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Isolation and Identification of *Acinetobacter Baumannii* from Different Clinical Sources and Determine Antibiotic Resistance in Karbala City

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Abstract

Background: *Acinetobacter baumannii* one of the important multidrug-resistant opportunistic nosocomial pathogens, in part due to its high capability of acquiring resistance to different antibiotics groups.

Objective: The objective of this study was to determine the bacterial infection with *A.baumannii* and antibiotics sensitivity pattern in Karbala city.

Patients and Methods: Two hundred different Clinical specimens were collected from various sources from patient who admitted to Imam Hussein Medical City, the study beginning from the period January 2024 till the August 2024. The specimens which included sputum, wound, urine, blood and fluid and all specimens was collected as 40 cases.

Results: The collection specimens from patients included 40(20%) positive specimens distributed as 25 (62.5%) from females, and 15 (37.5%) from males and they were divided into: 17(42.5%) sputum, 10(25%) wound, 7(17.5%) urine,5(12.5%) blood ,1(2.5%) fluid specimen. After cultured on Blood and MacConkey agar, the isolates were identified via VITIK 2 compact system (Biomérieux, France). All isolates were tested for their resistance to 18 different antibiotics and the results exhibited that highest level of resistance in *A.baumannii* isolates to total antibiotics used in this study except Minocycline, Colistin and Tigecycline . Most isolates were resistant to Ticarcillin 100%, Ticarcillin/ Clavlanic Acid, Piperacillin and Meropenem (39)97.5%, Piperacillin/Tazobactam, Cefotaxime, and Ceftazidime (38) 95%, Imipenem, Ciprofloxacin and Ampicillin /Sulbactam showed resistance rate 37 (92.5%) and Cefepime 35(90%), Amikacin and Tobramycin34(85%), Gentamicin 82.5%, Trimethoprim/sulfamethoxazole 75%. Our study showed the Colistin, Minocycline and Tigecycline were sensitive in the rate36 (90%), 35 (87.5) and 30(75%) respectively.

Conclusion: The study concluded that the most common cases of infection with *A.baumannii* bacteria in sputum, and that this bacteria has a high resistance to most antibiotics.

عزل وتشخيص بكتريا *A. baumannii* من مصادر سريرية مختلفة وتحديد مقاومة المضادات الحيوية في مدينة كربلاء

دعاء حسين كاظم الوحيلي، هيام عبد الرضا العواد

الخلاصة

المقدمة: تعد بكتيريا *Acinetobacter baumannii* واحدة من أهم مسببات الأمراض الانتهازية المقاومة للأدوية المتعددة، ويرجع ذلك جزئيًا إلى قدرتها العالية على اكتساب المقاومة لمجموعات المضادات الحيوية المختلفة.

الهدف: الهدف من هذه الدراسة هو تحديد نمط العدوى البكتيرية وحساسية المضادات الحيوية في مدينة كربلاء.

العينات وطرائق البحث: تم جمع 200 عينة سريرية من مصادر مختلفة من المرضى الذين ادخلوا الى مدينة الامام الحسين الطبية، وبدأت الدراسة من كانون الثاني 2024 حتى آب 2024. العينات التي شملت البلغم والجرح والبول والدم والسوائل وجمعت جميع العينات 40 حالة.

النتائج: شملت عينات الجمع من المرضى 40 (20%) عينة إيجابية، موزعة بواقع 25 (62.5%) من الإناث و15 (37.5%) من الذكور وتم تقسيمها إلى: 17 (42.5%) من البلغم، 10 (25%).) الجرح، 7 (17.5%) بول، 5 (12.5%) دم، 1 (2.5%) عينة سوائل. بعد زراعتها على أجار الدم وأجار ماكونكي، تم التعرف على العزلات باستخدام نظام VITIK 2 compact (Biomérieux، فرنسا). تم اختبار مقاومة جميع العزلات لـ 18 مضاد حيوي مختلف، وأظهرت النتائج أن أعلى مستوى مقاومة لعزلات *A.baumannii* لجميع المضادات الحيوية المستخدمة في هذه الدراسة ماعدا Minocycline وColistin وTigecycline. أظهرت معظم العزلات مقاومة لمضادات تيكارسيلين 100%، تيكارسيلين/حمض الكلافلانيك، بيبيراسيلين وميروبينيم (39)97.5%، بيبيراسيلين/تازوباكتام، سيفوتاكسيم، سيفتازيديم(38) 95%، إيميبينيم، سيبروفلوكساسين وأمبيسلين/سولباكتام بنسبة مقاومة 37 (92.5). ٪) و سيفيبيم 35 (90%)، أميكاسين وتوبراميسين 34 (85%)، جنتاميسين 82.5%، تريميثوبريم/سلفاميثوكسازول 75%. أظهرت دراستنا أن الكوليستين والمينوسيكلين والتيجيسايكلين كانت حساسة بنسبة 36 (90%)، 35 (87.5) و30 (75%) على التوالي.

الاستنتاج: استنتجت الدراسة إلى أن أكثر حالات الإصابة ببكتيريا *A.baumannii* في البلغم هي الأكثر شيوعاً، وأن هذه البكتيريا لديها مقاومة عالية لمعظم المضادات الحياتية.

1. Introduction

A.baumannii are characterized by abilities to spread, and capacities to survive in most ecological surfaces and it was surprising ease with which it obtain antimicrobial multiple resistances. Because *A.baumannii* is resistant to antibiotics, rapidly spreads, and possesses virulence factors, it is considered a cause of nosocomial infection. For the previous 30 years, strains of *A*. *baumannii* have acquired resistance to anew developed antimicrobial drugs; these strains are recognized as multidrug-resistant organisms (MDR) *A*. *baumannii*. It became prevalent in several hospitals all over the world and has been lately documented there as a leading nosocomial pathogen (Agyepong et al., 2023; Zhang et al., 2022). Antibiotics resistance has become a community health problem, with high morbidity and mortality rates affecting largely countries with developing economies. In the United States of America (USA) the Centers for Disease Control and Prevention (CDC) approximation that infections associated to antibiotic resistant microorganisms are accountable for at least 23,000 deaths per year. In 2050, infections related to antimicrobial resistance will be accountable for 10 million deaths each year according to the World Health Organization (WHO). The WHO published in February 2017, a list of antibiotic resistant microorganisms for which the advance of novel antimicrobial treatments is considered urgent. This list contains microorganisms from the ESKAPE group: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp (Ahmed et al., 2024; Luo et al., 2023; Zhang et al., 2022)*.

The aimed of study was isolation and identification of bacterial isolates *A.buamannii* from diverse clinical sample by culturing and VITEK-2 system as well as to investigate of the occurrence of MDR and antibiotic susceptibility in *A.baumannii* isolates by VITEK-2 system.

2. Materials and Methods

2.1. Study Design

A cross-sectional study from 40 cases were collected from different types of specimens obtained from 200 clinical cases as total which Includes wounds, urine, sputum, fluid and blood of infections from both male and female in different ages, diverse local regions, the study beginning between January 2024 to August 2024.

2.2. Clinical Specimens

All Specimens collection was directly transferred to the laboratory and inoculation on culture media, macckonkey agar and blood agar by using streaking method, the samples were cultured aerobically at 37°C for duration of 24 hrs, after incubation period, the growth was examined daily.

2.3. Inclusion and Exclusion Criteria

The inclusion criteria filled out by the patients participating in our study and included knowledge of their age, gender, symptoms**,** while the exclusion criteria for persons with Cancer and smokers.

2.4. Identification of Bacterial Isolates

The isolates from pure colonies were phenotypically identified based on morphological, cultural, and biochemical properties by using gram negative card (GN) cards (ID) of the VITEK 2 system (Biomérieux, France), Antibacterial sensitivity testing on isolated bacteria was also accomplished using the compact automated system VITEK 2 (Pincus, 2010).

2.5. Antibiotics Susceptibility Test

Antibiotics susceptibility testing (AST) for isolates was determines the susceptibility to a group of antibiotics. The cards were laden into the VITEK 2 compact system automatic reader-incubator afterward being inoculated via card (AST). Used turbidity meter to make sure the number and density of microorganisms inoculated into the VITEK 2 cards were right (Ambaraghassi et al., 2019; Funke et al., 1998).

2.6. Statistical Analyses

The results were analyzed statistically in SPSS version 22 to observe Chi-square and the Probability levels were less than 0.05 was significant ($p < 0.05$).

3. Results and Discussion

3.1. Isolation of *A.baumannii*

The results in the present study revealed that a total number of 40 (20%) specimens of *A. baumannii* were found from (200) clinical specimens including wounds, urine, sputum, fluid and blood of infections from both males and females, diverse ages, varied local regions, while 160 specimens showed negative result for *A.baumannii* Fig.1. The collection was from hospitalized patients from Al-Hussien Medical City in Karbala. Our study greed with previous studies, in which the researchers collected 200 clinical samples from three main hospitals in the province of Babylon, out of 40 *A.baumannii* isolates and it is different with (Odih et al., 2023; Pandey et al., 2021). On the other hand, it different with another study in hospitals of Mosul and Erbil cities/Iraq that only 41(14.4%) isolates were identified as *A. baumannii*, and generality of these isolates were from burns (36.5%), surgical wounds (34.1%), and sputum (14.6%). However, it was recognized in CSF, blood, and urine specimens with lesser percentages (7.3%, 4.8%, and 2.4%), respectively, (Raut et al., 2020; Vrancianu et al., 2020)was established that the isolation rate of *A.baumannii* was 13% and (Lee et al., 2022; Liu et al., 2016)was recognized that the isolation rate of *A.baumannii* was (3.09%), while (Khaled et al., 2021)found the isolation rate of *A.baumannii* was (55.6%), finally Chaudhury *et al*., (2018) found that the isolation ratio was (9.51%) who established that isolation ratio of *A. baumannii* was (84%).

The disparity in the isolation ratio levels for whole studies may be due to several factors such as collection place and date and collection period the percentage of isolation could be diverse rendering to variance in nearby patients' levels of contamination and ecological factors (Khaled et al., 2021; Lee et al., 2022; Liu et al., 2016; Vrancianu et al., 2020).

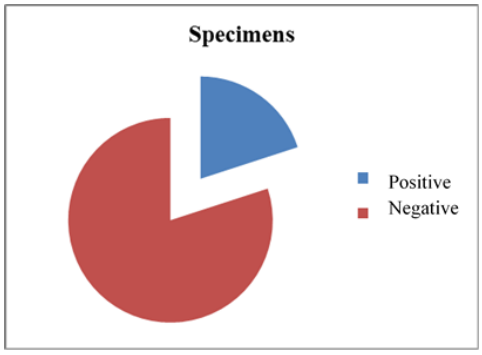

Figure1: Distribution of Tested Specimens Showing the Proportion of Positive and Negative Results. The blue segment represents **positive** specimens, while the red segment represents negative specimens. A larger proportion of the specimens tested negative.

3.2. Association Between The Occurance Of A.Baumannii With Types Of Specimen

The study found the isolation of *A.baumannii* from various clinical specimens, revealing important insights into its prevalence across different sources. Sputum specimens yielded the highest positivity rate at 42.5%, indicating a strong association with respiratory infections. This aligns with the known role of *A.baumannii* in respiratory tract infections, especially in patients who are critically ill or mechanically ventilated. On the other hand, Wound specimens followed, showing a positivity rate of 25%. This highlights the pathogen's significance in wound infections, particularly in hospitalized patients or those with surgical wounds, where *A.baumannii* can be a common contributor to complications. While, urine specimens had a positivity rate of 17.5%, suggesting that *A.baumannii* can also be involved in urinary tract infections, particularly in catheterized patients. While less common than in respiratory or wound infections, its presence in urine samples indicates a need for careful monitoring in these cases Table1. Blood specimens revealed a 12.5% positivity rate. Although lower than other specimen types, the isolation of *A.baumannii* from blood is concerning, as it can indicate serious conditions like bacteremia or sepsis, necessitating prompt clinical attention. Generally, Fluid specimens showed the lowest positivity rate at 2.5%. This suggests that *A.baumannii* is less frequently implicated in infections associated with body fluids, but its presence should still be regarded carefully in clinical assessments. Overall, the total positivity rate of 20% across all specimens underscores the significance of *A.baumannii* in this hospital setting. These findings emphasize the importance of choosing appropriate specimens for culture to ensure accurate diagnosis and effective treatment of infections caused by this pathogen Table1. The results indicated that sputum and wound specimens are the most common sources of *A.baumannii* infections. The Chi-square test result of 22.5 with a P-value of 0.00015 suggests a highly significant association between the specimen type and the presence of *A.baumannii.*

Table1: Types of Clinical Specimens with *A. Baumannii*

Types of specimens with *A. baumannii*						
Specimens	**Sputum**	**Wound**	**Urine**	**Blood**	**Fluid**	**Total**
Total	40	40	40	40	40	200
Positive	17	10	7	5	1	40
Percentage	42.5%	25%	17.5%	12.5%	2.5%	20%
Chi-square test: 22.5, P-value =0.00015, DF= 4						

In various studies, on *A.baumannii* isolation has shown the results are similar and different to our study, found the most common sources of *A.baumannii* was blood stream infections and lower percentage obtained from sputum ,urine and wound infections .The study in Hilla Teaching Hospital by Jabur, (Adewoyin et al., 2021; Blasco et al., 2019; Cartwright, 2010) found the highest percentage of isolation was obtained from urine samples ,the other source was wound , burn and sputum samples were low percentage of isolation, When analyzing the strains of *A.baumannii* antibiotics in patients, it was discovered that patients with specimens from the lung are more than those who have specimens from urine, blood, or even wound fluid. This raises the likelihood of these patients being harboring infections of the respiratory tract. It has been noted that the organism is associated with pneumonia due to introduction

of bacteria through mechanical ventilation towards seriously ill patients which could account for the larger number of pathogens isolated from the lungs, and moreover the prevalence of *A.baumannii* may indicate a capacity to develop and retain antibiotic resistance which makes the treatment of the infected individuals difficult and may improve the chances of their morbidity and mortality. In addition, other reasons for the higher rates of sputum collection include the possibility of high cut-off points for effective mould infection control interventions specifically among areas with high influx and out flux of patients or high levels of contamination devoid of appropriate measures (Aldali, 2023; Baral et al., 2019).

3.3. Association of occurrence A.bumanni with gender

The results showed that females account for the majority at 62%, and males make up 38% of the group, the gender breakdown of the patients depicted in the pie chart reveals a clear predominance of female individuals, who account the most common of the sample. In contrast, male participants constitute a smaller proportion, form the total Fig.2. The higher rates of *A.baumannii* infections in women compared to men might have multiple causes, the biology could explain this difference, as women's immune systems may react to infections. Hormones like estrogen could affect how the immune system works. Also, things like age other health problems, and existing medical conditions might lead to this gap. If more women in the study had these risk factors, it could explain why they got infected more often, how much time people spend in hospitals matters too. If women went to the hospital more or had more medical procedures, they'd be more likely to get *A.baumannii* and the people act can make a difference. Women might have different ways of staying clean or going to the doctor, which could change their chances of getting infected (Dias et al., 2022).

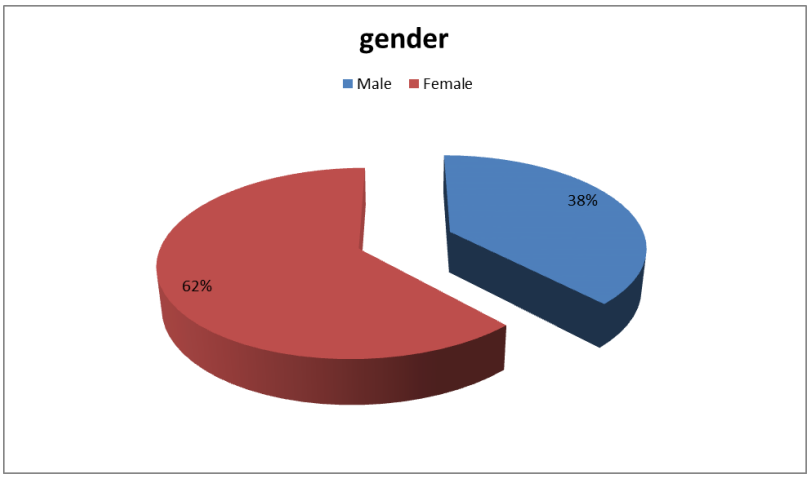

Figure2: Distribution of Participants by Gender. The chart illustrates the gender distribution of the study participants. Female participants represented 62% of the total sample, while male participants accounted for 38%.

3.4. Association of A.Bumanii Related With Seasonal Studies

This Table2 showed the variability in *A.baumannii* isolated from clinical samples during the study period has been reported. sputum consistently experienced the highest incidence of disease, ranging from 9.7% to 15.4% in different months. wound samples also showed a significant positive trend, at 13.6% in March. Urine and blood cultures showed low but consistent positivity, usually in the 1%. fluid sampling was the least overall, with only one case confirmed in

August. The overall quality across all sample types was 20%, with sewage being the most common source of *A.baumannii* isolates. These findings emphasize the importance of close monitoring of respiratory and wound infections in the management of this opportunistic pathogen.

Table2: Study Season Collecting Specimens According to The Study Season

Study Season	Total	Sputum	Positive (%)	Wound	Positive (%)	Urine	Positive (%)	Blood	Positive (%)	Fluid	Positive (%)
January	31	7	3 (9.7%)	5	2 (6.5%)	6	1 (3.2%)	7	1 (3.2%)	6	0
February	26	6	4 (15.4%)	6	2 (7.7%)	5	2 (7.7%)	6	1 (3.8%)	3	0
March	22	5	2 (9.1%)	5	3 (13.6%)	6	1 (4.5%)	3	0	3	0
April	29	6	3 (10.3%)	6	2 (6.9%)		2 (6.9%)	6	2 (6.9%)	7	0
May	18	4	2 (11.1%)	4	1 (5.6%)	4	0	4	1 (5.6%)	2	0
June	23	5	3 (13%)	5	0	5	1 (4.3%)	3	0	5	0
July	19	3	0	4	0	6	0	6	0	0	0
August	32	4	0	5	0	4	0	5	0	14	1 (3.1%)
Total	200	40	17 (42.5)	40	10 (25%)	40	7 (17.5%)	40	5 (12.5%)	40	1 (2.5%)
Chi-square test: 47.482 ; P value = 0.01217699; DF= 28											

Many *Acinetobacter* infections vary according to the season, which develops in damp conditions with more humid ambient air. Several outbreaks have been traced to liquid or wet environmental sources that have aided Acinetobacter species spread. There are numerous factors that are a significant cause of *A. baumannii* including environments, broad variety of PH, The link between humidity and infection rates points to *A.baumannii* thriving in wet environments. The experts have tracked outbreaks to wet or liquid sources in the environment. This shows that contaminated water and surfaces can harbor these bacteria. It underscores how crucial it is to watch and clean healthcare spaces to stop outbreaks as well as the, changes in seasons might affect how common infections are. This is because humidity levels go up at certain times of the year. This can make it easier for the bacteria to spread in hospitals and other medical facilities (Carvalheira et al., 2021). *A. baumannii* in places where cleaning isn't up to par. This is a big problem in intensive care units and surgical wards where patients are at higher risk and knowing these environmental factors

helps to create good infection control plans. To lower the chances of *A. baumannii* infections, hospitals can do a few key things. They can put strict cleaning rules in place, keep a closer eye on where germs might be hiding, and teach their staff why it's so important to keep things dry (Kyriakidis et al., 2021; Vázquez-López et al., 2020).

3.5. Determine The Sensitivity and Resistant Isolates In A.Baumanni

The specimen's data was entered on to a unique form, and they included: 17(42.5%) sputum, 10(25%) wound, 7(17.5%) urine,5(12.5%) blood ,1(2.5%) fluid specimen. After cultured on Blood and MacConkey agar, the isolates were recognized via Vitik 2compact system. All isolates were tested for their resistance to 18 different antibiotics and the results appeared that highest level of resistance in *A.baumannii* isolates to completely antibiotics used in this study except Minocycline, Colistin and Tigecycline . Most isolates were resistant to Ticarcillin 40(100%), Ticarcillin/ Clavlanic Acid, Piperacillin and Meropenem 39(97.5%), Piperacillin/Tazobactam, Cefotaxime, and Ceftazidime 38(95%), Imipenem, Ciprofloxacin and Ampicillin /Sulbactam showed resistance rate 37 (92.5%) and Cefepime 35(87.5%), Amikacin and Tobramycin 34(85%), Gentamicin 82.5%, Trimethoprim/sulfamethoxazole 75%. Our study showed the Colistin, Minocycline and Tigecycline were sensitive in the rate36 (90%), 35 (87.5) and 30(75%) respectively Table3.

Table 3: Analysis of Antimicrobial Susceptibility in Different Specimens

Specimens Antimicrobial	AST	Sputum	Wound	Urine	Blood	Fluid	Total
Ticarcillin	S	0	0	0	0	0	0
	R	17	10	7	5	1	40(100%)
Ticarcillin/ Clavlanic Acid	S	0	0	1	0	0	1
	R	17	10	6	5	1	39(97.5%)
Piperacillin	S	1	0	0	0	0	1
	R	16	10	7	5	1	39(97.5%)
Piperacillin/tazobactam	S	0	0	1	1	0	2
	R	17	10	6	4	1	38(95%)
Ceftazidime	S	0	0	1	1	0	2(5%)
	R	17	10	6	4	1	38(95%)
Cefepime	S	0	0	3	2	0	5(12.5%)
	R	17	10	4	3	1	35(87.5%)
Imipenem	S	0	0	2	1	0	3(7.5%)
	R	17	10	5	4	1	37(92.5%)
Meropenem	S	0	0	1	0	0	1(2.5%)
	R	17	10	6	5	1	39(97.5%)
Amikacin	S	0	4	0	2	0	6(15%)
	R	17	6	7	3	1	34(85%)
Gentamicin	S	3	0	2	2	0	7(17.5%)
	R	14	10	5	3	1	33(82.5%)
Tobramycin	S	2	2	2	0	0	6(15%)

	R	15	8	5	5	1	34(85%)
Ciprofloxacin	S	0	0	2	1	0	3(7.5%)
	R	17	10	5	4	1	37(92.5%)
Minocycline	S	16	9	7	3	0	35(87.5)
	R	1	1	0	2	1	5(12.5%)
Colistin	S	16	9	6	4	1	36(90%)
	R	1	1	1	1	0	4(10%)
Trimethoprim/sulfamethoxazole	S	6	0	2	2	0	10(25%)
	R	11	10	5	3	1	30(75%)
Ampicillin /Sulbactam	S	0	0	2	1	0	3
	R	17	10	5	4	1	37(92.5)
Cefotaxime	S	0	0	1	1	0	2(5%)
	R	17	10	6	4	1	38(95%)
Tigecycline	S	16	7	0	6	1	30(75%)
	R	1	3	5	1	0	10(25%)

Table3. showed that elevated level of resistance in *A.baumannii* isolates to all antibiotics used in this study except Minocycline, Colistin and Tigecycline .Most isolates were resistant to Piperacillin/Tazobactam 97.5% , 95% respectively , which was similar with local study in Babylon province. Results of another study showed the clinical isolates of *A.baumannii* were determined to be 95.6% resistant to piperacillin, 89.1% to ceftazidime, 97.8% to ceftriaxone, 95.6% to cefepime, 80.4% to ciprofloxacin, , 63% to meropenem and 54.3% to tetracycline , Imipenem showed resistance rate 37 (92.5%) and resistance rate to Meropenem was 39(97.5%) similar with study from diverse hospital in Thailand by (Aminul et al., 2021; Bhatta et al., 2021; Thirapanmethee et al., 2020). Another study found that 83 out of 91 (91.2%) isolates were resistant to imipenem and meropenem. Results establish that *A.baumannii* clinical isolates developing 100% resistance to ceftriaxone, cefotaxime, 95.45% to cefepime, chloramphenicol, aztronam and 40.90% to imipenem. Upon these local studies, we can observe interestingly the increase of resistance to imipenem antibiotic in our hospitals. Imipenem and Meropenem are from the Carbapenem antibiotics set. The cause for the emergence of resistance via bacteria to the antibiotic of this set is the ability of the bacteria to yield two types of β-lactamase enzymes, those are Carbapenem hydrolyzing class D of β-lactamase and Metallo β-lactamase enzymes, that hydrolysis and destroy carbapenems antibiotics (Gallego, 2015; Kitano et al., 2019; Thirapanmethee et al., 2020). Scientists showed was Cefepime highest resistance rate 20(100%) which was similar to our study, Further more in this study, high resistant ratio *A.baumannii* to Amikacin, ticarcillin 85%, 100% respectively. The resistance for Ciprofloxacin also displayed elevated resistance rate 92.5%, (Kareem, 2020). And other study found resistance rate (78.19%) for Ciprofloxacin. Our study showed the resistance rate to Colistin (10%) which was less than the result of found resistance rate to Colistin (66.96%). In our study the percentage of Ceftazidime (90%). These results were like and quite an agreement to the earlier studies in Iraq (Kareem, 2020; Stewart et al., 2018; van Duin & Bonomo, 2021). In conclusion: The study concluded that the most prevalent cases of infection with *A.baumannii* were found in sputum

samples. Additionally, it was noted that this bacterium exhibits high resistance to most antibiotics, posing significant challenges for treatment.

4. Conclusion

This study highlights the clinical and epidemiological significance of Acinetobacter baumannii infections in hospitalized patients at Al-Hussien Medical City, Karbala. The organism was isolated from 20% of clinical specimens, with sputum and wound samples being the most common sources. A significant association was found between the type of clinical specimen and the presence of A. baumannii, with the highest isolation rates observed in respiratory samples. Seasonal variation suggested increased prevalence during more humid months, emphasizing the potential role of environmental conditions in its transmission. The antimicrobial susceptibility profile revealed alarmingly high resistance rates to most tested antibiotics, particularly beta-lactams, aminoglycosides, and carbapenems, underscoring the multidrug-resistant nature of these isolates. Only Minocycline, Tigecycline, and Colistin retained considerable activity, indicating their potential utility as last-resort treatments. The emergence of resistance is likely driven by enzymatic mechanisms such as the production of carbapenemases and metallo-beta-lactamases. The findings underscore the need for continuous surveillance, strict infection control practices, and judicious use of antibiotics to combat the spread of multidrug-resistant A. baumannii. Additionally, targeted therapeutic strategies and further molecular studies are recommended to understand resistance mechanisms and guide effective clinical management.

5. Ethical Approval

Before the specimen was collected, written permission was obtained from each study patients, and all subjects involved in this experiment were informed. The university of Kerbala, College of Education for Pure Science Ethics Committee gave its approval to this study.

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