. 1 XRD pattern of the graphene nano-sheets

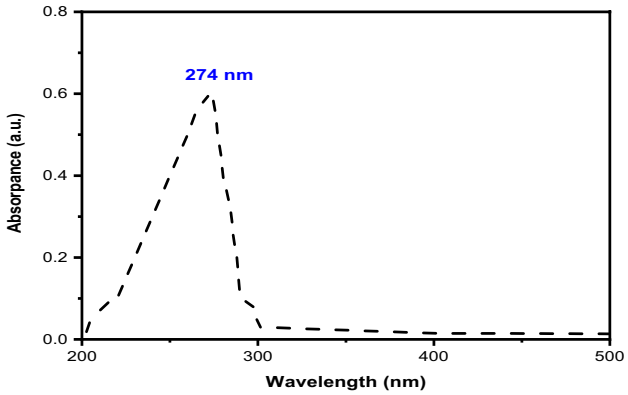

Fig. 2 The UV–visible spectrum of graphene nano-sheets

Results

Characterization of graphene nano-sheets

Powder XRD was used to analyze the produced graphene nano-sheet at diffraction 2θ range of 10°–80° as showed in Fig. 1. Diffraction peaks of graphene had adjusted about $2\theta = 26°$, and 42° corresponding to (002) and (100) was referred to as the structure of the carbon in graphene (Wang et al. 2020). The broad peak analogical to the (002) at $2\theta = 26°$ elucidates that the graphene nano-sheets were obtained. The broad X-ray hump obtained in the graphene reveals sp2carbon bonds in this electrolysis synthesized sample.

Figure 2 shows the UV–Vis absorption spectrum in the graphene nano-sheet with 0.5 mg/mL de-ionized ultrasonic dispersion for 15 min. In the UV–Vis spectra, a peak at 274 nm was observed, indicating $\pi \rightarrow \pi^*$transition of C–C bonds of the graphene sheet**.** It appears the graphene $\pi \rightarrow \pi^*$ network with the restoration of electronic conjugation of the sp2 hybridized carbon network (Selvakumari et al. 2020).

The nano-morphology of the synthesized graphene sheets can be analyzed with the TEM images shown in Fig. 3A. A significant number of scrolls and wrinkles appear on the sheet surface, and TEM images display a clear multi-layer formation. The graphene demonstrates multi-layered morphology. It says the layered graphene structure in sheet format**.** Figure 3B shows the SEM images of the synthesized graphene sheets show a layered structure that supplies ultrathin and homogeneous graphene films. The edges of the sheets of silk are crumbled and folded back. Hairy, as the surface of blurry arcs, are the graphene nano-sheets. It is made of a layered graphene sheet structure. The more layers that fit the XRD results are observed**.** The SEM images

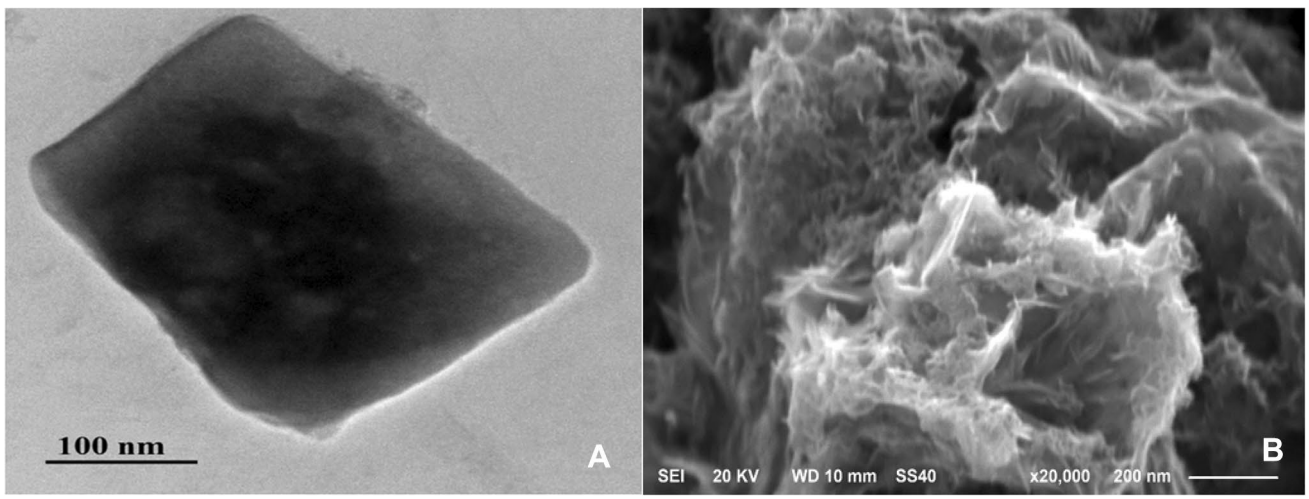

Fig. 3 Images of the synthesized graphene nano-sheets by **A** TEM and **B** SEM

Table 2 The demographic characteristics for the bacterial isolates

Specimen	ESβL-positive *E. coli* Number *N* percentage%	
Age (mean ± SD)	47 ± 17.5 (range 9–56 years)	
Female	412	80.1
Male	101	19.8
Children	116	22.41
Old age	399	77.58
History of interventions		
Nasogastric intubation	77	14.81
Intravenous line	318	62.18
Central venous line	10	2.14
Urinary catheters	218	42.3
Tracheotomy	9	1.75
Endotracheal intubation	24	4.39
Surgery	18	3.7

display a random orientation with a wavy look. The layered and flaky structures have a silky look, with the scale-like arrangement of nano-sheets (Selvakumari et al. 2020).

Bacterial demographic characterization and susceptibilities

Table 2 summarizes the demographic characteristics such as age, gender, reality, and any interventions. The average age of the total study participants was 9–56 years. The means ± SD age of patients with ESβL positive and negative was 47 ± 17.5 years and 47.5 ± 12.8 years, respectively ($p = 0.093$). Out of the 511 patients, 412 (80.1%) were females and 101 (19.8%) were males ($p = 0.0001$). The results have shown that the majority of patients were old and belonged to different Iraqi areas. During the research, a total of 511 *E. coli* were isolated. From those 511 positive cultures, 360 (69.9%) were extended-spectrum β-lactamase (ESβL) producing *E. coli* (Fig. 4). Males had a higher incidence of ESβL than females. Figure 5 depicts the frequency of extended-spectrum β-lactamase-producing *E. coli* isolates from various samples ($n = 359$). According to the findings, 64% of *E. coli* were resistant to amoxicillin and cefixime. Furthermore, a significant drop of 30–40% in susceptibility to all cephalosporins was observed. *E. coli*, on the other hand, demonstrated a disparate sensitivity rate to chloramphenicol, ofloxacin, gentamicin, and amikacin, with 94%, 98%, 97%, and 97%, respectively. Regarding the PCR detection of ESβL genotypes, it was discovered that all of the ESβL-positive *E. coli* isolates had one or more of the ESβL genes tested in the current study. Overall, one or more ESβL genes were found in 83.56% (300/359) of *E. coli* isolates. The PCR assay results revealed the following ESβL

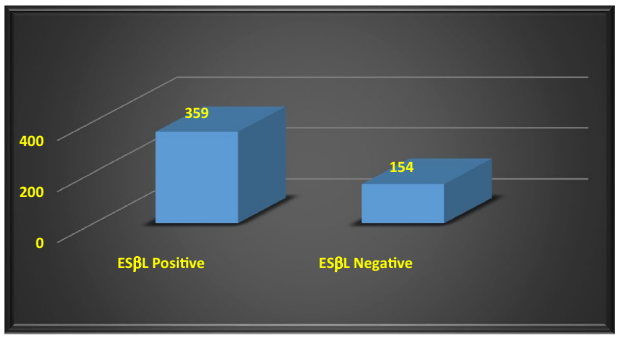

Fig. 4 Bar graph showing extended-spectrum β-lactamase-producing *E. coli*

genotype frequencies: 85% for the *blaCTX-M* gene, 26% for the *blaSHV* gene, and 28% for the *blaTEM* gene.

Data availability statement

The research data are not accessible because of institutional rules and regulation policy.

Antibacterial activity and MIC of graphene nano-sheets

The antibacterial activities of the graphene nano-sheets against ESβL-producing *E. coli* isolates has been investigated. The growth curve of *E. coli* isolates was determined in the presence of graphene nano-sheets or its absence as a control. The mean of inhibition zone for the graphene nano-sheets in the agar well-diffusion method was 15 mm in diameter as cleared in Fig. 6. Furthermore, the MIC demonstrate that graphene nano-sheets has high-antibacterial achievement mean values of 25 µg/mL. These results represent the remarkable enhanced antibacterial activity of the graphene nano-sheets.

Modulation of adherence-related genes expression

The expression of *fimH* and *papC* genes were measured with and without presence to graphene nano-sheets. In the presence of graphene nano-sheets, the expression of *fimH* and *papC* adhesion genes were significantly reduced by 4.6-fold ($p = 0.001$) and 3.4-fold ($p = 0.001$), respectively, as cleared in Table 3.

Discussion

The medical community depends on the published guidance and clinical expertise to help the physicians with different options in empirical therapy for the infections that

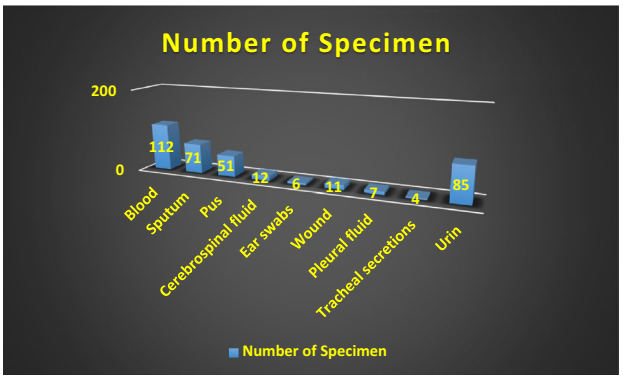

Fig. 5 Graph showing number of specimens of β-lactamase-producing *E. coli*

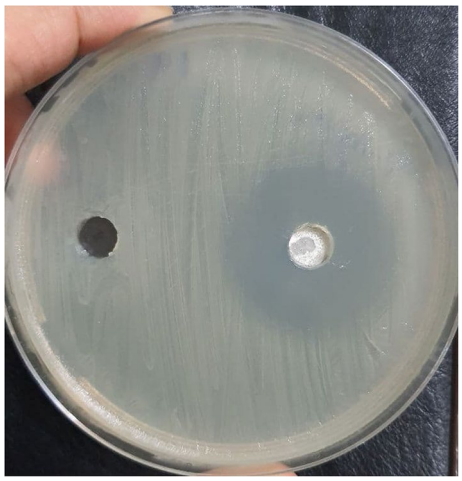

Fig. 6 Antibacterial activity in the agar well-diffusion method shows inhibition zone of G-sheet against ESβL-producing *E. coli* (in right) with normal saline as a control (in left)

Table 3 Gene expression of *fimH* and *papC* target genes of *E. coli*

Target gene	Control untreated bacteria	Graphene nano-sheets-treated bacteria	*p* value	Fold change
fimH	1 ± 0.156	0.22 ± 0.165	0.001*	4.6
papC	1 ± 0.234	0.29 ± 0.148	0.001*	3.4

are mostly based on system classification, including: urinary tract infections and community-acquired pneumonia. *E. coli* produce extended-spectrum β lactamases, which was firstly recognized from the late 1990s and appeared in the community settings as a significant reason of urinary tract infections (Pitout 2010). Extended spectrum β-lactamases are the major causes of βlactam antibiotics resistance, particularly in *E. coli*. The increasing frequency of the co-existence of ESβLs is a serious threat to successful treatment interventions of bacterial infections producing such defending enzymes (Adegoke et al. 2020; Nzima et al. 2020; Adekanmbi et al. 2020).

Gram-negative bacteria infections may be accompanied with the appearance of β-lactamase enzymes that play a major role in the spread of hospital infections, which represents a crucial concern. Some serious issues related to diagnosis and treatment have been concurrently occurred due to the prevalence of such strains that possess several resistance enzymes (Al-Kadmy et al. 2020; Marjani et al. 2020). Generally, AmpC β-lactamases and ESβL-producing isolates are susceptible to imipenem (Kuo et al. 2021). Ejaz et al. (2013) have reported a frequency of 68% for ESβL-producing gram-negative bacteria using the DDST method and a 100% using the CLSI method. These results altogether suggest that the method of detection might have an impact on the reported values as a result of the differences in the analytical sensitivity and specificity. Therefore, the need is pressing to give more attention to this kind of bacterial infections, and proper precautions should be taken to prevent the spread of them to other patients. ESβL-producing *E. coli* is a huge health concern, which is evolved primarily due to drug resistance, limited management options, increased mortality, and cost of management. In this context, most ESβLs evolved from genetic mutations in β classic lactamases (TEM-1, TEM-2 and SHV-1). These lactamases originated from the various ESβLs of TEM and SHV types (Liakopoulos et al. 2016). The present study has evaluated the frequency of extended-spectrum β-lactamases *E. coli* isolated from clinical samples in Iraq. The study has also documented an increased

frequency of extended-spectrum β-lactamase-producing *E. coli*. In the present study, the antimicrobial sensitivity arrays were detected in all isolates, and the results of the antimicrobial susceptibility test against *E. coli* revealed that isolated bacteria differed in their sensitivity to the tested antimicrobial drugs. Carbapenems are frequently the last choice of defense in treating infections caused by MDR Enterobacteriaceae (Elshamy and Aboshanab 2020; Zheng et al. 2021). Based on previous research, imipenem and meropenem, which have been identified as the most effective antibiotics against ESβL-producing isolates, demonstrated 100% sensitivity. This is an important finding from the current study because carbapenems can be used to treat a variety of infections. Our findings indicated that 33% and 35% of the ESβL-producing isolate were susceptible to cefotaxime and ceftazidime, respectively. We also observed that *blaTEM* and *blaSHV* genes were less common in our settings with 28% and 26%, respectively. In recent years, new approaches to antibacterial treatment have been developed, such as the combinations of antibiotics, and the formulations based on the antibacterial nanoparticles (Aziz et al. 2021; Mulani et al. 2019; Yeh et al. 2020; Hetta et al. 2021). Several studies have recently characterized the enhanced antimicrobial activity of graphene nano-sheets as a topically applied treatment versus pathogenic microorganisms. The roles of graphene nano-sheets against bacteria, including ESβL-producing isolates based on the direct action of graphene nano-sheets on the bacterial cell membrane because of the crystalline sharping corners and edges. Importantly, the current study considering the first in Iraq that proves the efficiency of graphene nano-sheets to reduce the gene expression of some adherence -related genes (*fimH* and *papC*). Our findings have demonstrated that 25 µg/mL of graphene nano-sheets significantly reduced the expression of adherence-related genes. However, further researches are recommended to evaluate the drug resistant patterns of bacterial isolates using rapid molecular diagnostic methods that are essential for effective clinical management. Moreover, continuous epidemiological surveillance is mandatory as well as necessary for the control of drug resistant *E. coli*. Finally, the inability of in vivo experiments are the limitations for the current study, which we recommend in future researches.

Conclusion

The current study has helped us to better understand the characteristics of ESβL-producing *E. coli*. The analysis of the isolates proved their higher antibiotics resistance, harboring a sheaf of adherence-related genes. On the other hand, the current study concluded the potential ability of graphene nano-sheets to reduce bacterial growth, multiplication, and the expression of adherence-related genes. To the best of our knowledge, few studies in Iraq showed the activity of graphene nano-sheets in inhibiting ESβL-producing *E. coli* isolates in vitro. Furthermore, the current study showed further step to understand the mechanisms by which graphene nano-sheets can conflict the bacterial virulence and resistance. However, further studies are necessary to understand the mechanisms by which graphene nano-sheets interfere with the microbial spread and resistance.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval The Ethics Committee of the Mustansiriyah University approved and oversaw this study.

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