Iraqi Journal of Biotechnology, 2026, Vol. 25, No. 2, 321-330

Isolation and Identification of *Enterobacter cloacae* with Phenotypic and Genetic detection

[1] Hawraa Ali Mahmood,[2]Sanaa Rahman Oleiwi

[1,2]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

Abstract

Background: The genus *Enterobacter* includes facultative anaerobic Gram-negative bacilli that are 2 mm long, are motile by means of peritrichous flagella, and belong to the family Enterobacteriaceae. The existence of genes in bacteria is problematic because *Enterobacter cloacae* resistance genes can be crucial to the pathogenicity of this organism and cause many bacteria to become resistant to many antibiotic groups. Clinical isolates containing biofilm and *papC* genes must be identified to control the bacteria's spread and reduce its pathogenicity. **Aim** of this research are isolation and identification of *Enterobacter cloacae* from clinical isolates and detection about resistant genes (*papC, csgAB ,bssb*) biofilm. **Method**: This investigation involved the collection of (225) clinical specimens from Baghdad and Al-muthana hospitals from various sources; (12) isolates, or 5.3 % of all isolates, were successfully identified as *Enterobacter cloacae*. **Results**: our result show exist only two of tested genes were founded in *E.cloacae* ,*papC* and Biofilm genes detected by PCR. These isolates were the most resistance for antibiotics. These show that there were differences between biofilm formation. **Conclusion**: The results highlight the importance of the bacterial biofilm phenotype as a potential virulence factor which may contribute to the clinical relapse of infections.

Keywords: *Enterobacter cloacae*, genes, PCR.

Corresponding author: (Gmail:hawraaali797@gmail.com, sanaeleiwi@yahoo.com.)

Introduction

The genus *Enterobacter* includes facultative anaerobic Gram-negative bacilli that are 2 mm long, are motile by means of peritrichous flagella, and belong to the family Enterobacteriaceae. To date, 22 species have been found in the genus *Enterobacter*: *E. aerogenes, E.amnigenus, E.arachidis, E.asburiae, E. carcinogenus, E. cloacae, E. cowanii, E. dissolvans,E. gergoviae, E. helveticus, E. hormaechei, E. kobei, E. ludwigii, E. mori, E. nimipressuralis, E. oryzae, E. pulveris, E. pyrinus, E. radicincitans, E. soli, E. taylorae, and E. turicensis* (1). *E. cloacae, E. aerogenes,* and *E. hormaechei* represent the most frequently isolated species described in clinical infections, especially in immunocompromised patients and those hospitalized in an intensive care unit (ICU), due to the adaptation of these species to antimicrobial agents and their behavior as opportunistic pathogens. These pathogens are frequently associated with a multidrug resistance (MDR) phenotype, mainly due to their adaptation to the hospital environment and their ability to easily acquire numerous genetic mobile elements containing resistance and virulence genes. Biofilms are populations of sessile bacteria that develop immersed in an extracellular matrix that they

generate and affixed to a surface or interphase matrix of extracellular polymeric biomolecules, such as DNA (2). Numerous bacteria that cause illnesses in the community and in healthcare facilities, such as *Escherichia coli*, *Klebsiella* species, and *Enterobacter* species, are members of the Enterobacteriaceae family (3,4,5). As the second or third most prevalent carbapenemase-producing bacteria, species of *Enterobacter cloacae* have caused increased worry in recent decades due to their growing association with the acquisition of carbapenemase-encoding genes. Enterobacteriaceae (CPE) (6,7,8,9) .EPS are primarily composed of polysaccharides, water channels, extracellular DNA, lipids, proteins, and compounds that resemble humic substances (10). Bacteria are strengthened against harsh environmental circumstances by the biofilm matrix that is formed (11). Because of their adaption to the hospital environment and their ease of acquiring a large number of genetic mobile elements encoding resistance and virulence genes, these infections are commonly linked to a multidrug resistance (MDR) phenotype. Because they exhibit a constitutive AmpC-lactamase, these species are naturally resistant to ampicillin, amoxicillin, first-generation cephalosporins, and cefoxitin (12).

Materials and Methods

Collection and Identification of Bacterial isolates

Two hundred and twenty five clinical samples of (wounds, sputum, and stool) were collected from patients in Baghdad hospitals (Al-Yarmouk Teaching Hospital) and Al-muthana Hospital, from the period between 17\1\2022 to 17\1\2023.

Samples were collected under sterile conditions and cultured in suitable media for the isolation of *Enterobacter cloacae* isolates are identified according to colonies' morphological structure, microscopic examinations, and biochemical tests. And the VITEK® 2 Compact system is dedicated to the identification of *Enterobacter cloacae.*

Antibiotics Susceptibility Test

The antibiotics sensitivity tests on the isolates to 12 antibiotics (Doxycycline 10µg, Doxycycline 30µg , Aztreonam 30µg, Cefixim 5µg , Ciprofloxacin 5µg, Ceftriaxone 30µg, Trimethoprim 5µg, Ticarcillin\clavunic acid 75\ 10µg, Naldixic acid 30µg, Tetracycline 30 µg, Amoxiclav 30µg ,Nitrofuration 100µg were determined on Mueller-Hinton agar. The zone diameter of inhibition was measured, and the marks were translated found on guidelines from the Scientific and Laboratory Values Institute.

Detection Biofilm of E.*cloacae* Formation by Microtiter plate method (13)

1. The medium used for biofilm growth was TSB supplemented with an extra 1% glucose. 2. Bacteria cultured in broth were used to create biofilm inoculums for cultivation. The culture was diluted 1:100 in TSB with 1% glucose added, and 200 µl was added to the well. Only broth (200 µl of TSB enriched with 1% glucose per well) is present in the negative control wells. Each strain was tested in triplicate.3. A lid was placed over the inoculation plate, and it was

incubated aerobically for 24 to 30 hours at 35 to 37 degrees Celsius under static circumstances.4. After decanting the contents of the wells and washing each well three times with 300 µl of PBS, the plates were to be drained upside down and fixed with 150 ml of methanol.5. at room temperature.6- 150 µl of 95% ethanol should be added to allow for dye resorption, and the microtiter plate was then sealed and allowed to sit at room temperature for at least half an hour without being shaken.7. A microtiter-plate reader (GloMax/Promega-USA) was used to measure the optical density (OD) of each well at 570 nm. The results showed that: OD ≤ ODc = no biofilm producer; ODc < OD ≤ 2 ODc = weak biofilm producer; 2 ODc < OD≤ 4 ODc = moderate biofilm producer; and 4.ODc < OD = strong biofilm producer. Three standard deviations (SD) above the mean OD of the negative control is the cut-off value, or ODc: ODc = average OD of negative control + (3× SD of negative control).

Extraction of bacterial Genomic DNA and molecular detection

The DNA genome of *Enterobacter cloacae* was isolated from bacterial growth. The polymerase chain reaction (PCR) was conducted in optimal laboratory conditions . The primer designs used in this inquiry were based on the global genome website (NCBI)

Table(1): Primer Pairs, Sequences, and Expected Size

Gene	Primer	Sequence	Size (bp)	
papC	Forward Reverse	CCCTGAAGACCGATGACAAT CGGAACGGAGGTTTGATAGA	148	(Flávia Roberta Brust *et al.,*2019)(16)
csgAB	Forward Reverse	ATGATGTTAACAATACTGGGTGC CGGCCATTGTTGTGATAAAG	1.3kb	(Sung-Min Kim *et al.,*2012)(17)
Biofilm	Forward Reverse	GATTCAATTTTGGCGATTCCTGC TAATGAAGTCATTCAGACTCATCC	225	(Engy A. Elekhnawy *et al*.,2021)(18)

Detection of genes

Enterobacter cloacae DNA genome was isolated from bacterial growth according to the protocol of EasyPure® Bacteria Genomic DNA Kit, and electrophoresis was carried out according to manufacturing company . The polymerase chain reaction has occurred in optimal laboratory conditions. The primers used in this study were designed based on the *Enterobacter cloacae* genome database as a reference. The PCR conditions for the 16S rRNA gene are shown in Table (2).

Table (2): The PCR conditions for the gene.

Cycle No.	Stage	Temperature	Time
1	Initial Denaturation	94	3 min.
35	Denaturation Annealing Extension	94 C Variables according to (primer"s TM) C 72	30 sec. 30 sec. 1 min.
1	Final Extension	72	5 min.

Results

Isolation of *Enterobacter cloacae*

Of the 225 samples collected from hospitals, 12 isolates were successfully diagnosed as *E. cloacae* representing 5.3 % of the total samples.

Identification of *E.cloacae*

In the laboratory, *E.cloacae* can grow on agar including nutrient agar and broth, blood agar, and MacConkey agar. On MacConkey agar medium the colonies of *E.cloacae* appear pink, that are 2 mm long, mucoid, lactose fermenting colonies. And in Blood agar *E.cloacae* large, smooth, flat colonies with entire margin without B hemolysis. According to biochemical testing, they behaved positively to the catalase, citrate utilization, and Voges Proskauer tests, but negatively to the oxidase, indole, methyl red, urease, and gelatin hydrolysis tests.

Identification of *E.cloacae* by Vitek2 system

Which is a new tool for the identification of bacteria from clinical specimens. The identification was performed by an automated Vitek2 system using GN-ID cards containing (64) biochemical tests. The results give that all (12) isolates for *E.cloacae* were confirmed with ID message (the percentage was 99%-95%). This system is distinguished by its ability to quickly identified of bacteria without the need for many culture media, and its ability to reduce culture contamination.

Antibiotics susceptibility test

The results show that all isolates were highly sensitive to Ciprofloxacin , Nalidixic acid and Aztreonam, while seven isolates resist to ampicillin , three isolates resist to trimethoprim-sulfamethoxazole, tow resist to tetracycline, and tow resist to Nitrofuration, but only one isolate was

resist to Ciprofloxacin and have both genes *papC* gene and Biofilm gene in table (3). This results show that the most resist isolate had genes responsible for biofilm formation.

Table (3): The antibiotics of *E.cloacae* clinical isolates(mm).

Clinical isolate	Tetracycline	Nalidixic acid	Aztreonam	Ampicilin	Trimethoprim-sulfamethoxanole	Ciprofloxacin	Ceftriaxone	Nitrofuration	L.S.D. value
EA80	12	20	29	13	24	32	27	17	4.87 *
EB81	13	20	30	10	25	33	25	14	5.62 *
EC90	11	20	27	9	24	33	25	15	5.94 *
ED101	15	21	30	18	25	32	28	19	5.58 *
EF120	12	19	28	10	23	30	25	14	6.74 *
EG144	0	19	18	0	0	18	0	15	6.37 *
EH200	12	15	30	11	0	17	24	15	5.82 *
EK202	13	20	32	14	24	35	29	17	6.75 *
EN205	12	20	33	12	25	32	28	15	6.67 *
ER210	15	19	33	20	10	22	27	20	6.82 *
ES211	13	22	31	18	25	32	29	17	5.97 *
ET215	13	20	32	12	25	32	28	16	5.72 *
L.S.D. value	3.08 *	3.72 *	5.33 *	4.87 *	5.69 *	6.02 *	5.84 *	3.76 *	---
* ($P \leq 0.05$).									

Detection of Biofilm Formation by Microtiter plate method

Twelve E. *cloacae* isolate that were previously detected by VITK were used for detection of biofilm formation by used microtiter plate method, with crystal violet stain and the OD was measured by GloMax apparatus at 560 nm as shown in figure (1) and table (4) . These isolates were the most resistance for antibiotics. The results show that there were differences between biofilm formation**.** As recommended by many studies (14), MPM method is an accurate and reproducible screening method which can serve as a reliable quantitative tool for determining biofilm formation. The differences in results may be due to the kind of specimens and samples, the number of isolates, the antibiotic relation with biofilm formation of the selected isolates, and the circumstances that affect the biofilm formation. The results highlight the importance of the bacterial biofilm phenotype as a potential virulence factor which may contribute to the clinical relapse of infections.

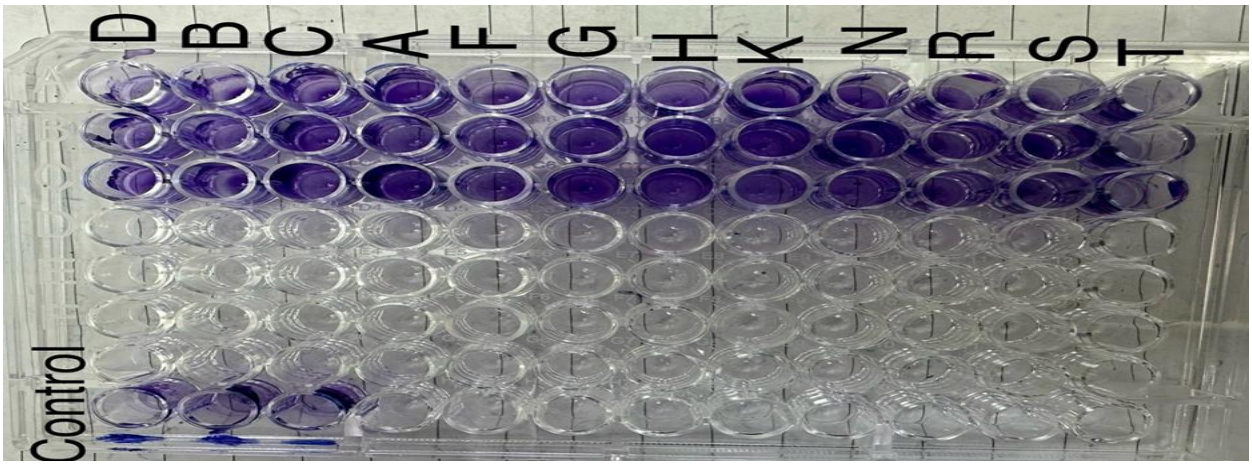

Figure (1): Detection of biofilm formation of *E.cloacae* isolates by microtiter plate method using crystal violet stain.

Table (4): The Optical density (OD) values of *E.cloacae* clinical isolates before treatment.

Clinical bacterial isolates	OD1	OD2	OD3	Average	ODC	2XODC	4XODC	Isolate OD	Result	Source of clinical isolation
EA80	0.331	0.330	0.343	0.334	0.072	0.144	0.288	0.262	Moderate	Clinical
EB81	0.420	0.453	0.399	0.424	0.072	0.144	0.288	0.352	Strong	Burn
EC90	0.321	0.320	0.324	0.321	0.072	0.144	0.288	0.249	Moderate	Urin
ED101	0.301	0.310	0.323	0.311	0.072	0.144	0.288	0.239	Moderate	Clinical
EF120	0.330	0.298	0.339	0.322	0.072	0.144	0.288	0.250	Moderate	Urin
EG144	0.442	0.472	0.330	0.414	0.072	0.144	0.288	0.342	Strong	Lung fluid
EH200	0.297	0.280	0.339	0.305	0.072	0.144	0.288	0.233	Moderate	Clinical
EK202	0.228	0.299	0.217	0.248	0.072	0.144	0.288	0.176	Moderate	Clinical
EN205	0.279	0.263	0.256	0.266	0.072	0.144	0.288	0.194	Moderate	Urin
ER210	0.274	0.228	0.288	0.263	0.072	0.144	0.288	0.191	Moderate	Clinical
ES211	0.353	0.355	0.288	0.332	0.072	0.144	0.288	0.260	Moderate	Urin
ET215	0.247	0240	0.244	0.243	0.072	0.144	0.288	0.171	Moderate	Clinical
Control	0.075	0.071	0.070	0.072	-	-	-	-	-	-
L.S.D. value	--	--	--	0.154 *	--	--	--	0.107 *	-	-
* (P≤0.05).										

Detection of genes

Extraction of DNA

The DNA extraction and purification by DNA extraction kit. The result was detected by electrophoresis on 1% of agarose and exposure to ultraviolet light in which the DNA appears as compact bands. The result was found to be as shown in Figure (2).

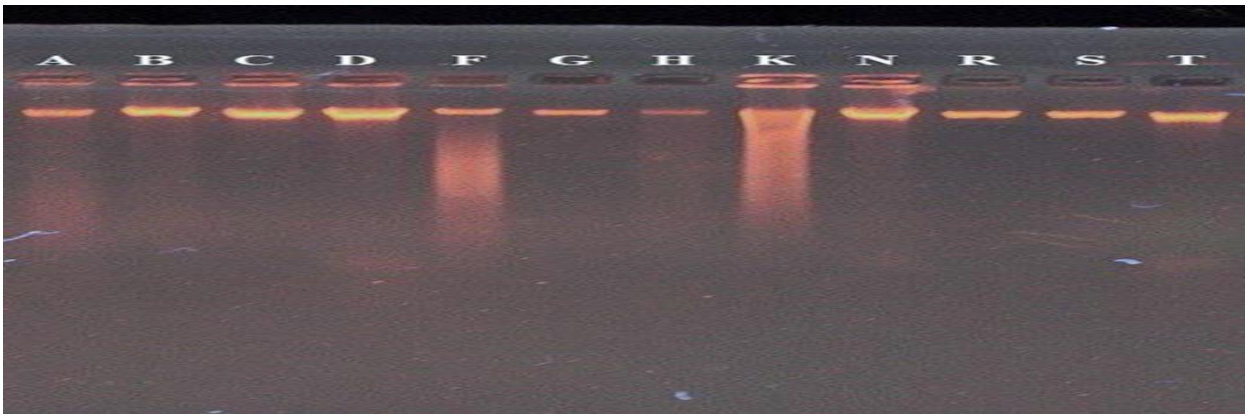

Figure. 2: Agarose gel electrophoresis stained with Ethidium Bromide dye showed a clear bands which represent for DNA molecules that are extracted from *Enterobacter* bacteria.

Detection of *papC*, *csgAB*and Biofilm genes

The isolates that were subjected to an examination to detect the presence of the *papC*, *csgAB*, Biofilm genes. The isolate's DNA was amplified by PCR technique to detect the *papC* and Biofilm genes and absence of *csgAB* gene. The PCR amplification results were confirmed by electrophoresis analysis. After analysis, the DNA strands that resulted from successful binding between the selected *papC*, Biofilm primer basis and the extracted DNA template appear as a single band under UV light. Light using ethidium bromide as a specific form of DNA dye. The results showed that (75%) isolates were positive for the *papC* and (8.3%) for Biofilm gene Figure (3&4).

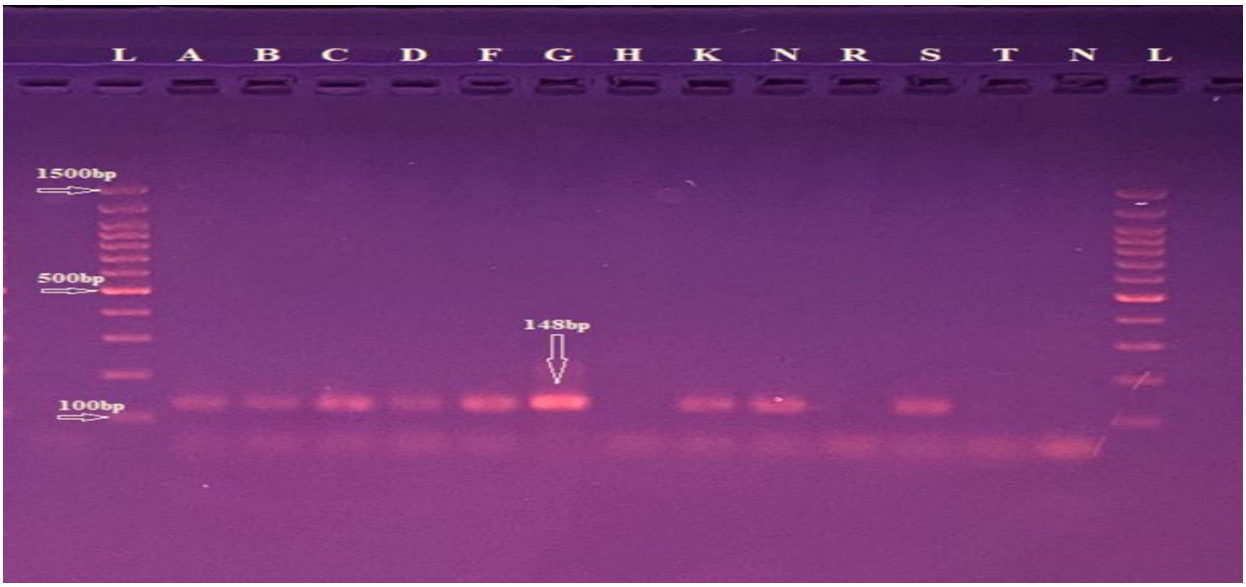

Figure. 3: Gel electrophoresis for PCR product of (*papC* primer) which show 148 bp Primer TM at (57°C), (Agarose 1.5 %, 15min. at 110 voltage and then lowered to 75 volts, 60min.).Visualized under U.V light after staining with ethidium bromide Lane L : DNA ladder (1500-100)bp , Lanes (A-G) represented positive results, Lanes (H,R and T) represented Negative results and Lane (N) represented Negative control .

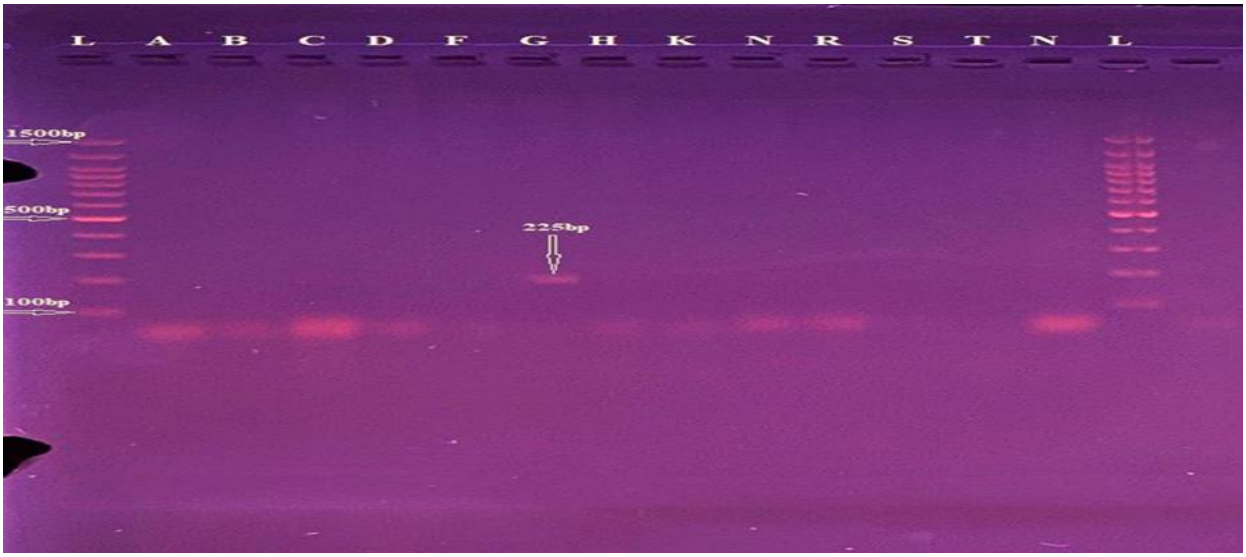

Figure (4) :- Gel electrophoresis for PCR product of (*bssS* primer) which show 225 bp Primer TM at (63°C), (Agarose 1.5 %, 15min. at 110 voltage and then lowered to 75 volts, 60min.).Visualized under U.V light after staining with ethidium bromide Lane L : DNA ladder (1500-100)bp , Lanes (A-T) represented Negative results, except Lane (G) which represent positive result, and Lane (N) represented Negative control

Discussion

The 12 isolates were successfully diagnosed as *E. cloacae* representing 5.3 % of the total samples.

The results give that all (12) isolates for *E.cloacae* were confirmed with ID message (the percentage was 99%-95%).

These isolates were the most resistance for antibiotics. The results show that there were differences between biofilm formation**.** The results highlight the importance of the bacterial biofilm phenotype as a potential virulence factor which may contribute to the clinical relapse of infections.

The results showed that (75%) isolates were positive for the *papC* and (8.3%) for Biofilm gene**.**

Conclusion

In our study we found *Enterobacter cloacae* have two genes *papC* ,Biofilm responsible for biofilm formation .Also these bacteria have ability to form strong and mucoid colonies on MacConkey agar.

References

1- Hoffmann, H., & Roggenkamp, A. (2003). Population genetics of the nomenspecies Enterobacter cloacae. Applied and environmental microbiology, 69(9), 5306-5318.

2- Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin-Scott, H. M. (1995). Microbial biofilms.

3- Peleg, A. Y., & Hooper, D. C. (2010). Hospital-acquired infections due to gram-negative bacteria. New England Journal of Medicine, 362(19), 1804-1813.

4- Morgan, D. J., Lomotan, L. L., Agnes, K., McGrail, L., & Roghmann, M. C. (2010). Characteristics of healthcare-associated infections contributing to unexpected in-hospital deaths. Infection Control & Hospital Epidemiology, 31(8), 864-866.

5-Van Duin, D., & Paterson, D. (2016). Multidrug resistant bacteria in the community: trends and lessons learned. Infectious disease clinics of North America, 30(2), 377.

6- Chavda, K. D., Chen, L., Fouts, D. E., Sutton, G., Brinkac, L., Jenkins, S. G., ... & Kreiswirth, B. N. (2016). Comprehensive genome analysis of carbapenemase-producing Enterobacter spp.: new insights into phylogeny, population structure, and resistance mechanisms. MBio, 7(6), 10-1128.

7- Moradigaravand, D., Reuter, S., Martin, V., Peacock, S. J., & Parkhill, J. (2016). The dissemination of multidrug-resistant Enterobacter cloacae throughout the UK and Ireland. Nature microbiology, 1(12), 1-5.

8- Lazarovitch, T., Amity, K., Coyle, J. R., Ackerman, B., Tal-Jasper, R., Ofer-Friedman, H., ... & Marchaim, D. (2015). The complex epidemiology of carbapenem-resistant Enterobacter infections: a multicenter descriptive analysis. infection control & hospital epidemiology, 36(11), 1283-1291.

9- Jean ShioShin, J. S., & Hsueh PoRen, H. P. (2017). Distribution of ESBLs, AmpC β-lactamases and carbapenemases among Enterobacteriaceae isolates causing intra-abdominal and urinary tract infections in the Asia-Pacific region during 2008-14: results from the Study for Monitoring Antimicrobial Resistance Trends (SMART).

10- Jefferson, K. K. (2004). What drives bacteria to produce a biofilm?. FEMS microbiology letters, 236(2), 163-173.

11 - Flemming, H. C., Neu, T. R., & Wozniak, D. J. (2007). The EPS matrix: the “house of biofilm cells”. Journal of bacteriology, 189(22), 7945-7947. 12-. Davin-Regli, A., & Pagès, J. M. (2015). Enterobacter aerogenes and Enterobacter cloacae; versatile bacterial pathogens confronting antibiotic treatment. Frontiers in microbiology, 6, 392.

13- Davin-Regli, A., Masi, M., Bialek, S., Nicolas-Chanoine, M. H., & Pagès, J. M. (2016). Antimicrobial drug efflux pumps in Enterobacter and Klebsiella. Efflux-Mediated Antimicrobial Resistance in Bacteria: Mechanisms, Regulation and Clinical Implications, 281-306.

14- Stepanović, S., Vuković, D., Hola, V., Bonaventura, G. D., Djukić, S., Ćirković, I., & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. Apmis, 115(8), 891-899.

15- Oliveira, J. S., Araujo, W., Lopes Sales, A. I., Brito Guerra, A. D., Silva Araújo, S. C. D., de Vasconcelos, A. T. R., ... & Freitas, A. T. (2015). BioSurfDB: knowledge and algorithms to

support biosurfactants and biodegradation studies. Database, 2015, bav033.

16-- Elekhnawy, E. A., Sonbol, F. I., Elbanna, T. E., & Abdelaziz, A. A. (2021). Evaluation of the impact of adaptation of Klebsiella pneumoniae clinical isolates to benzalkonium chloride on biofilm formation. *Egyptian Journal of Medical Human Genetics*, *22*, 1-6.

17- Kim, S. M., Lee, H. W., Choi, Y. W., Kim, S. H., Lee, J. C., Lee, Y. C., ... & Kim, J. (2012). Involvement of curli fimbriae in the biofilm formation of Enterobacter cloacae. The Journal of Microbiology, 50, 175-178.

18- Brust, F. R., Boff, L., da Silva Trentin, D., Pedrotti Rozales, F., Barth, A. L., & Macedo, A. J. (2019). Macrocolony of NDM-1 producing Enterobacter hormaechei subsp. oharae generates subpopulations with different features regarding the response of antimicrobial agents and biofilm formation. Pathogens, 8(2), 49.