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Research Article

Molecular and Biochemical Identification of Acinetobacter baumannii isolated from Different Clinical Sources in Kerbala and Najaf Governorates

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Abstract:

Acinetobacter baumannii has emerged as the best recognized and very important pathogen responsible for healthcare-associated infections as a result of its ability to survive under harsh environmental conditions. To investigate the prevalence of *A.baumannii* across various clinical sources and to evaluate selected virulence factors associated with this pathogen, a total of 150 clinical swabs were collected from burns, wounds, urine and sputum samples. These were cultured on blood agar and MacConkey agar, then incubated at 37°C for 24–48 hours. Bacterial isolates were identified based on colony morphology, cultural characteristics and standard biochemical tests, including catalase, oxidase and Simon's citrate tests. Virulence factors, including hemolysis, protease production, and polysaccharide capsule formation, were also evaluated using qualitative methods. The results showed that out of 120 bacterial isolates, 30 (25%) were identified as *A. baumannii*, whereas the remaining 90 (75%) were belonging to other bacterial genera. The *A. baumannii* isolates were Gram-negative, non-motile and exhibited no hemolytic activity on blood agar. Biochemical testing confirmed that all the isolates were catalase and citrate positive. Confirmation of *A. baumannii* was performed using the VITEK 2 system and polymerase chain reaction (PCR) by targeting a specific gene namely blaOXA-51 (353 base pairs in size), The results confirmed that 30 (100%) of the isolates were *A. baumannii*. The identified bacteria was also investigated for various virulence factors, the results revealed that all the isolates were incapable of producing hemolysin, whereas 25 (83.3%) of the tested becteria were positive for the protease qualitative test and all the isolates produced a polysaccharide capsule.

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Introduction

Subsequent to the United States invasion of Iraq in 2003, US military surgeons began documenting an “invisible enemy" they referred to as "*Iraqibacter*." The name referred to the bacterium *A.baumannii*, which was significantly infecting injured US military personnel [1]. The bacteria is one of the pathogens that comprise the ESKAPE group: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A.baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*.

A. baumannii is a Gram-negative, non-motile, strictly aerobic coccobacillus that is catalase-positive and oxidase-negative [2]. *A. baumannii* is a member of the Moraxellaceae family and is classified within the Acinetobacter calcoaceticus baumannii complex [3, 4]. *A. baumannii* is significantly associated with an increased mortality risk due to its multidrug resistance [5, 6]. A prevalent pathogen in intensive care units, associated with hospital-acquired pneumonia and ventilator-associated pneumonia in patients with prolonged hospitalizations [7]. *A. baumannii* is implicated in hospital-acquired infections, including wound infections, burn infections, skin ulcers, septicemia, urinary tract infections, secondary meningitis, and infective endocarditis [8].

Wound infections include clinical wound infections, severe soft tissue infections, bite wound infections, burn wound infections, diabetic foot ulcers, and pyogenic wound infections. Moreover, wounds may require an extended duration to heal and may proliferate due to human behaviors. They are exacerbated in individuals with diabetes, obesity, or cardiovascular disease. Burns are recognized as the fourth most prevalent type of global shock that damages bodily tissues, especially the skin [9]. Moreover, burn injuries result in prolonged hospitalization and these patients have a higher susceptibility to nosocomial infections. [10]. Diabetic foot infections are characterized by the invasion and proliferation of bacteria in host tissues, producing an inflammatory response and subsequent tissue destruction [11]. The bacteria possesses multiple virulence factors, including biofilm formation, lipopolysaccharides (LPS), vesicles, proteins, capsules, phospholipases, proteolytic enzymes, siderophores, and efflux pumps [12]. This study aimed to the isolation and identification of *A.baumannii* from different clinical sources, as well as the investigation certain virulence factors that contribute to its pathogenicity.

Materials and Methods

Collection of Specimens

This study involved the collection of 150 distinct clinical samples from patients at Imam Hussein Medical City and several private clinics within the Holy Governorate of Karbala and Najaf in Iraq. The samples included wounds, burns, sputum, and urinary tract infections from both sexes over the period from August 2024 to October 2024. The samples were obtained using sterile cotton swabs with transport media to maintain microbial viability during transit and were gently rotated over the affected region. The swabs were subsequently transported to the microbiology laboratory for isolation and testing.

Isolation and identification of microorganisms

The collected samples were spread on the MacConkey and blood agar and incubated at 37°C for 24 to 48 hours, The isolated bacteria were differentiated and the target species were diagnosed based on cultural, morphological and biochemical characteristics. In addition, *A.baumannii* isolates identification was confirmed using the Vitek 2 compact system and PCR assay.

Microscopic examination:

On the surface of a sterilized glass slide, a tiny quantity of cultivated bacteria was applied together with a few drops of distilled water by using a sterile loop. The cells were spread on the slide surface and the slide was dried over a flame. They were then Gram stained; a differential dye was used to stain them. After that, they were examined at 100X magnification using an optical microscope to observe the morphology of the bacterial cells and their reaction with the Gram stain. [13]

Biochemical tests

The following biochemical tests were carried out on the isolated colonies of *A.baumannii*:

Oxidase test

A piece of filter paper is moistened with a solution of tetramethylphenylenediamine dihydrochloride solution (BDH-England), then the tested bacterium streaked firmly on the filter paper with a sterilized glass rod. The positive result is indicated by the appearance of violet or purple color within 10 seconds [14].

Catalase test

From 18-20 hour isolated bacterial growth, single colony picked up from blood agar by a sterile loop or wooden tooth pick and placed on a sterile microscope slide, then a drop of 3% H_2O_2 placed on the bacteria by using a dropper. The formation of gaseous bubbles indicated the positive result [15].

Urease test

Colonies from each isolate were added to tubes containing 5 ml of urea medium (Oxoid, England), and the tubes were then incubated for 24 hours at 37°C. The medium color turning pink, a sign that bacteria can synthesize the urease enzyme, indicates a positive response [16].

Motility test

A single fresh colony of each *A. baumannii* isolates was picked from blood agar using the tip of a plastic loop and stabbed into nutrient agar medium (Himedia, India) in a polystyrene test tube, followed by incubation for 24 hours at 37ºC. After growth, motile bacteria would spread, representing the positive result [17].

Indole production test:

Fresh colonies of *A. baumannii* added to tubes filled with peptone water. After that, the tubes incubated for 24 hours at 37°C, then 4-5 drops of Kava's reagent added. The appearance of a red ring at the top of the medium , which denotes the breakdown of amino acid (tryptophan) and the generation of indole, this indicates a positive outcome [18].

Voges-Proskauer test:

After incubation of bacterial cultures in 5 ml of MR-VP broth at 37°C for 24 hours , 0.6 ml of alpha-naphthol solution (BDH, England) and 0.1 ml of potassium hydroxide solution were added into each tube. Afterward, the tubes were shaken and then left for thirty minutes. The medium turning red indicates a successful reaction and demonstrates that bacteria are capable of fermenting glucose to produce acetyl methyl carbonyl [19].

Methyl red test:

Two to three colonies of bacteria were added to a tube containing MR/VP broth (Oxoid-England), After 24 hours of incubation, 4-5 drops of methyl red reagent should be added. The medium's ability to turn reddish pink, indicate the tested bacteria are capable of producing acids, which is a sign of a positive result [19].

Citrate Utilization Test:

A.baumannii colonies were inoculated in tubes containing Simmon citrate medium (Oxoid-England) slant in order to perform this test. After that, incubation for 24 hours at 37ºC. The medium's hue changing from green to blue shows that the bacteria can successfully use sodium citrate as its only carbon source and indicates a positive result [20].

Coagulase test

A tube containing 1ml of human plasma inoculated with the bacterial colonies . Following that, the tube incubated at 37ºC and checked at half or one hour intervals. Then, any degree of coagulation is considered a positive test for the free coagulase enzyme [21].

Diagnosis of bacteria using the Vitek2 compact system

A.baumannii isolates in this investigation were identified using the Vitek 2 system (Biomerieux, France) in accordance with the manufacturer's guidelines. Colonies cultivated on the prepared medium for 18 to 24 hours were transferred into a tube containing three milliliters of sterile saline solution to formulate the bacterial suspension. The turbidity of the bacterial suspension was determined using the Densi device to match with the standard McFarland solution turbidity (0.5), then the tube was placed into the equipment beside the Salicram GN-ID diagnostic card for microorganisms. The diagnostic results were displayed on the computer screen after four to twelve hours [22].

Molecular detection of *A. baumannii* isolates

DNA extraction was performed using the Genomic DNA extraction kit (Microgen-Korea). The PCR was performed using specific primers (Macrogen, Korea), as shown in Table 1. The reaction volume was set at 25 µL; a master mix comprising 10 µL of Taq master mix kit (Bioneer-Korea). Two microliters of forward and reverse primers at a concentration of 10 pmol/µL, three microliters of target DNA and eight microliters of nuclease-free water. The PCR technique included an initial denaturation phase of 3 minutes at 95°C, followed by 33 cycles consisting of template DNA denaturation for 30 seconds at 94°C, primer annealing for 33 seconds at 53°C, and an initial extension of 55 seconds at 72°C. The procedure conducted with a last 5-minute extension at 72°C to guarantee complete synthesis of the DNA strands. Electrophoresis was performed on a 1% agarose gel containing ethidium bromide (Bioneer-Korea) at 50 volts for 1 hour, followed by examination under UV light to confirm the presence of PCR products.

Table 1: The primers used to diagnose *A.baumannii* by the polymerase chain reaction (PCR)

Primer	Target Gene	Primer sequence	Product size (bp)	Annealing Tem.	References
Forward Reverse	blaOXA-51	TAATGCTTGATCGGCCTTG TGGATTGCACTTCATCTTGG	353	53	[23]

Screening phenotypes for specific virulence factors:

Production of hemolysin

To evaluate hemolytic activity, single colony of each isolate were streaked onto blood agar medium supplemented with 5% human blood and cultured for 24 hours at 37°C. Following the growth, surrounding area of the colony was examined [24].

Capsule Staining

A few bacterial colonies were collected, combined with a drop of nigrosine, then spread over a microscope slide to form a thin film. Following air drying, slides were dyed with 1% Crystal Violet (Merck, 115940) for approximately 1 minute. After that, the slides were allowed to air dry. Photographs of dyed bacteria were captured using an Olympus IX53 inverted and light microscopes at a magnification of 100x . [25].

Production of protease

Skim milk agar medium (Himedia, India) was used to evaluate the protease production capacity of *A. baumannii*, using a 5 mm cork borer (Kotterm, Germany) to form three wells in the skim milk culture medium. Following the extraction of the agar discs, 0.1 ml of the bacterial culture broth was introduced into the well using a micropipette (Afco-Dipo Jordan), the plates were incubated at 37°C for 18-24 hours. The ability to synthesize protease was assessed by the presence of a distinct halo zone around the colonies [26].

The results

Diagnosis of bacterial isolates

150 swabs were obtained from clinical sources, including wound swabs, burns, urine and sputum. The samples were collected from 86 male patients (57.33%) and 64 female patients (42.66%). Out of the total samples, 120 microbial growth (86.66%) were collected, whereas the remaining samples exhibited no growth. Wound swabs constituted the biggest quantity with 75 samples in both males and females, burn swabs with 40 samples, urine with 20 samples and sputum samples with 15 samples in both sexes.

One hundred and twenty bacterial isolates were identified on the genus level, comprising 30 (25%) *Acinetobacter spp*, 36 (30%) *Pseudomonas spp*., 17 (14.16%) *Staphylococcus spp*., 10 (8.33%) *Escherichia coli*, 26 (21.66%) *Klebsiella spp*., 27 (22.5%) *Proteus spp*., 10 (8.33%) *Burkholderia spp*, 9 (7.50%) *Enterobacter spp*., and 14 (9.33%) *Aeromonas spp*., as shown in Figure 1.

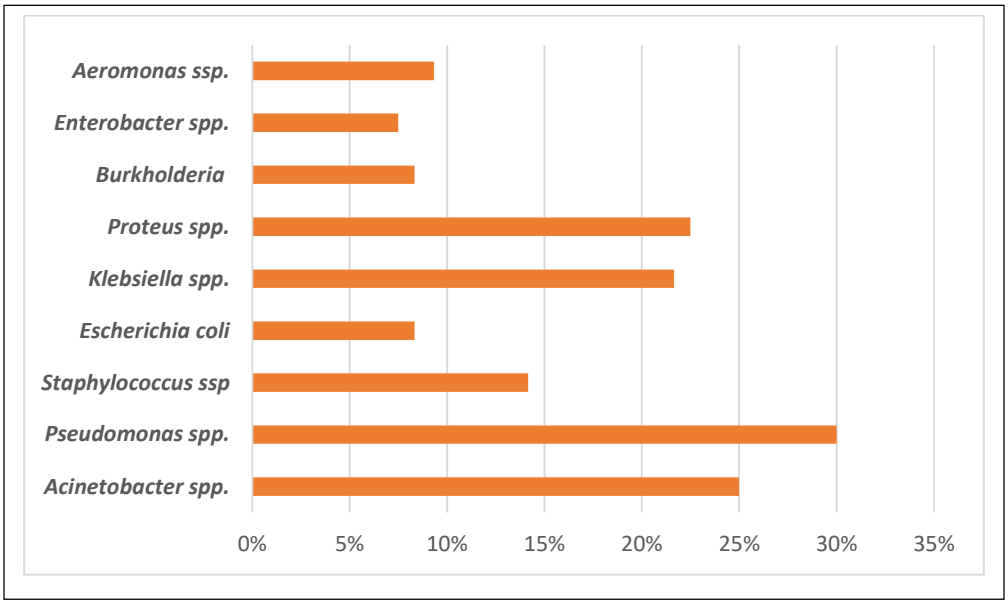

Figure 1: prevalence of different bacterial genera isolated in this study**.**

After that, 30 isolates of *A.baumannii* were identified, representing 25% of the total, with 14 out of 75 (18.66%) isolates resulting from wounds, 11 out of 40 (27.5%) from burns, 3 out of 20 (15%) from urine and 2 out of 15 (13.33%) from sputum, as illustrated in Figure 2.

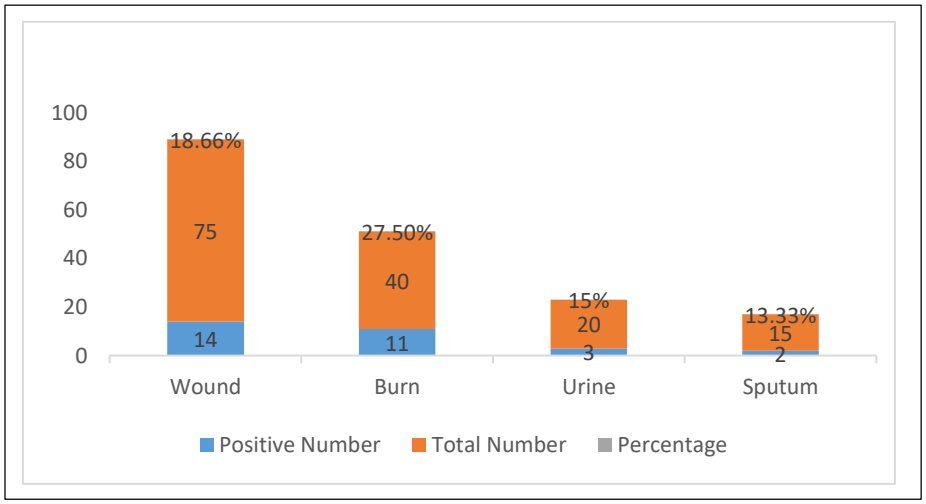

Figure 2 : Distribution of *A.baumannii* according to clinical specimen.

The identification results indicated that *A. baumannii* isolates grow on blood agar and MacConkey agar. On blood agar, it produces nonhemolytic, opaque, round, greyish colonies that are shiny and mucoid in appearance, smooth in texture, with a diameter of 1–2 mm after 18–24 hours of incubation at 37°C.

The bacteria generates pale yellow to pink colonies on MacConkey agar that are glossy, mucoid, opaque, and round, demonstrating its non-lactose-fermenting characteristic.

The microscopic analysis revealed that the isolated bacterial cells are small, exhibit a spherical bacillus morphology, arranged in pairs or may exist singly and Gram-negative.
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The bacteria were cultivated on a solid culture medium, and the procedure was replicated for each isolate, initially at 44°C. The findings indicated that 30 isolates proliferated at 44°C, a trait indicative of *A. baumannii*.

Chemical tests showed positive results for catalase, and citrate consumption tests, whereas negative results were reported for the oxidase, the Voges-Proskauer, methyl red and indole tests. The urease test exhibited variable results and the growth outcomes on TSI medium indicated that the bottom of the tube turned yellow, as illustrated in Table 2. The diagnosis was subsequently verified using the VITEK 2 system.

Table 2: Biochemical tests for *A.baumannii*

Test	Result
Lactose Fermentation	-
Hemolysis Production	-
Growth in 44C	+
Gram Stain	-
Oxidase	-
Catalase	+
Urease	**Variable**
Indole	-
Methyl red	-
Vosges_proskaure	-
Citrate utilization	+
Motility	-

In the molecular identification results, PCR technique using the *blaOXA-51* gene primer pair specific to *A. baumannii* produced PCR products (353 base pairs in size). The assays were performed on 30 isolates of *A. baumannii* identified by conventional methods.

The PCR results are summarized in figures 3 and 4.

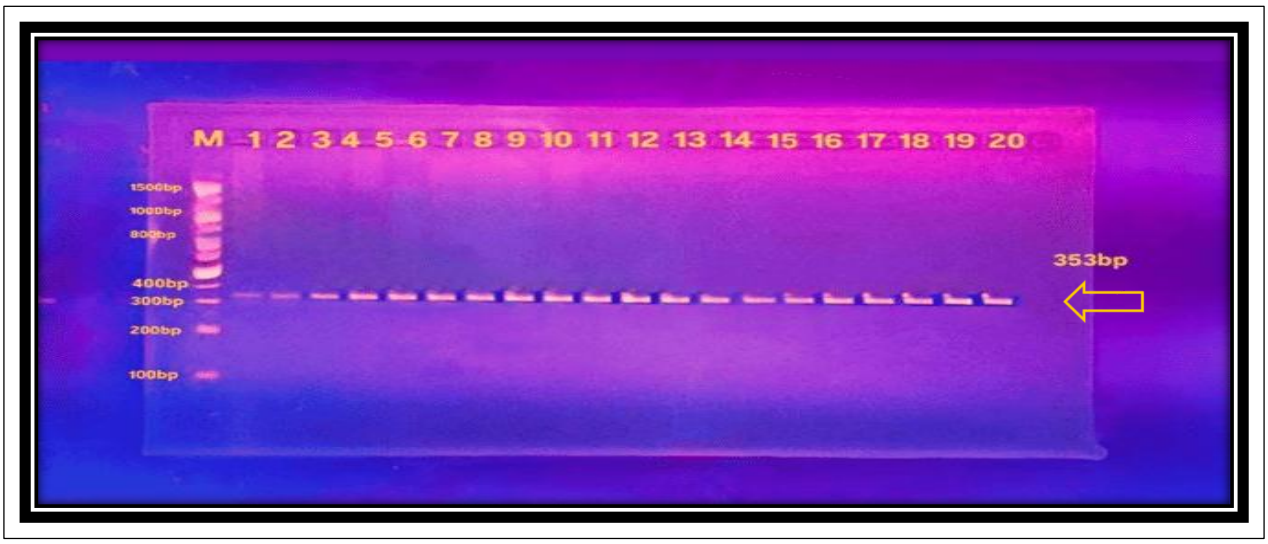

Figure 3: Electrophoresis of PCR products of S1-S20 *A. baumannii* isolated from different clinical sources using the blaOXA-51 gene-specific primer (353 bp) in 1% agarose gel at 50 V for 1 hour.

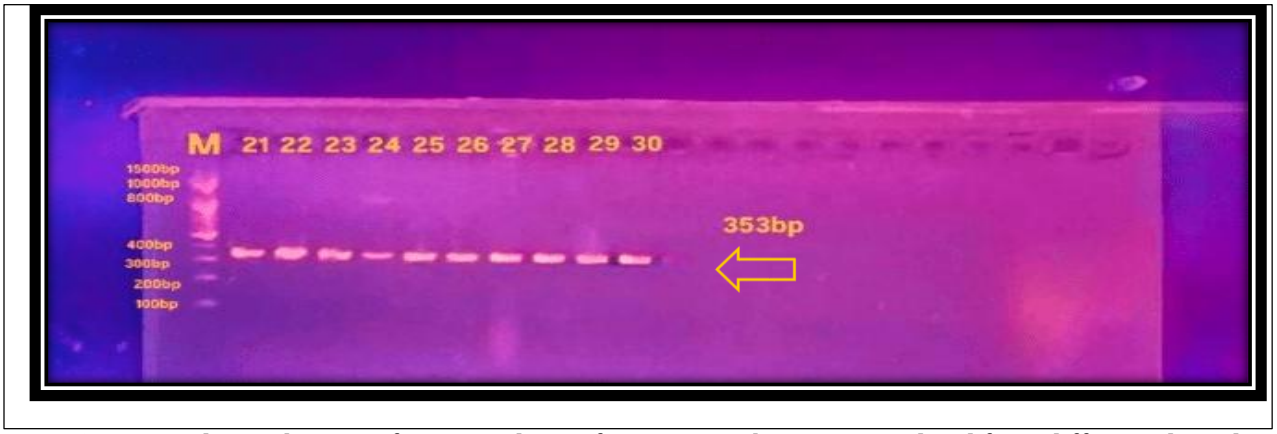

Figure 4: Electrophoresis of PCR products of S21-S30 *A. baumannii* isolated from different clinical sources using the blaOXA-51 gene-specific primer (353 bp) in 1% agarose gel at 50 V for 1 hour.

A. baumannii was examined for various virulence factors. The findings indicated that all isolates were classified as non-hemolytic species (figure 5A). Additionally, 25 out of 30 (83.3%)isolates tested positive for protease production, evidenced by a transparent zone surrounding the colonies due to casein degradation (figure 5B). Furthermore, all thirty isolates (100%) produced a polysaccharide capsule for environmental protection (figure 5C).

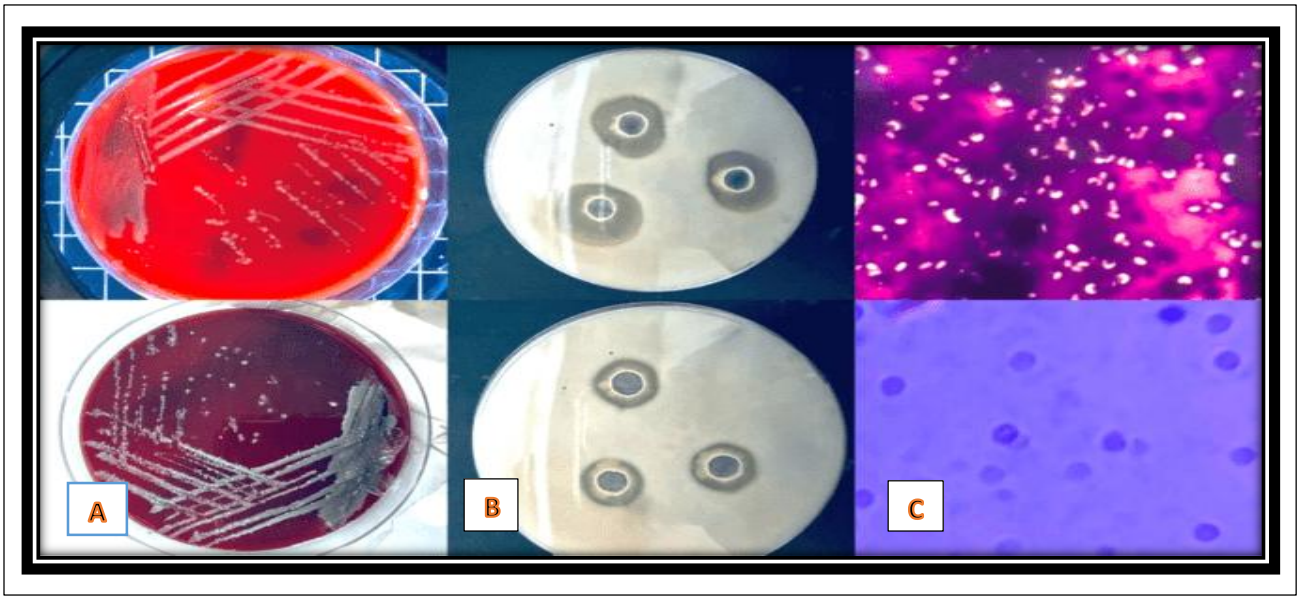

Figure 5: A. Growth of non-hemolytic *A.baumannii* on a blood agar plate. B. Protease enzyme test on skim milk agar. C. *A.baumannii* capsule observed under both light and inverted microscopes, showing a clear halo around the cell.

Discussion

A. baumannii a prominent opportunistic pathogen in nosocomial infections and its importance has increased with infection rates, especially among patients returning from conflict zones, in addition to the emergence of multiple drug-resistant strains and its dangerousness is due to ability to survive in the hospital environment and growing resistance to antibiotics, making it a leading cause of infection in intensive care units [27, 28].

A. baumannii was isolated from 25% of the clinical samples (Figure 1), including wounds, burns, sputum, and urine. This finding is consistent with studies, highlighting the bacterium persistence in hospital environments [29, 30, 31]. However, the isolation rate disagree with another study [32]. Burn sample showed the highest *A.baumannii* isolation rate (Fiigure2) this agree with [34]. Wound and sputum isolation rates varied across studies, ranging from 15% to 29% [35,36]. Differences in *A.baumannii* isolation rates result from variations in sample types, geographic regions, infection control measures, diagnostic methods, and the season of sample collection [37].

PCR was used in this study to detect the *blaOXA-51* gene ,one of the important genes used to identify *A.baumannii* [23]. Figures 3 and 4 show the electrophoresis of PCR products, from which it can be seen

thoroughly that the primer of the gene was successfully amplified 353bp product in 100% of the isolates, indicating high sensitivity and specificity compared to biochemical tests. The results agree with the majority of other investigations that identified the *blaOXA-51* gene in all clinical isolates of *A. baumannii*, but it was not observed in other *Acinetobacter spp* [38, 39]. The high prevalence of the *blaOXA-51* gene is associated with its widespread nature, innate characteristics and chromosomal localization, which reveals its function in the exact identification of *A. baumannii* [40, 41]. The *blaOXA-51*-like gene's presence is not correlated with carbapenem resistance levels in *A. baumannii* isolates, as it is regulated by the insertion sequence ISAbal upstream of this gene, providing a promoter for *blaOXA-51* gene expression. Alternatively, these genes may only be incapable of expression when the bacteria exist within the patient's body [42,43].

As shown in figure 5 *A. baumannii* is exhibited no hemolytic activity on blood agar, consistent with its classification as a non-hemolytic species [44]. This agree with study evaluating 195 isolates, revealing 3.6% with weak hemolytic activity, There results confirm that hemolysis is neither common nor characteristic of this species [45].

This study revealed that *A. baumannii* exhibited an 83% protease production rate, this finding agree with study that demonstrated the important enzymatic capacity of this bacterial species [46], A study confirmed that the production of protease by *A. baumannii* is affected by variable factors such as pH, temperature and carbon source type, as a result emphasizing the importance of the conditions employed in this study that resulted in a notable production rate [26]. Moreover, the results were compared between two investigations ,the percentage of protease production on skim milk agar were 60% and 100%, respectively [47, 48].

A. baumannii is a highly adaptable and antibiotic resistant Gram-negative bacterium, with its most significant virulence factor being the capsule, a polysaccharide coating bacterial cells, protect them from phagocytosis, antibiotics and dehydration [49]. Several researchers have emphasized the importance of the capsule in increasing *A.baumannii* survival and pathogenicity [50]. The capsule has been identified as a critical virulence factor, playing a central role in evading host immune defenses, Genetic heterogeneity at the K locus, which regulates capsule biosynthesis, adds further complexity [51], who investigated the variability in polysaccharide composition across different *A. baumannii* strains. *Acinetobacter* species possess a polysaccharide coating that safeguards cells against direct lysis by complement or antimicrobial agents, as well as from phagocytic destruction [52].

Conclusion

A. baumannii was identified in clinical samples, confirming its role as a pathogen associated with healthcare-associated infections. Isolates showed no significant differences in prevalence between sample types or between genders. The prevalence of *blaOXA-51* was recorded in all isolates, confirming its importance as a reliable molecular marker for identifying *A. baumannii*. The isolates showed some virulence factors, including protease production and capsular formation, whereas lacking hemolytic activity, emphasizing the need for continued monitoring and research on this pathogen and to develop accurate diagnostic strategies and improve infection control.

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