Medical Science Journal for Advance Research

Vol. 5, No. 3, September 2024
e-ISSN: 2774-4892

First Identification of phospholipase C (*PlcH*) gene in Multi-Drug Resistant *Acinetobacter baumannii* Isolated from Clinical Specimens in Iraq

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ABSTRACT

Background: *Acinetobacter baumannii is gram negative opportunistic bacteria increasingly associated with various epidemics, representing a serious concern according to the broad spectrum of antimicrobial resistance and clinical manifestations, with high mortality rates, especially in patients who reside in intensive care units.* ***Objective:*** *The current study aimed to molecular detection of PlcH gene and other virulence genes.* ***Subjects and methods:*** *Cross-sectional study was conducted for five months, starting in September 2023 and finishing in January 2024. Two esteemed establishments, Baghdad Teaching Hospital and Al-Sadder Medical City, located in the provinces of Baghdad and Najaf, respectively, provided a total of sixty-five clinical samples. The samples were put through a thorough processing technique that comprised morphological approaches, biochemical tests, and Vitek2 systems to ensure the accuracy of the diagnosis. 22 distinct antibiotics from 9 antimicrobial groups had their susceptibility to antibiotics assessed. For the colistin antibiotic the Colistin Broth Disk Elusion Method was used and the disk diffusion method for other antibiotics.Conventional PCR analysis using monoplex and multiplex techniques is utilized to identify particular virulence genes.* ***Result:*** *The investigation involved the examination of 65 specimens obtained from patients ,which included of sputum 31(47.6%), burn exudate 11(16.9%), wound abscesses 8(12.3%) and blood 7(10.7%), buccal swab 4(6.1%), urine 3(4.6%) , pus 1(1.5%) from the total .The majority of the specimens were collected from hospitalized patients 55 (84.6%) ,while there10 (15.3%)indicating a substantial contrast in comparison to outpatients .The rates of resistance were as follows: Ampicillin and Pipracillin (100%) ,Ampicillin-sulbactam(95%), Pipracillin tazobactam (91%), Ticarcillin clavulanic acid (92%). Ceftazidime (95%), Cefotaxime, Ceftriaxone and Cefepime (100%), Imipenem (98%), Meropenem (92%), Amikacin (98%), Gentamicin (91%), Tobramycin (77%), Netilmicin (55%), Doxycycline (40%), Minocycline (20%), Ciprofloxacin (94%), Levofloxacin (92%), Gatifloxacin (28%), Trimethoprim/sulfamethoxazole (92%), Colistin (71%). PCR amplification revealed the following genes bla $_{OXA-51}$ 52(80%), 4(8%) of isolates was positive for the presence of PlcH gene, Omp-A 47(90%) and Bap 19 (37%), FimH 13(25%), FimA (0%), epsA 19(37%), ptk 11 (21.1%).*

Conclusions: *A higher percentage of A.baumannii isolated from sputum samples. High rates of biofilm formation genes (Omp-A and Bap), followed by capsular polysaccharide genes (EpsA, ptk), Adherence genes (FimH) and FimA was not detected. Tissue destruction gene (PlcN), First reporet of phospholypase gene (plcH) in Iraq.*

Keywords: *A. baumannii, Gene, Phospholipase, Virulence.*

Article Information
Received: June 16, 2024; Revised: August 10, 2024; Online: September, 2024

https://doi.org/10.46966/msjar.v5i3.196

INTRODUCTION

Acinetobacter baumannii is a gram-negative, aerobic coccobacilli, non-motile. *A.baumannii* is one of the six "ESKAPE" pathogens that exhibit virulence and multidrug resistance. This group may avoid the biocidal impact of antimicrobial drugs and is mostly responsible for nosocomial infections. *A.baumannii* infections are considered a severe issue due of their extensive antibiotic resistance and high molarity mortality rate . *A. baumannii* infections are more common in hospitalized and susceptible patients because of the organism's capacity to pierce skin and airway abnormalities. Furthermore, the bulk of infections caused by this bacterium are likely to affect patients in the critical care unit (ICU). *A. baumannii* is the causative agent of many infections, including those of the skin and soft tissues, wounds, bacteremia, endocarditis, urinary tract infections (UTIs), meningitis, and pneumonia (1).

Acinetobacter baumannii has a distinct array of virulence factors that provide fitness advantages at different phases of pathogenesis, including attachment, host cell death, and host immune response survival (2). Several novel virulence factors have been discovered over the course of the years, despite the fact that the significance of bacterial persistence and resistance has been widely regarded as an essential component of *A.baumannii's* virulence strategy. Many of these factors have been discovered (3) .Extracellular polysaccharide capsules, phospholipases, biofilm formation, siderophore-mediated iron-acquisition system, adhesion and invasion in host cells, and host cell death are examples of virulence factors that have a substantial impact on bacterial pathogenicity (4).Phospholipases (PLs) with the ability to hydrolyze phospholipids contributed to the pathogenicity of gram-negative bacteria by facilitating the rupture of the host cell membrane(5).PLs categorized into four groups based on the cleavage site: phospholipase A (PLA), phospholipases B (PLB), phospholipases C (PLC), and phospholipase D (PLD). While PLA and PLB hydrolyze the fatty acids from the glycerol backbone, PLC cleaves the phosphorylated head group from the phospholipid. PLDs catalyze the breakdown of the phosphodiester bond, which is the connection between the phosphate and the phospholipid headgroup (6). Phospholipid degradation affects the stability of host cell membranes, and the cleaved head group might trigger alterations in cellular communication that change the host immune response (7). The two distinct genes that produce hemolytic and non-hemolytic forms of the PLC, respectively, are *plcH* and *plcN*. Both of these enzymes have the ability to work in tandem and sequentially. PLC-H would aid in the degradation of the phospholipid elements of the erythrocyte membrane, including sphingomyelin and phosphatidylcholine, which are located on the outer layer. The inner layer would become visible as a result of this procedure. The inner leaflet contains phospatidylserine, which PLC-N can enzymatically degrade. High doses of pure PLC-H in mice result in increased vascular permeability, organ malfunction, and mortality, according to the experimental study. This validates PLC-H's important function as a virulence factor (8). OmpA is an example of an outer membrane protein (OMP) that aids in the attachment to and penetration of host epithelial cells. Furthermore, OmpA prompts the host cell to secrete apoptotic factors, triggering the process of apoptosis and finally resulting in cell death (2). Protein tyrosine kinases (Ptk) and the gene producing polysaccharide external membrane protein (epsA) were identified as essential genes by a transposon mutagenesis screen used to identify genes that are crucial for the proper development of *A.baumannii* in inflammatory human ascites fluid .Mutations in the genes *ptk* and *epsA* affect the synthesis and assembly of capsules

in bacteria present in human serum, resulting in abnormal growth and poor capsule formation(9).Fimbriae, sometimes referred to as pili, are thin protein structures that extend from the surface of many commensal and pathogenic bacteria. Pili serves multiple functions in bacterial physiology, including adherence to and invasion of host cells, horizontal transfer of DNA and proteins, formation of biofilms, facilitation of cell motility, and execution of various other tasks (10).

SUBJECTS AND METHODS

Over a span of 5 months, from September 2023 to January 2024, a cross-sectional study was conducted. A total of 65 clinical samples were gathered from two prominent institutions, namely Baghdad Teaching Hospital and Al-Sadder Medical City, located in the provinces of Baghdad and Najaf. Investigation of *A. baumannii* was carried by morphological by culture on blood, MacConkey agar, CHROM Agar, and biochemical test and VITEK-2 system.

Detection of virulence genes in *A. baumannii*

The manufacturer's instructions for the DNA extraction procedures were followed (Magen, Canda). The bacterial isolates were cultured in LB medium for 24 hours at 37 oC to guarantee their viability and growth. To precipitate the material, add one milliliter of broth culture to the column and centrifuge at 10,000 rpm for one minute. The precipitated cells were mixed with 650 ul of Buffer GW1 and centrifuged at 10,000 rpm for one minute after the flow through was disposed of. Next, take off the supernatant and add 650 ul of Buffer GW2, centrifuging at 10,000 rpm for a minute. The column was contained in a sterile 1.5 ml micro centrifuge tube. After adding 100 ul of AE buffer and allowing it to settle at room temperature for two minutes, centrifuge for one minute at 10,000 rpm. The Plus Biophotometer (Eppendorf, Germany) was used to measure the optical density at 260nm and 280nm in order to determine the concentration and purity of the DNA. A total of 25 µl was used to prepare the PCR reaction mixture, which included 10 µl of Solg™mix PCR master mix, 2.5 µl of each primer, and 2 µl of the extracted DNA after the volume was adjusted to 25µl using sterile deionized distilled water. The Biometra PCR equipment (T3000 Thermocycler) was used to determine the presence of the genes by using specific primers and procedure that explained inTab1 and Tab2.

Table 1: The Primers used in the study.

Primer Name	Primer sequence	Product(bp)	References
bla OXA-51	F:TAATGCTTTGATCGGCCTTG R:TGGATTGCACTTCATCTTGG	353	(11)
PlcH	F: GCACGTGGTCATCCTGATGC R: TCCGTAGGCGTCGACGTAC	608	(12)
plcN	F: TCCGTTATCGCAACCAGCCCTACG R: TCGCTGTCGAGCAGGTCGAAC	481	
FimH	F:TGCAGAACGGATAAGCCGTGG R: GCAGTCACCTGCCCTCCGGTA	506	(13)
FimA	F: CGGACGGTACGCTGTATTTT R: GCTTCGGCGTTGTCTTTATC	500	
ompA	F: CGCTTCTGCTGGTGCTGAAT	531	

	R: CGTGCAGTAGCGTTAGGGTA		
Bap	F:TACTTCCAATCCAATGCTAGGGAGGG TACCAATGCAG R:TTATCCACTTCCAATGATCAGCAACCA AACCGCTAC	1225	(14)
ptk	F: ATTTCAGGGCTTATTGGTC R: TCATAAGCAGCAACGGCAG	141	(15)
epsA	F: ACAAACTTCTTCTGTAGCACC R: AAAAATACTCTGCCATAGGG	271	

Table 2: PCR Cycling Conditions.

Gene	Initial Denaturation Temp/Time	Denaturation Temp/Time	Annealing Temp/Time	Extension Temp/Time	Final Denaturation Temp/Time	Cycle number
bla$_{\text{OXA-51-like}}$	95 /15 min	94 /30 sec	52/1 min	72 /90 sec	72 / 10 min	30
plcH	94/3 min	94/30 sec	55 /1 min	72/1.5 min	72 /5 min	30
plcN	94/3 min	94/30 sec	55 /1 min	72/1.5 min	72 /5 min	30
Bap	96/5 min	96/1 min	56.5/1 min	72/1 min	72/10 min	35
ompA	94/5 min	94/1 min	55/1 min	72/45 sec	72/5 min	35
fimH	95/2 min	94 /30 sec	57/30 sec	72/1 min	72/7 min	30
fimA	95/2 min	94 /30 sec	55/30sec	72/1 min	72/7 min	30
ptk	94/5 min	94/1 min	51/ 1 min	72/45sec	72/5 min	30
epsA	94/5 min	94/1 min	51/ 1 min	72/45sec	72/5 min	30

Statistical Analysis:

In the present study, statistical analysis was performed using Microsoft Office Excel 2019 for certain calculations.

RESULTS

The clinical specimens comprised of sputum 31(47.6%), burn exudate 11(16.9%), wound abscesses 8(12.3%) and blood 7(10.7%), buccal swab 4(6.1%), urine 3(4.6%), pus 1(1.5%) from the total.

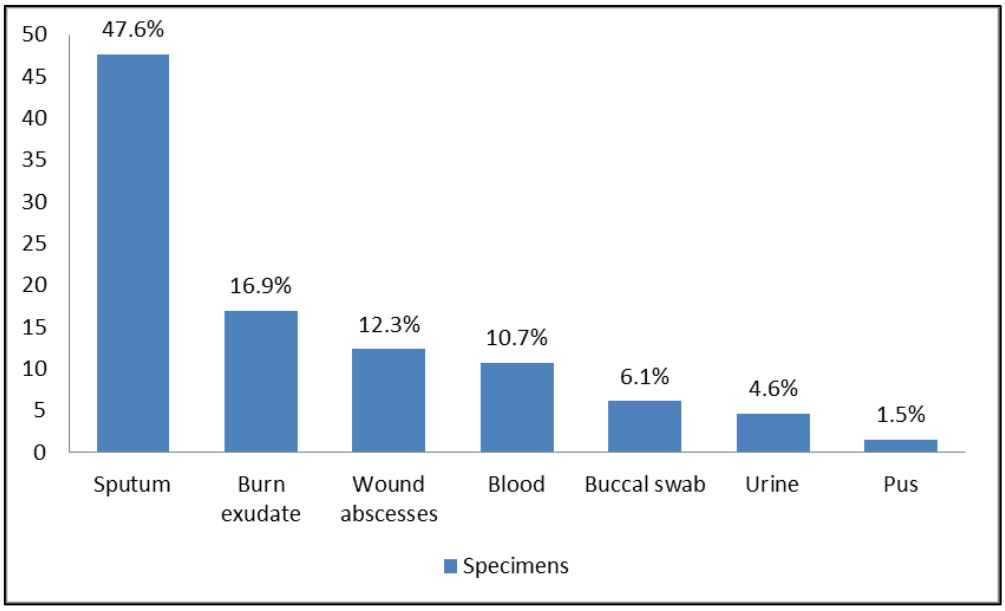

Figure 1: Distribution of *A.baumannii* in several clinical specimens.

Antibiotics susceptibility patterns

A.baumannii's susceptibility to 22 antibiotics from nine different antimicrobial groups was evaluated. The findings showed that 100% of the specimens were completely resistant to pipracillin and ampicillin. The findings showed that 95% of ampicillin-sulbactam, 91% of pipracillin-tazobactam, and 92% of ticarcillin-clavulanic acid combinations were resistant to beta-lactam combinations. *A.baumannii* demonstrated a significant rate of resistance to cephalosporins III and IV in the current investigation, exhibiting rates of 95% for ceftazidime and 100% for cefotaxime, ceftriaxone, and cefepime. The resistance rates to carbapenem antibiotics were very high, at 98%, as opposed to 92% for imipenem and meropenem. correspondingly.

In addition to about 98% of *A.baumannii* were resistant to amikacin, 91% to gentamicin, 77% to tobramycin, and 55% to netilimicin when it came to aminoglycoside antibiotics. For tetracyclin class that inclouded both doxycycline and minocycline, resistance rates were 40%, 20% respectively. 94% of ciprofloxacin, 92% of levofloxacin, and 28% of gatifloxacin were resistant to fluoroquinolones. colistin had a resistance rate of 71% and trimethoprim/sulfamethoxazole exhibited a resistance rate of 92%.

Molecular Characterization of virulence Genes

PCR analysis was performed on 65 specimens to verify the precision of our tests and procedures for genus identification using the $bla_{\text{OXA-51}}$ 52(80%) gene. Only samples with the gene $bla_{\text{OXA-51}}$ were taken into account for additional molecular examination. Monoplex and multiplex PCR procedure were subjected for 52 specimens to determine the frequency of genes encoding virulence factors. The outcome demonstrated the following: the genes responsible for tissue degradation *plcH* gene 4(8%) and *PlcN* 3 (6%) from the total number of specimens was positive ,high frequency of *Omp-A* (biofilm production gene) at 47 (90%) and *Bap* 19 (37%). Adherence genes *FimH* was found in 13 (25%) of the specimens , while *FimA* was negative in all of them .The capsular polysaccharide genes *epsA* 19 (37%), *ptk* 11 (21.1%).As explained in Fig1.

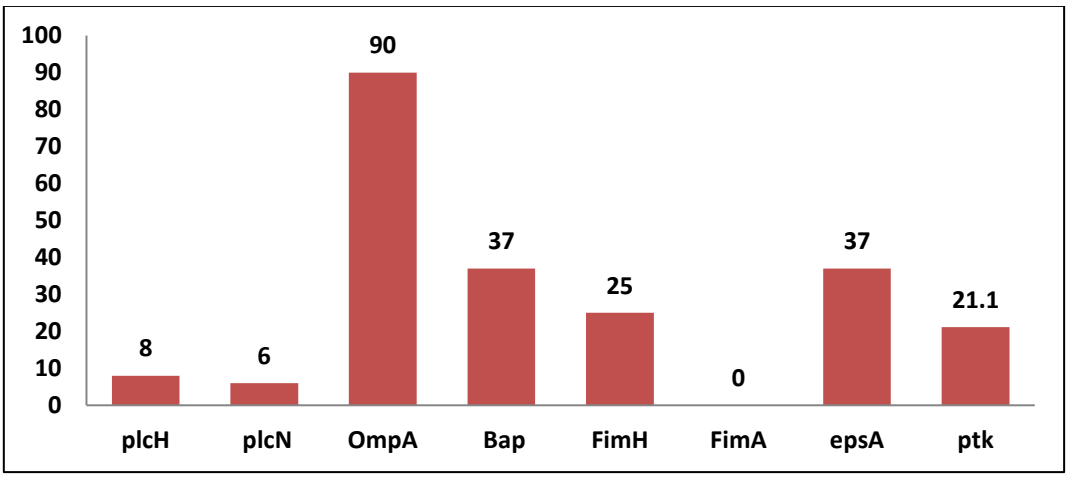

Figure1: Distributions of virulence genes in *A. baumannii*.

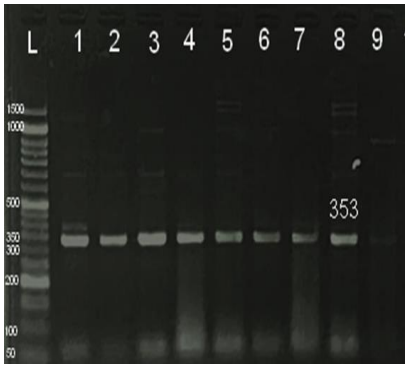

Figure 2-a

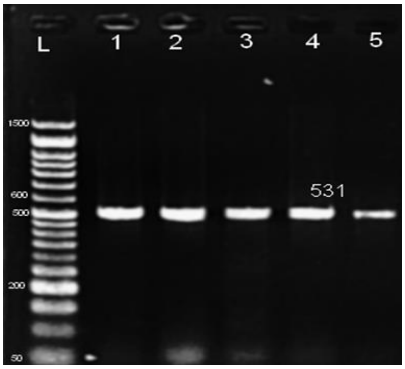

Figure 2-b

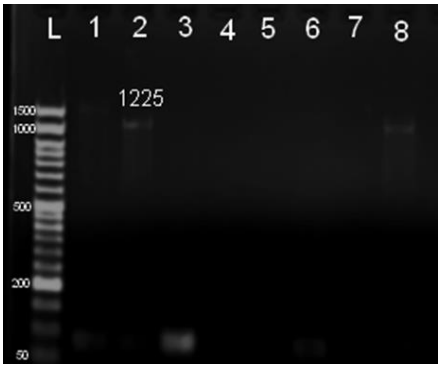

Figure 2-c

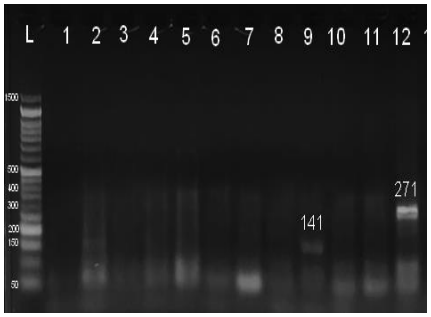

Figure 2-d

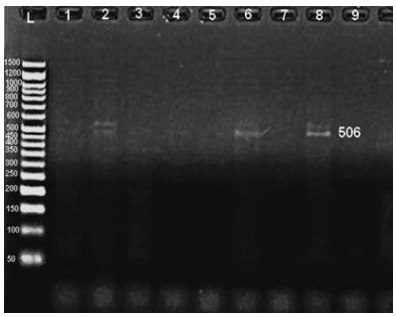

Figure 2-e

Figure 2-f

Figure (2a-f) *Agarose gel that has been stained with ethidium bromide, 1.5 grams of agarose powder were added to 100 milliliters of TBE buffer. The gel displays the outcome of a monoplex and multiplex PCR reaction carried out with DNA isolated from A. baumannii. The electrophoresis was conducted with a voltage of 65 volts for a duration of 1 hour. Lane (L), DNA molecular size marker (50 bp ladder).(a) amplified with* bla_{OXA51} *genes primers ,Lanes (1,2,3,4,5,6,7,8,9) show positive result with* bla_{OXA51}*(353 bp),(b) amplified with ompA genes primers ,Lanes (1,2,3,4,5) show positive result with ompA (531bp) ,(c) amplified with Bap genes primers ,Lanes (2,8) show positive result with Bap (1225bp),(d) multiplex amplification with epsA and ptk genes primers , Lanes (12) show positive results with epsA 271 bp , Lanes(9) show positive results with ptk 141bp ,(e) amplified with FimH gene primers ,Lanes (2,6,8,) show positive result with FimH (506bp), (f) amplified with plcH and plcN genes primers ,Lanes (7) show positive result with both genes plcH (608 bp), plcN (481bp) .*

DISCUSSION

Acinetobacter baumannii possesses virulence factors, which are substances that aid the bacteria in bringing about infection (16). A significant number of nosocomial infections, such as pneumonia linked to ventilator use, endocarditis, meningitis, urinary tract infections, and surgical site infections in critically ill patients, are caused by the most significant species, *A. baumannii* (17). They highlight the nosocomial character of *A. baumannii* infections, community-acquired illnesses, and the need for effective infection control practices in healthcare settings to prevent the spread of these diseases (18). This outcome is in line with previous studies conducted by (19). The aim of thr current study was to determine the presence and prevalence of *PlcH* gene and other virulence genes in *A. baumannii's* specimens. The present results indicated that these genes are distributed in a varied manner among the specimens. The $bla_{\text{OXA-51-like}}$ gene, was detected in *A. baumannii* specimens. These findings validate previous research that suggest the identification of the $bla_{\text{OXA-51-like}}$ gene can serve as a genetic diagnostic tool for detecting *A. baumannii*, as supported by supplementary methods. In this study about 52(80%) of specimens was positive for $bla_{\text{OXA-51-like}}$ gene agreement with (20).

Phospholipase is a crucial enzyme that contributes to the pathogenicity of gram-negative bacteria. It is accountable for breaking down phospholipids present in the membranes of host cells, leading to the creation of chemicals that disrupt the membrane. This leads to the destruction of the cell and the liberation of its internal contents. According to the findings of the current investigation, *plcH* was positive in 4(8%) of specimens, it is important to refer to that this study was the first detection of *PlcH* gene in *A.baumannii* specimens in Iraq ,which may be connected to the phospholipase activity of bacteria ,and 3(6%) of the specimens had *plcN*, which may be connected to the phospholipase activity of bacteria, *plcH* and *plcN* genes lead to the synthesis of hemolytic and nonhemolytic variations, respectively. It was the first study by (21) to uncover critical virulence factor genes that encode for PLCN, a specific form of phospholipase C, in Iraqi specimens of *A. baumannii* that showed 23.3% of the *A. baumannii* specimens tested positive for *plcN* using PCR analysis. According to a different study about 20% of samples have the *plcN* gene (22). A study conducted in Romania that found both the *PlcN* and *PlcH* genes were found in other gram-negative bacteria, but all *A. baumannii* specimens tested negative (23). The *A.baumannii* isolates in the clinical specimens exhibited a high frequency of virulence genes .The *OmpA* gene amplification, revealed 47(90%) of specimens was poisitive , which confirmed by study conducted in Sulaimani, Iraq(24). The main surface protein found in *A. baumannii's* outer membrane proteins is the *OmpA* gene. *OmpA* functions as the main disease-causing component of *A. baumannii* and control the pathogen's capacity to adhere to surfaces, exhibit aggressiveness, and develop biofilms. Another factor that raises the risk of death in nosocomial pneumonia and bacteremia cases is the increased production of *OmpA* by *A. baumannii* (25). A biofilm-associated protein that is encoded by the *Bap* gene is essential for bacterial cell accumulation, intercellular adhesion, and biofilm formation (26). In this study the *Bap* gene was found in 19(37%) of the specimens, responsible for biofilm formation, agreement with study that conducted by (27). The capacity to form biofilms and antibiotic resistance were highly correlated. When exposed to particular

medications, some antibiotic-resistant microbes have demonstrated higher capacity for biofilm formation. The detection results of the *epsA* gene indicate that 19(37%) of *A. baumannii* specimens tested positive for having this gene. Dis agreement with study in Iran report that all isolates had *epsA* gene (28). In the present study, 11 (21.1%) of the specimens had the *ptk* gene identified. In contrast, the results of (15) showed that only two specimens contained *epsA* and one specimen was positive for the *ptk* gene; according to (29) *epsA* was 91.9% and ptk was 80%. These genes required for capsule polymerization and assembly in *A. baumanii* which in turn helps the organism to resist host immune mechanism. *Ptk* encodes a putative protein tyrosine kinase (PTK) and *epsA* encodes a putative polysaccharide export outer membrane protein (EpsA). The *epsA* gene, derived from EPS, encodes a cell adhesion protein that promotes the formation of a compact biofilm. This biofilm is situated within the extracellular matrix of the mitochondria. Between 50 and 90 percent of all organic carbon is found in biofilm, and this carbon is essential to the survival and resistance of bacteria .The creation of bacterial aggregates during the biofilm-forming process depends on the generation of EPS (30). The *FimH* gene is essential for the pathogenicity of bacteria because it makes it possible for the bacteria to adhere to the surface of host cells and so cause harm. Within the parameters of this study, it was found that 13 (25%) of the specimens revealed positive for *FimH* presence. *FimH* was (81.8 %) in different study (31). An Egyptian study found that 60% of people had positive *FimH* (32).53.3% of specimens had *FimH* by (33) .*FimA* was not present in any of the bacterial specimens, which is consistent with the results of the study conducted in Romania by (23) and the province of Wasit by (34) in which none of the specimens produced a positive result .Variations in geographic location or climate, customs, and public health could all contribute to the diversity in the distribution of virulence genes (35).

CONCLUSIONS

A higher percentage of *A.baumannii* isolated from sputum samples. High rates of biofilm formation genes (*Omp*-A and *Bap*), followed by capsular polysaccharide genes (*EpsA*, *ptk*), Adherence genes (*FimH*) and *FimA* was not detected. Tissue destruction gene (*PlcN*), First reporet of phospholypase gene (*plcH*) in Iraq.

RECOMMENDATIONS

Utilization of genomic bacterial strain typing methods such as pulsed field gel electrophoresis (PFGE) , multilocus sequences typing (MLST), whole-genome sequencing (WGS).Concurrent typing of clinical and environmental specimens of *A. baumannii* is a vital technique for identifying the genesis and pathways of transmission of this epidemic bacterial infections ,and to investigate the genetic structures ,molecular mechanism of virulence and antibiotic resistance determinants of this pathogen.

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