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# Antibiogram and Molecular Detection of *Klebsiella pneumoniae* Biofilm-Forming Genes Isolated From Meat Products in Qalyubia Governorate, Egypt

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**Abstract**

INCIDENCE of foodborne pathogens in meat and meat products poses significant health risks to humans and results in financial losses for producers due to market recalls. This study aimed to isolate and identify of pathogenic *Klebsiella pneumoniae* from meat and its products by using conventional culture and biochemical characters. In addition, molecular technique was applied for detection of the isolated *Klebsiella pneumoniae* using *gapA* gene. Overall, total of one hundred (n=100) random samples of beef products including 10 from each {minced meat, kofta, sausage, luncheon, burger, bastrma, frankfurter, liver, chicken and shawarma} were collected from Benha markets in Qalubia Governorate, Egypt, for bacteriological examination**.** The antimicrobial resistances were investigated against 7 different classes of antimicrobial groups. The results showed that incidence of isolated from meat products were 10 isolates *Klebsiella.* The antimicrobial sensitivity test revealed that high sensitivity to imipenem and gentamicin and high resistance to cefotaxime, levofloxacin, amoxicillin & clavulanic acid, erythromycin and trimethoprime & sulfonamide. Further, molecular identification of *gap*A gene revealed that 5 *Klebsiella pneumoniae* at about 391 bp. Also, screening of 3 virulence genes *(mrk*D, *uge*, *lux*S) of 5 *K. pneumoniae (*n=5*)* which responsible for biofilm production showed the positive result at (226pb, 535pb, 477pb) for the examined strains. Finally; this study concluded that meat products have significant health hazards due to bad sanitary quality, increase the load of pathogenic Klebsiella and the presence of multidrug resistant (MDR).

**Keywords:** Food preservation, Minced meat, Nanoemulsion.

## Introduction

A significant opportunistic bacterium called *Klebsiella pneumoniae* causes a range of infectious illnesses in humans, such as pneumonia, diarrhea, septicemia, and liver abscesses [1]. The hospital-acquired infection is widely recognized and has been linked to elevated rates of patient morbidity and mortality. [2]. Apart from clinical settings, *K.pneumoniae* has been identified as a significant food-borne pathogen and is commonly detected in foods such as meat, raw vegetables, powdered infant formula, fish, and street foods [3] . There have been numerous instances of outbreaks in food caused by *K. pneumoniae* in various countries in recent years [4].

For most people around the world, meat is the primary source of protein and essential vitamins, playing a crucial role in supporting daily activities as well as the growth, repair, and maintenance of body cells. Despite its nutritious significance, fresh meat is extremely susceptible to contamination. Consuming contaminated meat can lead to illnesses or even death. Both developed and developing nations indicate that food-borne illnesses may impact at least 10% of the population [5].

The convenience of meat and meat products, such as minced beef, contributes to their popularity. However, due to their large exposed surface area, these products degrade quickly, resulting in a short shelf life. Such factors, as the meat's composition, the hygiene of cutting, grinding, and preparation processes, and the storage conditions all play a role in how rapidly the meat spoils. The main drivers of meat spoilage are microbial contamination and growth, which significantly affect both the safety and quality of the product [6].

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Biofilms create conditions that physically shield cells from harsh environmental factors, antimicrobials, and immune system components, leading to many chronic infections, especially those linked to implanted medical devices [7]. In these sessile communities, where bacteria live in close proximity, there are enhanced opportunities for chemical signaling and gene transfer between bacterial cells of the same or different species [8] .This genetic mechanism is a key factor driving the rise in antibiotic-resistant phenotypes and associated infections [9].

Several genes contribute to the virulence of *Klebsiella pneumoniae,* including fimbrial and non-fimbrial adhesion genes such as the *mrkD* gene. *mrkD* is thought to function as a type 3 fimbrial adhesin, playing a role in biofilm formation in *K.pneumoniae.* Biofilm formation is a critical virulence factor, as it enhances the pathogen's ability to cause chronic infections [10]. Lipopolysaccharides (LPS) play a vital part in the dissemination of pathogens via bloodstream, contributing to sepsis, and they discuss the protection of *Klebsiella pneumoniae* against bactericidal effects and phagocytosis of human serum [11]. A key gene involved in the production of the LPS core oligosaccharide is the gene, encoded the enzyme uridine diphosphate (UDP) galacturonate 4-epimerase [12]. This enzyme converts uridine diphosphate glucuronic acid (UDP-GlnA) into uridine diphosphate galacturonic acid (UDP-GalA). GalA serves as the first outer core LPS residue in K.pneumoniae, which is vital in maintaining LPS structural stability [12]. The absence of the uge gene results in K. pneumoniae being entirely avirulent in models of infection, due to a truncated core oligosaccharide at the GalA residue [12]. This highlights the essential role of the uge gene in the LPS biosynthesis pathway.

The enzyme luxS produces autoinducer-2 (AI-2), a quorum sensing (QS) signaling molecule [13]. The luxS/AI-2 QS system controls a variety of bacterial properties, including surface attachment, biofilm formation, motility, and pathogenicity [14]. However, the relationship between this gene and many physiological activities in Klebsiella pneumoniae, particularly in bacteria with different mucoid phenotypes, is not well characterized. More outstandingly, QS is identified to detect cell density in order to govern bacterial biological processes [15], but the specific regulatory mechanism by which luxS interacts with cell density in K. pneumoniae strains with varying mucoid phenotypes still unidentified.

The study's goal was to record the incidence and antimicrobial resistance patterns of *Klebsiella* isolates and assesses biofilm production ability genotypically by detecting *mrkD, luxS and uge* genes.

## Material and Methods

### *Sampling*

A total of 100 frozen beef product samples, packaged in plastic, were collected from Benha markets in Qalubia Governorate, Egypt. The samples included 10 each of minced meat, kofta, sausage, luncheon, burger, bastrma, frankfurter, liver, chicken, and Shawarma. They were kept in ice bags and promptly transported to the Microbiology Laboratory for bacteriological examination. Ten grams (10 g) of each beef product sample were aseptically weighed and placed into a sterile homogenizer flask containing 90 mL of 0.1% sterile peptone water. The mixture was homogenized at 14,000 rpm for 2.5 minutes and then left to stand at room temperature for approximately five minutes. A 10-fold serial dilution of the homogenate was prepared, extending up to $10^{-6}$ from the initial 1:10 dilution. These dilutions were used for microbiological examination [16].

### *Isolation and identification*

The isolation of Klebsiella adapted by culturing on (Nutrient broth, Nutrient agar, MacConkey,s agar and Eosin Methylene Blue agar media then identified by using Gram staining and motility test further, biochemically (oxidase test sugar fermentation test ,Indole test , Methyl red (MR) test ,Voges proskauer (VP) test, Citrate utilization test, urea test, Lysine decarboxylase test and H2S test) . (HiMedia-India) [17].

### *Antimicrobial susceptibility testing* [17]

Mueller-Hinton agar (MHA) (HiMedia-India) was tested for antimicrobial sensitivity using the Kirby-Bauer disk diffusion technique. The test used seven antimicrobial disks: cefotaxime (CTX, 30 µg/disk), amoxicillin/clavulanic acid (AMC, 20/10 µg/disk), imipenem (IPM, 10 µg/disk), gentamicin (CN, 10 µg/disk), erythomycin (E, 15 µg/disk), levofloxacin (LEV, 5 µg/disk), and trimethoprime/sulfamethoxazole (COT, 1.25/23.75). Bacterial zone sizes were compared with the Clinical and Laboratory criteria Institute's resistance and sensitivity criteria [18].

### *Molecular identification of Klebsiella pneumoniae isolates*

### *DNA extraction*

The QIAamp DNA Mini kit (Qiagen Germany GmbH) was used to extract DNA from bacterial cultures, according to the manufacturer's instructions.

### *Polymerase Chain Reaction (PCR)*

Using a GS-96 gradient thermocycler (Hercuvan, Malaysia), specific primers prepared by [19] were employed in a partial amplification of the gapA gene

to identify K. pneumoniae at the molecular level. The reaction volume was 25 µl and included 12.5 µl of 2x COSMO PCR RED Master Mix (Cat. W1020300X, Willofort Co., UK), 1 µl of target DNA, and 0.5 µl (10 µM) of gapA forward primer.5/ AAGCATTGTTCACCTCTTCG3. And gapA reverse primer 5/GTGATGGGCGTTAATGAGAG3/. The initial stage in the PCR process was denaturation. We employed partial amplification of the gapA gene. for two minutes at 95 degrees, followed by 35 cycles of denaturation (30 seconds at 95 degrees), annealing (30 seconds at 56 degrees), extension (45 seconds at 72 degrees), and final extension (7 minutes at 72 degrees). The PCR amplicons were validated by putting them onto a 1.5% agarose gel containing 100 bp and performing the electrophoresis for 30 minutes at 120 volts. Finally, the Ethidium bromide-stained DNA bands were visible under a UV lamp using the InGenius3 gel documentation system (Syngene, UK), and gel images were obtained.

*Molecular identification of virulence genes for Klebsiella pneumoniae strains*

For identifying the biofilm formation genes (mrkD, luxS, uge). The SimpliAmp™ Thermal Cycler (Cat. No. A24811, Applied Biosystems, USA) was used to execute PCR reactions in a final volume of 25µl. The reaction mixture included 12.5µl of 2× EasyTaq® PCR SuperMix (Cat. AS111-01/11, Trans Co., China), 0.5µl (10 µM) of each primer, and 2µl of target DNA. The PCR results were separated by electrophoresis on 1.5% agarose gel, photographed, and evaluated using the Syngene InGenius3 gel documentation system (UK). Table 1 shows the primers used and the cycle conditions.

## Results

The incidence of Klebsiella isolated from meat products samples:

Based on cultural, staining, and biochemical characteristics *Klebsiella* appears large, circular, mucoid and pink colonies on MacConkey agar due to lactose fermented and large, mucoid and pink to purple colonies on EMB media. Upon Gram staining show red short rods, non-motile positive (lysine, urea, Simmons citrate, Voges prokour) tests and negative (oxidase, indole, TSI )tests biochemically .

The incidence of *Klebsiella pneumoniae* in examined samples of meat products is (10/100) 10% in meat products as shown in figure (1).

*Antimicrobial sensitivity*

Antimicrobial sensitivity testing on K. pneumoniae isolates indicated whether they were susceptible, intermediate, or resistant. All isolates were evaluated for antibiotic resistance to seven distinct types of antibiotics., and it was discovered that Klebsiella was resistant to Amc 90%, E 100%, CTX 90%, LEV 100%, COT 90%, sensitive to IPM 100%,CN 80%, and intermediate to Amc 10%, CN 20%, COT 10%, CTX 10%, E-coli resistance to Amc 80%, CTX 100%, LEV 90%, E 90%, COT 100%, sensitive to CN 100%, IPM 90%, and intermediate to Amc 20%, IPM 10%, E 10%, LEV 10%. The presence of MD poses major health concerns.

*Molecular detection*

PCR revealed 5 strains of *K .pneumoniae* at *gapA* gene (391bp) at agarose gel electrophoresis.

In terms of biofilm generation genes, all five K.pneumonae isolates tested positive for PCR targeting virulence genes mrkD, lux, and uge (Figs. 3, 4, and 5), with specific PCR products of 226bp, 477bp, and 535bp, respectively.

## Discussion

*Klebsiella Pneumoniae* is an opportunistic bacteria associated with community-acquired and healthcare-associated infections, such as bacteremia, pneumonia, and urinary tract infections (UTIs) [22]. Two key mechanisms contributing to multidrug resistance (MDR) in *K. Pneumoniae* are efflux pump systems and biofilm formation [23]. Identifying the prevalence of MDR strains and biofilm formation in *K. Pneumoniae* isolates can improve infection management and guide the development of new preventive strategies

In the current study the occurrence of *Klebsiella* was 10/100 (10%) in a total, while [24] isolated *Klebsiella* spp with an incidence of (12%) on another side [25] showed a higher rate (*Klebsiella* spp. (33.33%).

Our results also included occurrence of *Klebsiella* in kofta (20%), sausage (10%) and minced meat (20%), which was higher than findings of [26] who recoded presence of *Klebsiella* in kofta (16%), sausage (4%) and shawerma (12%).

[27] had isolated *Klebsiella* spp from sausage in incidence of (47.23%). Further, [28] has isolated *Klebsiella* spp from sausage and basterma samples in incidence of (20%), [29] isolated 24% of *Klebsiella oxytoca*, and these are higher than the data reached from the present work.

The widespread use of antimicrobials has resulted in a significant rate of resistance in K. pneumoniae [3]. Our study's food isolates were highly resistant to levofloxacin, cefotaxime, amoxicillin/clavulanic acid, erythromycin, and sulfonamide/trimethoprime, as well as very sensitive to gentamycin and carbeneme. These findings were similar with recent data from [30], which showed strong resistance to ampicillin. Resistance to piperacillin, cephalosporin, streptomycin, and tetracycline was also widespread. [31] discovered that 47% of K. pneumoniae isolated from shrimp were resistant to trimethoprim/sulfamethaxazole and chloramphenicol.

*GapA* gene act as one of the house keeping genes for identifying *K. pneumoniae* strains using Multilocus sequence typing (MLST) to get its subtypes [32] . In our study; PCR revealed 5 strains of *K. Pneumoniae* at *gapA* gene (391bp) at agarose gel electrophoresis.

Klebsiella species produce chromosomally encoded β-lactamases, including penicillinases, resulting in natural resistance to penicillins and certain cephalosporins (Arakawa, 2020; [33]). Similarly, researchers showed that biofilm formation in clinical Klebsiella pneumoniae is regulated by certain genes, including type I fimbriae (fimA and fimH) and type III fimbriae (mrkD and mrkA) [34].

Type 3 fimbrial adhesins can facilitate K. Pneumoniae attachment to a variety of human cells, including endothelium cells, epithelial cells of the respiratory and urinary tracts. Fimbrial-related gene (mrkD) plays a vital role in the microorganism's attachment to collagen molecules. The capacity of a microbe to create biofilm is an essential virulence factor, and these biofilms are the primary cause of many chronic illnesses [10].

Type 1 fimbriae (fimH-1) and Type 3 fimbrial adhesion (mrkA and mrkD) are the most frequent bacterium cell adhesive agents that induce K. pneumoniae to connect to urinary epithelial and endothelial cells, resulting in urinary tract infection [35].

In this study, the PCR assay showed a 100% prevalence of the *mrk*D gene in the isolates, which aligns with the findings of [36]. However, [37] reported lower prevalence rates of the *mrk* D gene.

In the current investigation, the mrkD gene was found in all isolates (100%), which is consistent with [38] (100%), [39] (92.2%), and [40] (83.0%). On the other hand, [41] reported a low frequency of the mrkD gene at (18%), whereas [42] observed (0%).

Previous animal studies have shown that *K. pneumoniae* strains with the uge gene are more virulent [12].

The current results revealed detection of *Uge* gene in all examined isolates. The occurrence of *uge* gene in *K. pneumoniae* has varied broadly (41.6% to 96%) between different authors: [43] who found (96%), [44] who reported (93.75%) from clinical isolates, [45] who found (86%) from clinical samples, [46] who found (62.5%), [30] who said (56.5%) from food and [42] who reported (41.6%) from clinical isolates.

Bacterial properties such as surface attachment, biofilm formation, motility, and virulence are controlled by LuxS/AI-2 quorum sensing system [14].

The LuxS/AI-2 system is recognized as an essential quorum sensing pathway that regulates a range of physiological activities, including bacterial growth, biofilm development, virulence, and metabolic functions across various bacterial species [47].

Analysis of biofilm-related genes demonstrated that the *luxS* gene was recorded in all (100%) of isolates, consistent with findings by [48] who revealed (98%) occurrence in clinical isolates.

*Conclusion*

Our findings revealed that the beef samples under investigation were either unsuitable for consumption or pose a potential health risk. This is attributed to the presence of bacteria that not only accelerate meat spoilage but can also cause foodborne illnesses if ingested. Effective prevention and control of such infections require proper handling of animal products and responsible use of antimicrobials. Furthermore, environmental factors and nutrient availability play a crucial role in bacterial biofilm formation, polysaccharide production, and metabolic activity, highlighting the importance of rigorous safety protocols.

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*Declaration of Conflict of Interest*

The authors state that there is no conflict of interests.

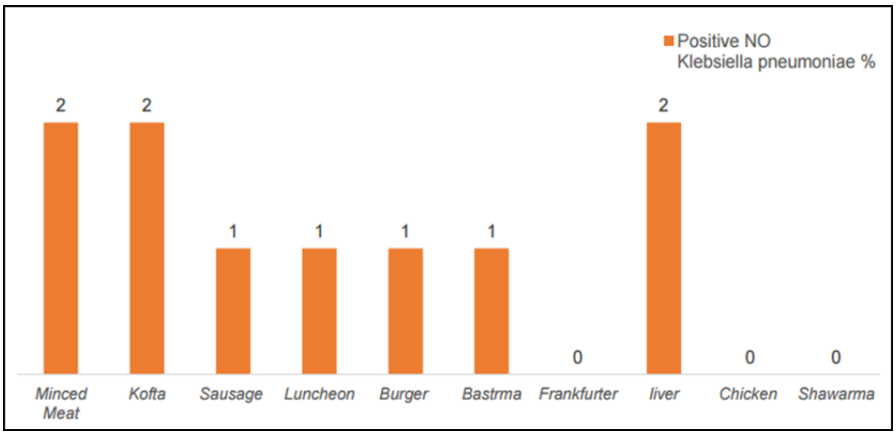


**Fig.1. Incidence of *Klebsiella pneumoniae* in the examined samples of meat products.**

**TABLE 1. Cycling conditions for the detection of genes& PCR primers.**

| Gene | Init.Denat. | Denat. | Anneal. | Extention | Final Ext. | Cycles | Sequence (5´-3´) | Amplicon size (bp) | Refrence |
|---|---|---|---|---|---|---|---|---|---|
| ***mrkD*** | 95°C 3min | 95°C 30sec | 54°C 30sec | 72°C 45sec | 72°C 10min | 35 | CCACCAACTATTCCCTCGAA ATGGAACCCACATCGACATT | 226bp | 20 |
| ***luxS*** | 95°C 3min | 95°C 30sec | 53°C 30sec | 72°C 45sec | 72°C 10min | 35 | GCC GTT GTT AGA TAG TTT CACAG CAG TTC GTC GTT GCT GTT GATG | 477bp | 21 |
| ***uge*** | 95°C 3min | 95°C 30sec | 55°C 30sec | 72°C 45sec | 72°C 10min | 35 | TCT TCA CGC CTT CCT TCA CT GAT CAT CCG GTC TCC CTG TA | 535bp | 20 |

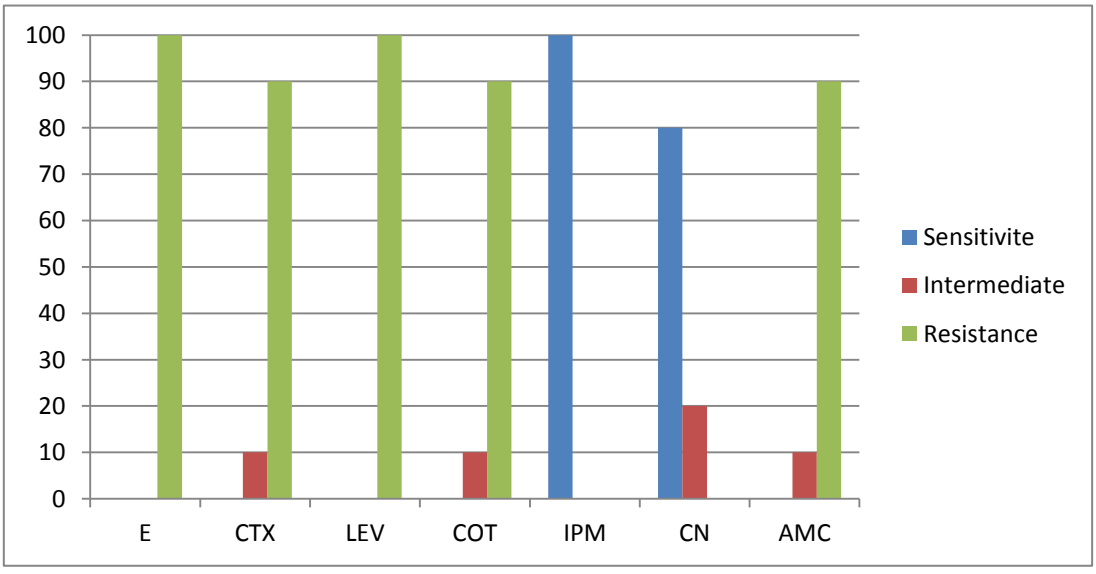


**Fig.2. Result of antimicrobial sensitivity of *K.pneumoniae* against different antibiotic disks. amoxicillin/clavulanic acid (AMC), imipenem (IPM), gentamicin (CN), erythomycin (E) , levofloxacin (LEV), trimethoprime & sulfamethoxazole(COT), cefotaxime (CTX).**

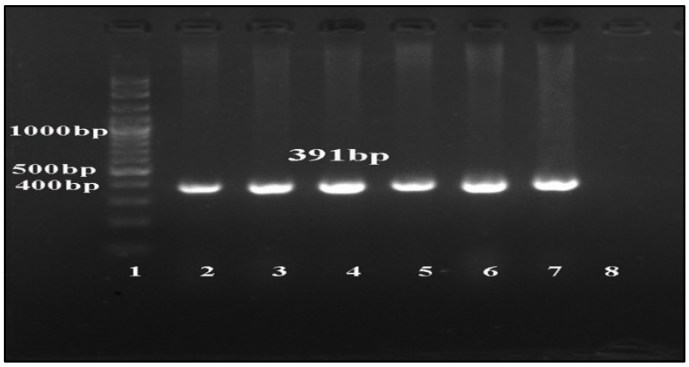


**Fig. 3. Agarose gel electrophoresis of PCR product amplified from *K. pneumonae gap A* gene (391bp). Lane 1, 100 bp DNA Ladder; Lane 2, positive control ;k.pneumoniae ST627 Lanes 3-7, tested samples; Lane 8, negative control:pcr reaction without DNA.**

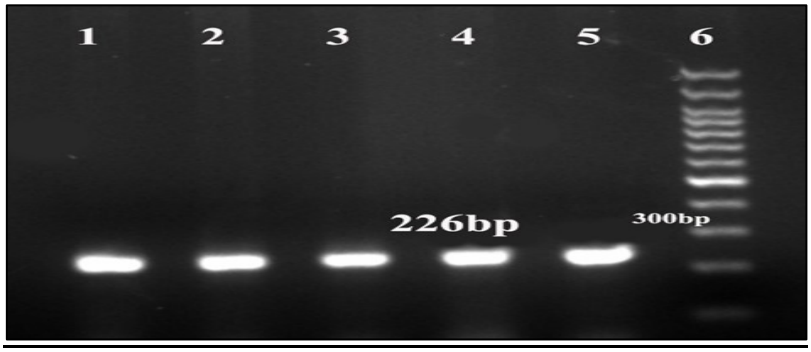


**Fig. 4. Agarose gel electrophoresis of PCR product amplified from *K.pneumoniae mrkD* gene (226bp). Lane 6, 100 bp DNA Ladder; Lanes 1-5, positive *K.pneumonae* isolates.**

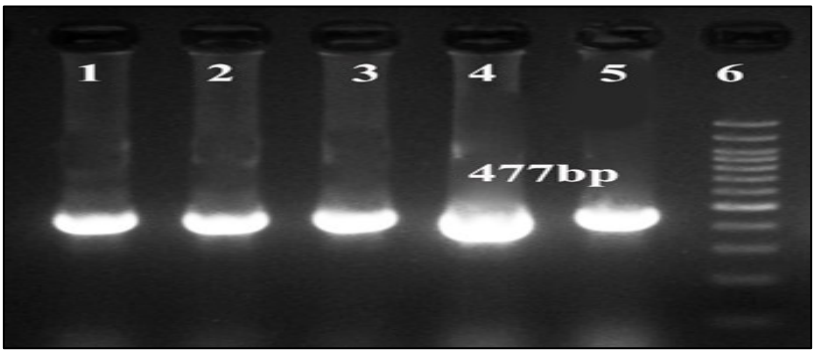


**Fig. 5. Agarose gel electrophoresis of PCR product amplified from *K.pneumoniae luxS* gene (477bp). Lane 6, 100 bp DNA Ladder; Lanes 1-5, positive *K.pneumonae*isolates.**

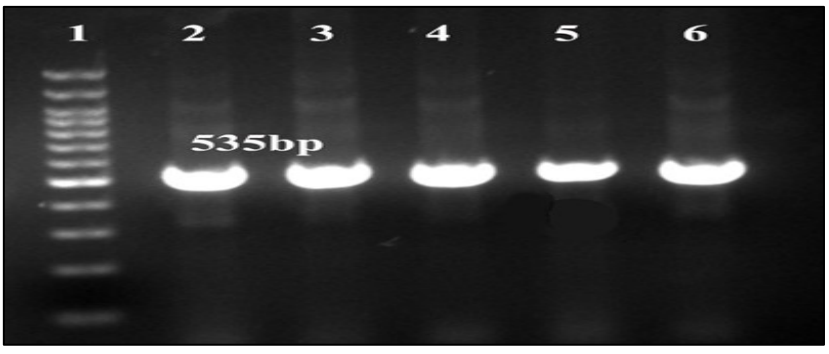


**Fig. 6. Agarose gel electrophoresis of PCR product amplified from *K.pneumoniae uge* gene (535bp). Lane 1, 100 bp DNA Ladder; Lanes 2-6, positive *K. pneumoniae* isolates.**

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# دراسة أنماط مقاومة المضادات الحيوية والكشف الجزيئي لجينات تكوين الأغشية الحيوية لبكتيريا كليبسيلا الرئوية المعزولة من منتجات اللحوم بمحافظة القليوبية، مصر

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**الملخص**

يشكل انتشار مسببات الأمراض التي تنتقل عن طريق الغذاء في اللحوم ومنتجاتها مخاطر صحية كبيرة على البشر ويؤدي إلى خسائر مالية للمنتجين بسبب سحبها من الأسواق. هدفت هذه الدراسة إلى عزل وتحديد مسببات الأمراض كليبسيلا نيومونيا من اللحوم ومنتجاتها باستخدام الطرق التقليدية والخصائص الكيميائية الحيوية. بالإضافة إلى ذلك، تم تطبيق التقنية الجزيئية للكشف عن كليبسيلا نيومونيا المعزولة باستخدام جين gapA. تم جمع مائة (ن = 100) عينة عشوائية من منتجات لحوم البقر ( 10 ) عينة من اللحم المفروم، الكفتة، السجق، الغداء، البرجر، البسطرمة، فرانكفورتر، الكبد، الدجاج والشاورما لكل منهم من أسواق بنها بمحافظة القليوبية، مصر، للفحص البكتيري. تم التحقيق في مقاومة مضادات الميكروبات ضد 7 فئات مختلفة من هذه المضادات. أظهرت النتائج أن انتشار المعزولات من منتجات اللحوم كان 10 معزولات من الكليبسيلا. أظهر اختبار حساسية المضادات الحيوية وجود حساسية عالية للإيميبينيم والجنتاميسين ومقاومة عالية للسيفوتاكسيم والليفوفلوكساسين والأموكسيسيلين وحمض الكلافولانيك والإريثروميسين والتريميثوبريم والسلفوناميد. علاوة على ذلك، كشف التعريف الجزيئي لجين الفجوة A عن وجود 5 كليبسيلا نيومونيا عند حوالي 391 زوجًا أساسيًا. كما أظهر فحص 3 جينات ضراوة (mrkD و uge و luxS) لـ 5 كليبسيلا نيومونيا (ن = 5) المسؤولة عن إنتاج الأغشية الحيوية النتيجة الإيجابية عند (pb226 و pb535 و pb477) للسلالات التي تم فحصها . أخيرًا؛ خلصت هذه الدراسة إلى أن منتجات اللحوم لها مخاطر صحية كبيرة بسبب سوء الجودة الصحية وزيادة الإصابة بالكليبسيلا المسببة للأمراض ووجود عترات متعددة المقاومة للمضادات الميكروبية (MDR).

**الكلمات الدالة:** الأغشية الحيوية، منتجات اللحوم; كليبسيلا، جينات الضراوة وجينات متعدد المقاومة للمضادات الميكروبية.