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Isolation and Molecular Diagnosis of *Acinetobacter Baumannii* Strains from Various Clinical Samples and Their Cultural Properties

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ABSTRACT

Objective: *For the optimal and accurate isolation of Acinetobacter baumannii bacteria, which are considered bacteria are increasingly serious health problem in the healthcare community, especially immunosuppressed people and residents' patients' hospital, due to its high resistance to antibiotics.* ***Methods:*** *Accordingly, 300 clinical samples were collected from different hospitals in Mosul city from January to June 2023. They were grown on culture media such as MacConkey and Hichrome Agar for isolation purposes, the number and percentage of all isolated strains for each medium were recorded. Molecular screening was also done by recA gene and 16S rRNA to confirm the diagnosis. Morphological and physiological characteristics were observed on other different media.* ***Results:*** *the 130/43.3% of A. baumannii were isolated from MacConkey medium and 119/91.5% were isolated on Hichrome medium. However, the isolation rate decreased to 15/11.5% in the case of molecular diagnosis with conservative recA gene. The highest isolation rate was from wound samples at 8.3%, followed by urine and burns at the same rate of 7.5%, then throat swabs at 6.6%, followed by sputum at 1.4%, while the bacteria were not isolated from blood, CSF and medical equipment. The morphological characteristics of the bacteria varied greatly on many media such as Eosin –methylene blue EMB medium, Mueller medium, and Nutrient Agar medium in addition to the previous media.* ***Novelty:*** *This research proves the molecular diagnostic ability of the recA gene in A. baumannii bacteria. While highlighting the isolation of these bacteria at the same time on a selective diagnostic medium and comparing the data of these results with each other.*

INTRODUCTION

Acinetobacter baumannii is a ubiquitous, Gram-negative, nonflagellated coccobacillus bacterium commonly isolated from the environment [1]. The danger of Acinetobacter disease is due to the site infection within the patient's body and its immunity state. Therefore immunocompromised persons, intensive care unit (ICU) patients, children, pregnant women, and hospital residents are more sensitive to these bacteria. Acinetobacter considered main cause of morbidity and mortality in patients [2], [3]. Recently, it has been noted that it is frequently associated with an increased rate of mortalities [4], [5].

Diagnostic media are an important means of trying to diagnose many pathogenic bacterial species. They are a diagnostic guide for a specific bacterial species based on certain characteristics, whether physiological, biochemical or morphological [6], [7]. However, at the same time, they do not provide conclusive evidence for the final diagnosis of a specific bacterium [8], [7]. Therefore, molecular diagnosis remains the conclusive and decisive evidence in diagnosing these bacteria. From this standpoint,

testing the ability of some conservative genes in diagnosis is of utmost importance, especially since bacteria with high variability in terms of the results of biochemical and morphological tests [9]. One of these genes is the recA gene. It has an important function in the bacterial cell, as it is responsible for coding the RecA protein, which maintains DNA [10], [11]

RESEARCH METHOD

A. Specimens' Collection and Culture Condotions

Samples were collected from December to June 2023 from various hospitals of Mosul city as. From 300 different clinical specimens, they were as follows: 106 urine, 67 sputum, 30 blood, 15 throat swab, 53 burn samples, 5 CSF, 12 wound infections, and 12 swabs from clinical devices and equipment.

Various samples were collected using Swap Gel ready-made or sterile liquid nutrient as transport media. In both cases, the specimens were activated by inoculating the swap into a sterile broth liquid medium and incubating it at 37°C/24 h. Clinical specimens were cultured on solid media as nutrient, McConkey, and blood Agar at 37°C/24 h. This was done to investigate the morphological and physiological characteristics of the grown isolates [12].

B. DNA Extraction

The extraction of genomic DNA was done by Genaid/ USA kit according to the attached protocol. The DNA concentration was measured using the NanoDrop device at 260/280 nanometer, and kept in the deep freezing until use.

C. Primers Properties

The primers described in Table (1) were used. They are primers specifically designed for the current study from the NCBI/ Pick Primer (except for the 16S rRNA gene). Their annealing temperature calculated by NEB TM Calculator program, from the website (https://tmcalculator.neb.com/). These primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in nuclease-free water to give a final concentration of 100pmol/µl as a stock solution.

Table 1. Primers that used in this study with their annealing temperature.

Gene name	Primer name	Product size bp	Sequence (3'-5')	Annealing temperature
Recombinase	*rec*A	369	F: ACCATGCACCAGCTTTCTGT R: CACGTCTTATGAGCCAGGCA	53
16S rRNA	27F 1552R	1495	F: AGAGTTTGATCMTGGCTCAG R: AAGGAGGTGATCCARCCGCA	55

D. PCR program

The same PCR program was performed for the two genes under study (*rec*A and 16S rRNA) but with different annealing temperatures for each gene (Table 2).

Table 2. PCR program that used with all gene primers under study.

Stage	No of cycles	Temperature C°	Time (minutes)
Initial denaturation	1	95	3:00
Denaturation		95	00:30
Annealing	30	***	00:30
Extention		72	1:30
Final extention	1	72	3:00

*** Mean: annealing temperature that mentioned in table (3-9) according to each primer

RESULTS AND DISCUSSION

A. Distribution of *A. baumannii*

The fifteen (15) *A. baumannii* strains were isolated in the current study at (31.3%) isolation percentage to the total number of samples. The isolation rate of *A. baumannii* is varied from different sources of samples as (Table 3). So, the highest isolation rate was from wound samples at 8.3%, followed by urine and burns at the same rate of 7.5%, then throat swabs at 6.6%, followed by sputum at 1.4%, while the bacteria were not isolated from blood, CSF and medical equipment (Table 3).

The results revealed that 187 (62.3%) of the specimens showed bacterial growth, while 113 (37.6 %) showed no growth. On MaCconkey agar, 57 of the bacteria were lactose fermented, while the 130 were non lactose fermented and oxidase negative.

There is a difference in the isolation percentages between the Hichrom Agar, MacConkey media and *rec*A gene (Table 3, Figure 1). Gene *rec*A gave the lowest isolation percentage at 15/ 11.5%, followed by the Hichrom medium with a high percentage that reached 119/91.5%, but it is somewhat lower than the MacConkey medium, which gave an isolation percentage of 130/43.3%.

Table 3. The percentage of *A. baumannii* that isolated from different sources.

No.	Specimen type	The total No. of samples	Isolation rate on MacConkey agar medium (No. of A. baumannii) % (no. of specimen for each source)	Isolation rate on Hichrom Agar Medium (No. of A. baumannii) % (no. of specimen for each source)	Isolation rate by recA (No. of A. baumannii) % (no. of specimen for each source)
1	Urine	106	53/50	49/ 46.2	(8)/ 7.5
2	burns	53	31/58.5	30/ 56.6	(4)/ 7.5
3	Sputum	67	19/28.35	17/25.4	(1) /1.4

4	Wounds	12	10/83.3	8/66.6	(1)/ 8.3
5	Throat swab	15	12/80	10/66.6	(1)/ 6.6
6	blood	30	2/6.6	2/6.6	(0)/ 0
7	Csf	5	0/0	0/ 0	(0)/ 0
8	Medical devices	12	3/25	3/ 25	(0)/ 0
	Total	300	130/43.3	119/91.5	(15)/ 11.5

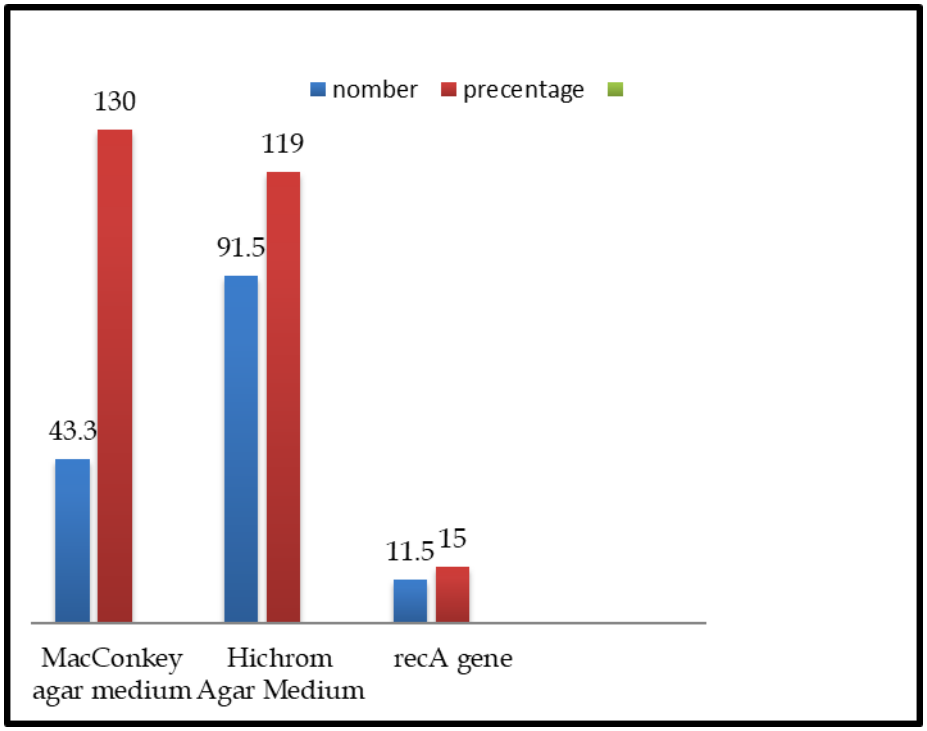


Figure 1. The difference of rate isolation of *A. Baumannii* among macconkey agar, medium, Hichrom Agar Medium, and reca gene.

There is a difference in the isolation percentages between the Hichrom Agar, MacConkey media and *rec*A gene (Table 3, Figure 1). Gene *rec*A gave the lowest isolation percentage at, followed by the Hichrom medium with a high percentage that reached 119/91.5%, but it is somewhat lower than the MacConkey medium, which gave an isolation percentage of 130/43.3%.

The *rec*A gene gave the lowest isolation percentage at 15/ 11.5%, but at the same time it was very accurate, as all isolates that did not show any association with this gene were excluded (Figure 1). The isolation percentage on the Hichrom medium was decreased compared to the MacConkey medium by (about 49%). The negative strains for the *rec*A gene, which were 8 times more than the positive *A. baumannii* strains for the same gene, gave growth on this medium. This indicates that the Hichrom Agar medium is not a selective medium enough to give false positive results. This is consistent with [8], [7].

The contaminated burns and wounds with *A. baumannii* increase mortality especially among little ages or elderly patients in hospitals [14], [15], [16]. It is also due to damaged skin, the first immune barrier and the long period of stay in the hospital, which leads to infection with MDR *A. baumannii* [17]. So, the highest isolation rate was from wound samples at 8.3%. The current study indicated a high rate of *A. baumannii* isolation from urinary infections at 7.5% (Table 3), which is what many recent studies have indicated especially *A. baumannii* [18], [19]. This is due to the excellent ability of *A. baumannii* strains to form biofilm more than other bacterial genera [15], [20].

The community acquired pneumonia (CAP) that caused by *Acinetobacter* spp record by many reports, infection with this resistant bacteria, if it occurs in combination with other infections such as Covid-19 or *Klebsiella pneumoniae*, in addition to a prolonged stay in the hospital, can lead to major complications [21], [22]. This indicates the high rate of isolation also such as throat swabs at 6.6% and sputum at 1.4% (Table 3)**.**

This bacterium was not isolated from blood and CSF due to the fact that the bacteria did not reach these biological fluids with distant anatomical location. They are also considered sterile body fluids that do not become infected unless exposed to contaminated medical devices or surgical procedures [23], [24]. However, this bacterium was not isolated from the medical devices perhaps for the low number of samples, which are only 12 samples or this due to the sterilization by the medical staff before the collection process. The reason for contamination of this equipment by *A. baumannii* is the formation of biofilm on it, and one of the prevention biofilm means is to prevent the first stage, which is the attachment stage whether by sterilizers, detergents, enzymes, patient isolation, reinforced hand hygiene, cleaning and more efficacious disinfection [25], [26].

B. Molecular diagnosis by recA and 16S rRNA

The *rec*A gene has demonstrated high efficiency in diagnosis of suspected isolates using primers that designed for this gene as 2, 3, 8, and 9 lanes in figure 2. As for the remaining suspected as (1, 4, 5, 6, 7, 10, 11, and 12 lanes) isolates which didn't give any amplification with *rec*A primer (Figure 2). We selected 10 isolates randomly from them and amplified with the 16S rRNA gene primer. It was later proven that it belongs to the following bacterial genera by sequencing analysis: *Klebsiella*, *Enterobacter*, *E. coli*, and *Serratia*. Thus, screening of strains that do not belong to *A. baumannii* was confirmed.

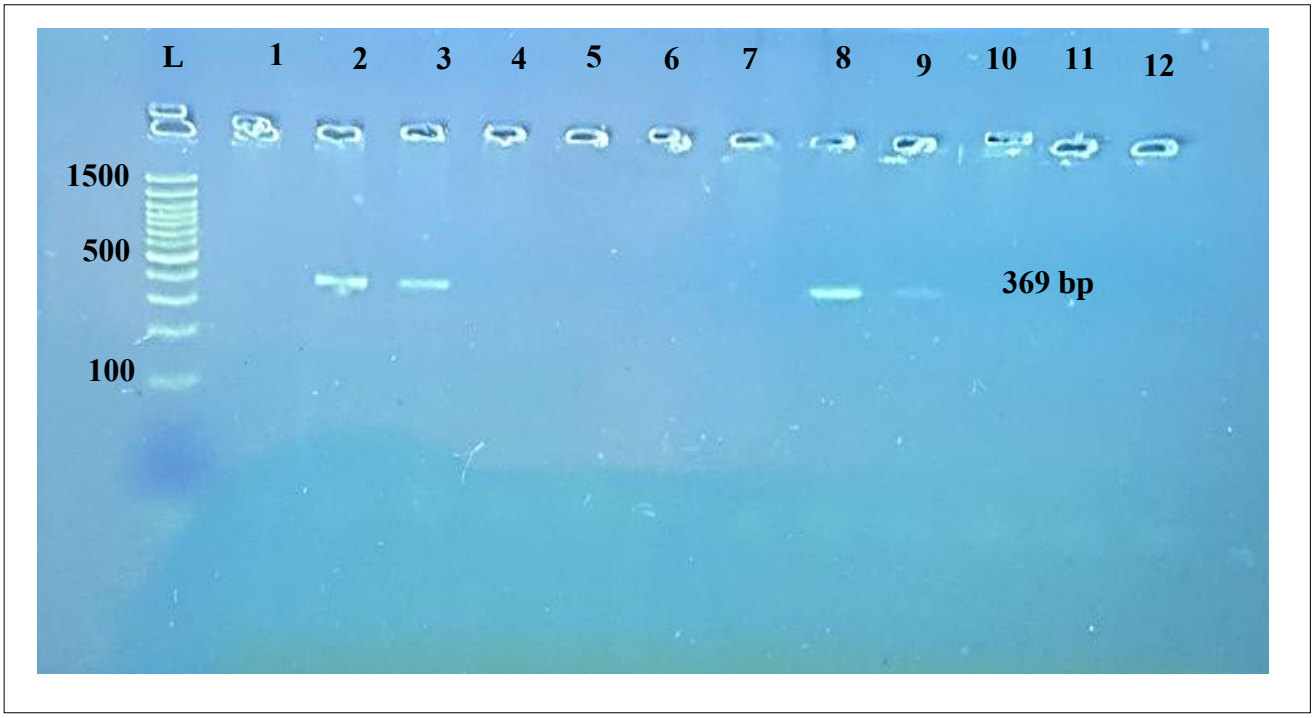


Figure 2. Diagnosis of *A. baumannii* by *rec*A gene at 369 bp.

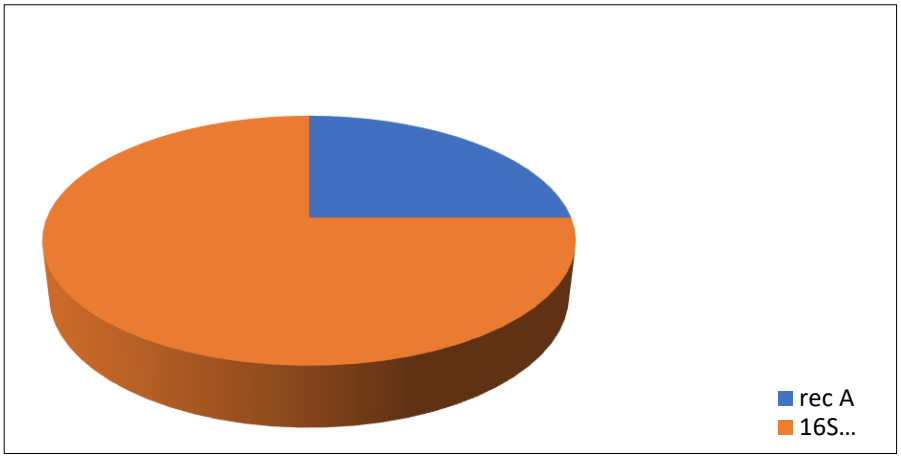


Figure 3. Rate of diagnosis by *rec*A and 16S rRNA from 60 suspected strains.

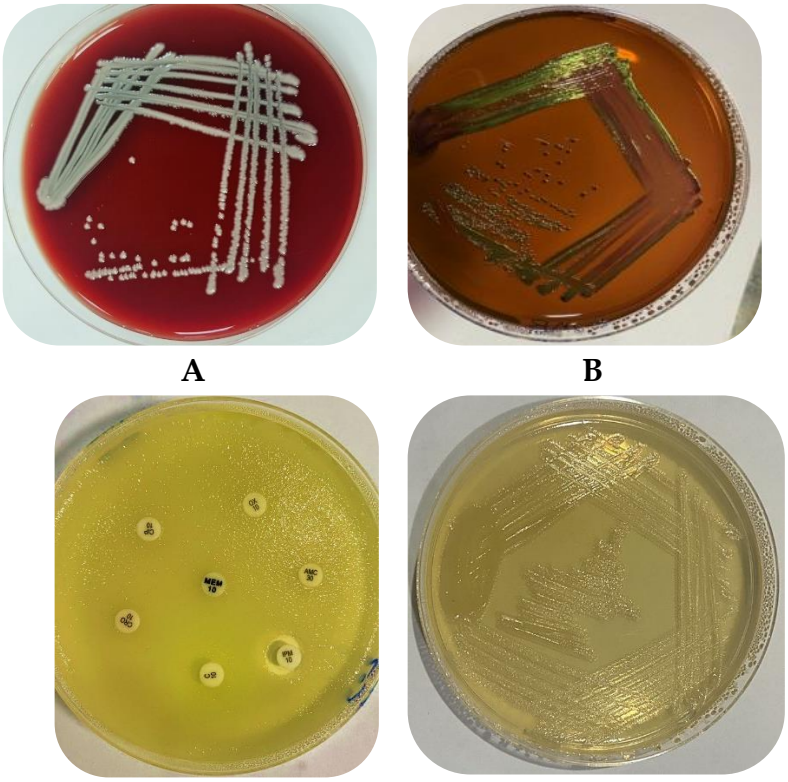
The 60 suspected isolate only 15 from them were identified by *rec*A. It was found that all of the above-mentioned strains belong to *Acinetobacter baumanni*, with high matching rates of up to 99% using NCBI. The *rec*A gene is considered one of the conservative genes that specialize repairing, reconstructing DNA errors, and diverting the process path to the correct one [13], [10], [11]. However, it was very sufficient to prove that the isolated bacteria were *A. baumanni* because the design confirmed by *A. baumanni* strains sequences analysis.

C. Culture media and conditions

The fifteen *A. baumannii* strains molecularly characterized by the *rec*A gene showed high variation in colony morphology, texture, color and appearance as shown in Table 3 and Figure 4.

Table 4. Morphological characteristics of *A. baumannii* strains on different media.

Medium type	Morphology on it	No./ percentage
Hichrom agar	Strong growth	(7)/46.7
	Medium growth	(5)/33.3
	Weak growth	(3)/20
Macconkey	Non fermenate	(6)/40
	Late fermenate	(9)/60
Blood agar	Grey colonies	(12)/80
	White colonies	(3)/ 20
EMB	Sheen metal colonies	(5)/ 33.3
	Brown colonies	(10)/66.7
Nutrient agar	Creamy white color	(11)/73.3
	Small transparent colonies	(4)/26.7
Muller Hinton agar	Light yellow color	(13)/86.7
	Light phosphorus green color	(2)/13.3



A **B**

C D

Figure 4. The colonies of *A. baumannii* strains on: A Blood agar, B: EMB agar, C: Nutrient agar, D: Muller Hinton agar.

The colonies of *A. baumannii* on the blood agar did not show any hemolysis and they were white or leaden colonies (Figure 4: A), which is consistent with [27], [28], noting that the incubation time was 24 hours and the bacteria were not incubated for more than this time. While, there are some studies indicate hemolysis pattern can be occur when blood agar plates of *A. baumannii* were incubated for six days or more [29].

The EMB medium contains the methylene blue dye, which is used to stain microorganisms (Figure 4: B). It is a good medium for the growth of *A. baumannii* [30]. It is also a very efficient medium in growing and diagnosing *A. baumannii*, especially if it is followed by other accurate examinations such as diamond spectrometry [31].

However, some of *A. baumannii* strains on Mueller Hinton agar appear as light phosphorus green growth (Figure 4:C), while other give light yellow or light brown on it. It is known that some strains of *A. baumannii* produce certain pigments, especially on Mueller-Hinton medium (Figure 4: C). The pigments production in these bacteria is still under study by researchers [32], [33], [34].

On a nutrient agar it gives some time a small creamy white color or mostly they gave small circular transparent colonies and the bacteria were preserved on it during the study (Figure 4:D).

CONCLUSION

Fundamental Finding : The study demonstrates that the recA gene is highly effective in diagnosing *Acinetobacter baumannii* strains, surpassing the accuracy of selective Hichrom Agar medium, which occasionally isolated other bacterial genera despite its intended specificity. **Implication :** These findings underscore the importance of molecular diagnostic methods like the recA gene in enhancing accuracy and reliability, especially in clinical and microbiological settings, where accurate identification of *A. baumannii* is critical. **Limitation :** The study's reliance on culture media variability and the unexpected selectivity issues of Hichrom Agar highlight the need for broader evaluations, as these limitations may affect the reproducibility and applicability of the results across different laboratories. **Future Research :** Future studies should focus on exploring additional molecular markers alongside recA and investigating improved culture media formulations to ensure greater specificity and reliability in isolating *A. baumannii*.

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