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Molecular Characterization of ESBLs and Carbapenemases in *Pseudomonas aeruginosa* Isolated from Different Food Products in Egypt

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Abstract

This research was undertaken to estimate the prevalence of Extended spectrum Beta-lactamases (ESBLs) and AmpC beta-lactamases (AmpC), and blaNDM in P. aeruginosa from various food products in Egypt. A number of 300 food samples were randomly collected from different markets. We used standard biochemical and bacteriological methods for isolating and identifying all of the samples. Out of these 300 samples, 240 (80%) isolates were identified as P. aeruginosa. Out of 240 isolates, 200 (80%) demonstrated resistance phenotypes to two or more antimicrobial agents. Overall, the complete resistance (100%) was observed against cefoxitin, ampicillin (90.8%), cefoxitin (88.3%), while susceptibility of isolates was observed against aztreonam and colistin. The PCR- and DNA-sequencing screening recognized many narrow- and ESBL and carbapenemase -encoding genes in 16 (6.7%) bacterial isolates.The BlaCMY-2 β-lactamase encoding gene was identified in 2 (0.8%) isolates. The blaNDM-1 was identified as carbapenemase-encoding gene, which identified in 16 (6.7%) isolates. The elevated incidence of β-lactamase resistant genes in different foodstuffs in Egypt constitutes a serious public health threat with the possibility of transfer of these strains to people.

Keywords: ESBLs, *bla*$_{NDM}$, Food, PCR, *Pseudomonas aeruginosa.*

Introduction

A variety of illnesses, such as soft tissue and skin infections, urinary tract infections, sepsis, and lower respiratory tract infections, can be caused by *Pseudomonas aeruginosa* (*P. aeruginosa*), an essential nosocomial pathogen [1]. *P. aeruginosa* is a common cause of spoilage in a variety of milk, and meat products [2]. The worldwide spread of multidrug-resistant (MDR) *P. aeruginosa* has become a threat to public health. Recent evidences have demonstrated that consumption of food contaminated with MDR *P. aeruginosa* can cause severe infections and spread antimicrobial resistance *in vivo*, especially in health care settings and immunocompromised patients [2]. Transmission of antibiotic-resistant bacteria and genes to humans occurs as a result of the excessive and inappropriate utilization of antibiotics in food animals for the treatment of illness or the promotion of growth [3]. Because β-lactams are frequently employed as primary antibiotics to treat *Pseudomonas* infections, resistance to them is particularly important [3]. The World Health Organization (WHO) has recognized *P. aeruginosa that are resistant to carbapenems* as a serious threat to healthcare [1]. There is importance that animals and people are susceptible to multidrug-resistant (MDR) bacteria from consuming meat and dairy products [4]. The global rate of carbapenem resistance has increased in recent years, which has caused significant concern due to the significant limitations on the therapeutic options available to patients [5]. Enzymes of the class A (GES and KPC), class B (NDM, IMP, and SPM), and class D (OXA-48 β-lactamases) may be present in the MDR *P. aeruginosa*, with the ability to hydrolyze the most of β-lactam drugs [6]. Difficult-to-treat resistance (DTR) *P. aeruginosa* was identified in the US in 2018. This strain was resistant to a wide variety of drugs, involving aztreonam, ciprofloxacin, cefepime, imipenem, meropenem, levofloxacin, and ceftazidime, and was related to a high mortality rate [7]. Extended spectrum beta-lactamase (ESBL)-producing *P. aeruginosa* is the second most prevalent gram-negative bacteria which contribute to urinary tract infections (UTIs) in hospitalized

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patients, among Gram-negative bacilli (GNB) [8]. New treatment options are required immediately for *P. aeruginosa* that are resistant to carbapenems, which was identified as a top most critical pathogen by the WHO [9]. Approximately 13–19% of MDR *P. aeruginosa* infections in hospitalized patients related to health care are reported annually in the US [10]. Poor outcomes, such as costs, elevated resource utilization, morbidity, and mortality in hospital settings, are associated with MDR P. aeruginosa infections [11]. The ESBLs are most important reason of *P. aeruginosa* resistance to β-lactam antibiotics, involving cephalosporins (such as cefotaxime) and penicillin (1st, 2nd, and 3rd generations). $bla_{\text{CTX-M}}$ and bla_{TEM} are the principal ESBLs genes that cause such type of resistance [12].

This research aimed to recognize the mechanisms of ESBL and carbapenemase resistance in clinical strains of *P. aeruginosa* that were obtained from a variety of foods. Additionally, the antimicrobial susceptibility profiles of these strains were observed.

Material and Methods

Sampling

Between May and August 2024, a number of 300 food samples including (100 beef meat, 100 raw milk, and 100 kareish cheese) were collected randomly from different butchers, slaughterhouses and local markets in Kafr El-Sheikh governorate, Egypt. Each sample was labeled, placed in cool boxes, and transferred immediately to the bacteriology laboratory, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, for bacteriological examination.

Isolation and identification

All samples were incubated in buffered peptone water overnight at 37°C then, plated on cetrimide agar and incubated at 37°C for 24 to 48 hr. After the incubation period, the plates were examined for yellow-green colonies which are characteristic of *P. aeruginosa* growths on cetrimide agar. Non-repetitive presumptive *P. aeruginosa* colonies were sub-cultured on cetrimide agar to obtain pure cultures for further characterization and storage. Presumptive *P. aeruginosa* isolates were recognized based on their Gram staining reaction, motility, and biochemical characteristics. Biochemical reactions performed include the catalase test, oxidase test, citrate utilization, indole, and methyl red test.

Antimicrobial susceptibility testing

Characteristics of bacteria's sensitivity to antimicrobials were determined using a Kirby-Bauer disk diffusion assay according to the standards and interpretive criteria defined by Clinical and Laboratory Standards Institute, 2020 [13]. Antimicrobial agents that utilized involving: amoxicillin-clavulanic acid (AMC), 20/10 µg; aztreonam (ATM), 30 µg; ampicillin (AMP), 10 µg; cefotaxime (CTX), 30 µg; cefoxitin (FOX), 30 µg; cefpodoxime (CPD), 10 µg; ceftazidime (CAZ), 30 µg; ceftriaxone (CRO), 30 µg; ciprofloxacin (CIP), 5 µg; imipenem (IPM), 10 µg; meropenem (MEM), 10 µg; gentamicin (GEN), 10 µg; oxacillin (OXA), 1 µg; sulfamethoxazole-trimethoprim (SXT), 23.75/1.25 µg and colistin (COL), 30 µg. *P. aeruginosa* ATCC 27853 was used as quality control (QC) strain. The antimicrobial disks were purchased from MTA Pharmaceutical Co. (Cairo, Egypt).

DNA extraction and molecular detection of resistance genes

Regarding the manufacturer's instructions, DNA was extracted from each isolate by employing the QIAamp DNA mini kit. The bacterial isolates were tested for TEM, SHV, CTX-M, CMY-2 and OXA-1, and NDM-1 β-lactamase encoding genes by PCR utilizing universal primers as previously revealed (Table 1). The total volume of 25 µl required for all PCR assays was as follows: 12.5 µl of Emerald Amp GT PCR mastermix (2x premix), 1 µl of 20 pmol of each forward and reverse primer, 5 µl of bacterial DNA template, and 5.5 µl of nuclease-free water. The T3 Thermal cycler (Biometra) was employed to conduct the PCRs as uniplex reactions. These are the conditions that were used for the PCR cycling: initial denaturation at 94 ºC for 5 min after that 35 cycles of denaturation at 94 ºC for 30 sec, annealing at 50 ºC for 30 sec and extension at 72 ºC for 30 sec followed by a final extension at 72 ºC for 10 min. Lastly, each amplified PCR product was electrophoresed on 1.5% agarose (Co., St. Louis, Sigma-Aldrich, MO, USA) prepared in 1X Tris Borate EDTA (TBE) buffer, and then stained with 0.5 µg/ml ethidium bromide (Co., St. Louis, Sigma-Aldrich, MO, USA). The gel was subsequently photographed and visualized using a UV transilluminator.

Results and Discussion

Global problems persist due to the spread of antimicrobial resistance (AMR) [14]. As per the 2019 Centers for Disease Control and Prevention (CDC) Antibiotic Resistant Threats Report, antimicrobial-resistant pathogens collectively result in over 2.8 million infections and over 35,000 deaths annually [14]. Although the availability of novel antibiotics to combat resistant infections has recently increased [15], resistance to numerous of these agents has been demonstrated [16]. These pathogens has classified as urgent or severe threats, according to the Centers for Disease Control and Prevention (CDC) [14].The CDC has classified MDR *P. aeruginosa* as an important risk for the past decade, with an estimated 2700 deaths, 32,600 cases, and US $767 million in annual attributable healthcare costs [17, 18]. In comparison to patients who had non-MDR-*P. aeruginosa* infections, patients with MDR-

P. aeruginosa respiratory infections exhibited higher mortality, a hospital length of stay that was approximately 7 days longer, higher readmission rates, and an excess cost of US $20,000 per infection, according to a national database study conducted in the US [18]. In this investigation, a number of 300 samples of different foodstuff of animal origin were screened for *P. aeruginosa*. Out of these 300 samples, 240 (80%) isolates were recognized as *P. aeruginosa* from different food products.(Table 2)

In this study, the antimicrobial sensitivity tests demonstrated that 200 out of 240 (83.3%) *P. aeruginosa* isolates showed resistance phenotypes to two or more antimicrobial agents. The resistance phenotypes most frequently documented were against oxacillin, ampicillin, amoxicillin-clavulanic acid, cefpodoxime, sulfamethoxazole/trimethoprim, and gentamicin. At the same time, lower resistance was observed against aztreonam and colistin (Tables 3,4).

It is possible that antibiotic overuse is a contributing factor to the resistance of all *P. aeruginosa* clinical isolates examined in our investigation to Amoxicillin/clavulanate and Amoxicillin. This resistance rate exceeds which noticed by Ahmad et al. [19], In Pakistan, the resistance to Amoxicillin was 73.4% and Amoxicillin/clavulanate was 67.7%. This could be attributed to the interval between the studies, and even the variation in the antibiotics and strains utilized.

In Egypt, 78.3 resistance to imipenem were previously revealed by Abaza *et al*. [20], which higher than our result. In fact, our result disagrees with Farhan *et al*. [21], and Elmaraghy *et al*. [22], from Egypt, who found that imipenem showed low resistance rates of only 8 and 14.9%, respectively. In contrast to our investigation, the studies from Egypt and Sudan discovered that the bacteria had low resistance rate and high susceptible to the medications listed previously [23]. These percentages are much higher than those described in this investigation, likely as a result of the differential antibiotic use rates, geographical location, and study time. While, lower resistance was observed against aztreonam (9.6%), colistin (8.3%). Colistin has the highest susceptibility rate of any antimicrobial agent tested against this particular group of isolates (91.7%). In the same way, in an Iranian study implemented by Malekzadegan *et al*. [24].

The following three types of Gram-negative bacteria that are resistant to antimicrobials present unique therapeutic difficulties: carbapenem-resistant *P. aeruginosa*, extend-spectrum β-lactamase–producing [17]. In this study, incidence of β-lactamase-encoding genes is 6.7%.(Table 2). The bla_{TEM} a narrow -spectrum β lactamase gene was detected in 16 (6.7%) isolates(Fig. 1). The bla_{OXA} were detected in 12 (5%) isolates(Fig. 2). The bla_{SHV} was identified in 8 (3.3%) isolates(Fig. 3). While, $bla_{\text{CTX-M}}$ were identified in 6 (2.5%) isolates. **Figure 4** The $bla_{\text{CMY-2}}$ β-lactamase encoding gene was identified in 2 (0.8%) isolates(Fig. 5). The $bla_{\text{NDM-1}}$ was identified as carbapenemase-encoding gene, which identified in 16 (6.7%) isolates(Fig. 6).

Our results are similar to results have been revealed by Ragupathi, P., et al [25] in United Arab Emirates, Jamali *et al.* [26], in India, Alam, Ali Nazari, *et al*. [27], in Iran, Devarajan N., *et al* [28], in Switzerland, and Gondal, A. J., *et al* [29], in Pakistan.

A higher prevalence rate was documented by Bahrami, M., *et al* [30], in Iran, the frequencies of $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} genes were 23.95%, 23.08%, and 57.29%, respectively. The research of Farzeali Shirehjini *et al.* demonstrated that P. aeruginosa strains had high resistance levels and 21.6% and 34.2% of the strains had $bla_{\text{CTX-M}}$, and bla_{TEM} genes, respectively [31]. The study by Komijani *et al*. [32] showed that among *P. aeruginosa* isolates, ceftazidime resistance was the highest at 77.64 percent. The bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, and bla_{OXA} genes were found to be present in 60.86%, 29.81%, 24.22%, and 14.28% of *P. aeruginosa* isolates, respectively, and 81.98% of them were ESBL-positive. Also, a higher results were revealed by Salah *et al*. [33], the prevalence of bla_{TEM}, bla_{SHV}, and $bla_{\text{OXA-1}}$ in Egypt, were 50%, 33%, and 17%, respectively and by Abdelrahman, Dina N. *et* al [34] found that bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, $bla_{\text{OXA-1}}$, and bla_{CYM} genes were 44.2%, 37.2%, 23.3%, 16.3%, and 81.5%, respectively.

Although the present study was significant, these limitations should be addressed. Inappropriate use of antimicrobials, including the widespread utilize of antibiotics in farms and veterinary healthcare, patient noncompliance, and the availability of medicines without a prescription for purchase over-the-counter, is the root cause of Egypt's high frequency of ESBLs and MBLs [35].

Conclusion

Monitoring drug resistance in health care settings requires phenotypic and genotypic detection of ESBLs and carbapenemases, in addition to analysis of the resistance pattern of the isolates, due to the high prevalence of resistance genes and the resistance pattern of the isolates. Isolates of *P. aeruginosa* exhibited a high rate of β-lactamase production, as well as co-resistance to other antibiotic classes. The resistance rate of *P. aeruginosa* to Ceftazidime was the lowest, followed by aztreonam and colistin. Third-generation cephalosporins resistance was insignificantly related to the synthesis of β-lactamases in *P. aeruginosa*.

Avoiding these limitations, a small sample size, coverage of other β-lactamase classes, phenotypic detection of β-lactamases, and gene sequencing is crucial for future research that aims to identify and confirm all the genes carried by *P. aeruginosa* strains in Egypt.

Author's contribution:

Conceptualization: D.E.N. & A.A.M., methodology: A.M.A. & D.E.N., Formal analysis and investigation: A.A.M. & A.M.A., Writing—original draft preparation: D.E.N., Writing—review and editing: A.A.M, D.E.N., supervision, validation and final editing: A.A.M., A.M.A. & D.E.N., all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Ethical of approval: This study follows the ethics guidelines of the Faculty of Veterinary Medicine, Kafr El-Sheikh University, Egypt.

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TABLE 1. Primers of β-lactamase-encoding genes

Gene	Sequence	Amplified product	Reference
bla_{TEM}	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp	Colom *et al*., [36]
bla_{SHV}	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	392 bp	
bla_{OXA-1}	ATATCTCTACTGTTGCATCTCC AAACCCTTCAAACCATCC	619 bp	
bla_{CMY2}	TGG CCA GAA CTG ACA GGC AAA TTT CTC CTG AAC GTG GCT GGC	462 bp	Pérez-Pérez and Hanson, [37]
bla_{NDM-1}	GGCGGAATGGCTCATCACGA CGCAACACAGCCTGACTTTC	287 bp	Xia *et al*., [38]
bla_{CTX-M}	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593 bp	Archambault *et al*., [39]

TABLE 2. Incidence of resistance genes in *Pseudomonas aeruginosa* isolates

Food products	*Pseudomonas aeruginosa* Isolates Total no.=240	Resistance genes					
		Narrow-spectrum β lactamase		Extended - spectrum β lactamase			Carbapenemase
		bla_{TEM}	bla_{OXA}	bla_{SHV}	bla_{CTX-M}	bla_{CMY-2}	bla_{NDM-1}
Raw milk	**90**	+	+	+	+		+
Cheese	**85**	+	+	+		+	
Meat	**65**	+	+	+	+		+

TABLE 3. Antimicrobial susceptibility testing of *Pseudomonas aeruginosa* isolates in dairy products

Antimicrobials tested[a]	Dairy products (n=160)	
	Milk (n=90)	Cheese (n=70)
FOX	75 (83.3%)	60 (85.7%)
AMP	80 (88.9%)	63 (90%)
AMC	85 (94.4%)	50 (71.4%)
OXA	90) 100%)	70 (100%)
SXT	45 (50%)	38 (54.3%)
CAZ	10 (11.1%)	25 (35.7%)
CPD	15) 16.7%)	33 (47.1%)
CRO	20 (22.2%)	22 (31.4%)
COL	9 (10%)	5 (7.1%)
CIP	25 (27.8%)	18 (25.7%)
IPM	45 (50%)	37 (52.9%)
MEM	43 (47.8%)	35 (50%)
CTX	15 (16.7%)	22 (31.4%)
GEN	65 (72.2%)	63 (90%)
ATM	9 (10%)	7 (10%)

Data represents as frequency (%), [a]) AMP, ampicillin; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; ATM, Aztreonam; CPD, Cefpodoxime; CIP, ciprofloxacin; CAZ, Ceftazidime; COL, Colistin; CTX, Cefotaxime; CRO, ceftriaxone; FOX, cefoxitin; GEN, Gentamicin; IPM, Imipenem; MEM, Meropenem; OXA, oxacillin; SXT, sulfamethoxazole/trimethoprim.

TABLE 4. Antimicrobial susceptibility testing of *Pseudomonas aeruginosa* isolates in meat products

Antimicrobials tested[a]	Meat products (n=80)
FOX	77 (96.3%)
AMP	75 (93.8%)
AMC	60 (75%)
OXA	80 (100%)
SXT	40 (50%)
CAZ	25 (31.3%)
CPD	35 (43.8%)
CRO	29 (36.3%)
COL	6 (7.5%)
CIP	33 (41.3%)
IPM	40 (50%)
MEM	35 (43.8%)
CTX	25 (31.3%)
GEN	75 (93.8%)
ATM	7 (8.8%)

Data represents as frequency (%), [a]) AMP, ampicillin; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; ATM, Aztreonam; CPD, Cefpodoxime; CIP, ciprofloxacin; CAZ, Ceftazidime; COL, Colistin; CTX, Cefotaxime; CRO, ceftriaxone; FOX, cefoxitin; GEN, Gentamicin; IPM, Imipenem; MEM, Meropenem; OXA, oxacillin; SXT, sulfamethoxazole/trimethoprim.

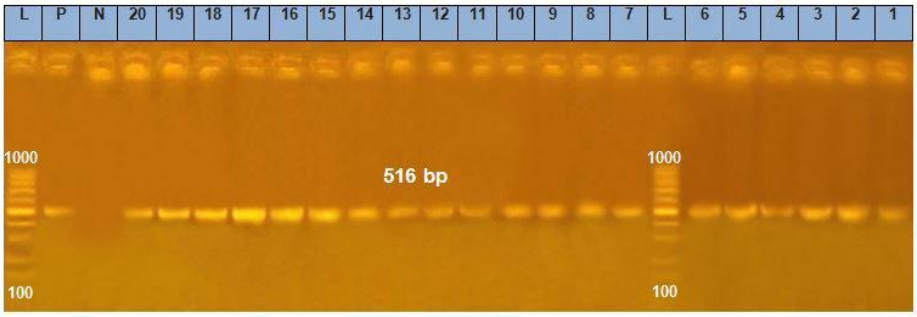

Fig. 1. Gel electrophoresis photos of PCR for detection of TEM gene are detected at 516 bp. P= control positive, N= control negative.

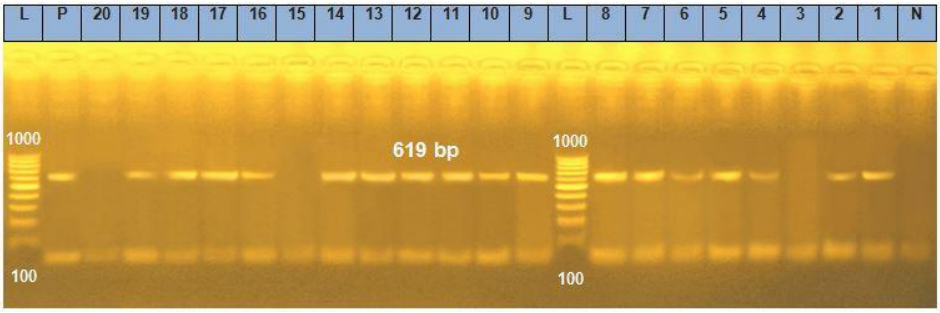

Fig. 2. Gel electrophoresis photos of PCR for detection of OXA-1 gene are detected at 619 bp. P= control positive, N= control negative.

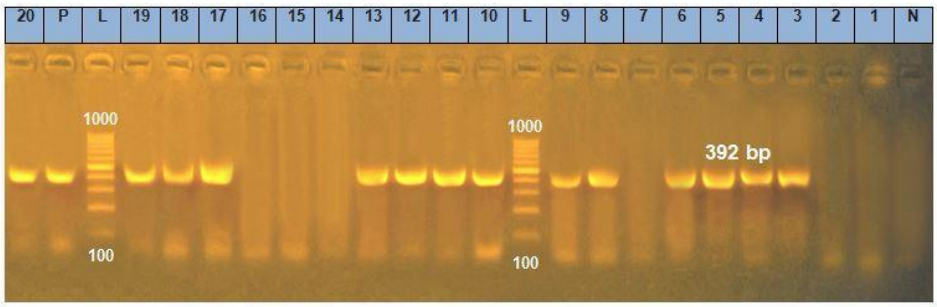

Fig. 3. Gel electrophoresis photos of PCR for detection of SHV gene are detected at 392 bp. P= control positive, N= control negative.

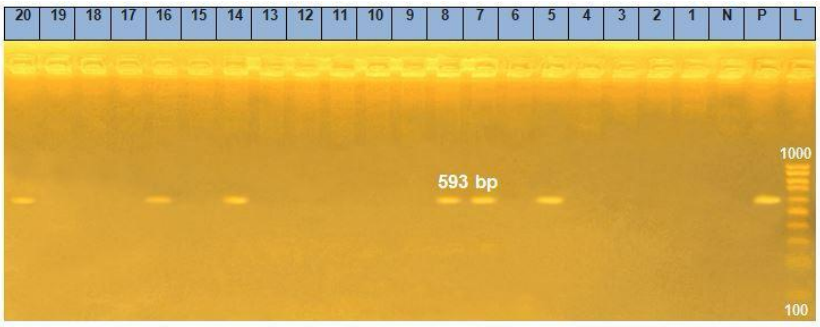

Fig. 4. Gel electrophoresis photos of PCR for detection of CTX-M gene are detected at 593 bp. P= control positive, N= control negative.

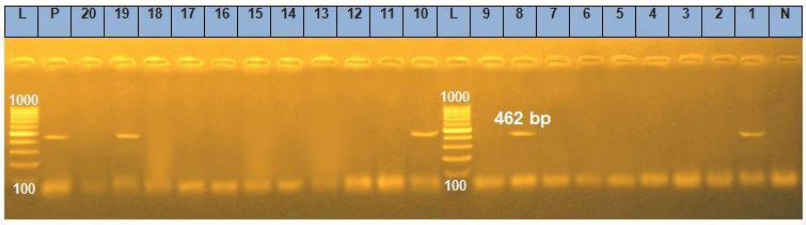

Fig. 5. Gel electrophoresis photos of PCR for detection of CMY2 gene are detected at 462 bp. P= control positive, N= control negative.

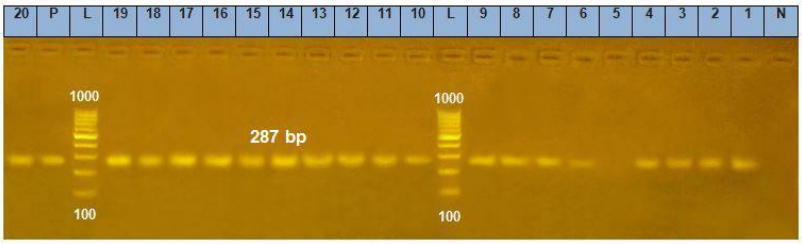

Fig. 6. Gel electrophoresis photos of PCR for detection of NDM-1 gene are detected at 287 bp. P= control positive, N= control negative.

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التوصيف الجزيئي للبيتا-لاكتاماز ذات النطاق الممتد وكاربابينماز في بكتيريا السيدوموناس ايروجينوزا المعزولة من منتجات غذائية مختلفة في مصر

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الملخص

أجريت الدراسة الحالية لتقدير انتشار AmpC وESBL وbla_{NDM} في المتصورة الزنجارية من مختلف المنتجات الغذائية في مصر. تم جمع ما مجموعه 300 عينة غذائية بشكل عشوائي من أسواق مختلفة. تم فحص جميع العينات من خلال سلسلة من التقنيات البكتيرية والكيميائية الحيوية التقليدية للعزل والتعرف عليها. من بين هذه العينات ال 300، تم تحديد 240 (80٪) عزلة على أنها المتصورة الزنجارية. من بين 240 عزلة، أظهرت 200 (80٪) أنماطا ظاهرية مقاومة لاثنين أو أكثر من العوامل المضادة للميكروبات. بشكل عام، لوحظت المقاومة الكاملة (100٪) ضد السيفوكستين، الأمبيسلين (90.8٪)، السيفوكستين (88.3٪)، بينما لوحظت قابلية العزلات ضد أزتريونام وكوليستين. حدد فحص تسلسل تفاعل البوليميراز المتسلسل والحمض النووي العديد من الجينات الضيقة البيتا-لاكتاماز ذات النطاق الممتد وكاربابينماز في 16 عزلة بكتيرية (6.7٪). تم تحديد جين ترميز ل bla_{CMY-2} في 2 (0.8٪) من العزلات. تم تحديد bla_{NDM-1} على أنه جين مشفر للكاربابينيماس، والذي تم تحديده في 16 عزلة (6.7٪). يشكل الانتشار المرتفع للجينات المقاومة للاكتاماز في المواد الغذائية المختلفة في مصر تهديدا محتملا للصحة العامة مع إمكانية انتقال هذه السلالات إلى البشر.

الكلمات الدالة: بيتا لاكتاماز ممتد الطيف ، بلاندم، الغذاء، تفاعل البلمرة المتسلسل ،السيدوموناس ايروجينوز.