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Molecular detection of some virulence gene in *Acinetobacter* spp. isolated from different drinking water samples in AL-Najaf Al-Ashraf Province

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Abstract---Contamination of drinking-water is a significant concern for public health throughout the world. Microbial hazards make the largest contribution to waterborne disease in developed and developing countries. Biofilm-producing bacteria were identified by the Congo Red Agar (CRA) method we obtained isolated of *Acinetobacter* spp. had ability to biofilm formation in medial , the antibiotic resistance screening test to a number of antibiotics on *Acinetobacter* spp. isolates. The results revealed that isolates of *Acinetobacter* spp showed a high resistance to amoxicillin, levofloxacin, cefoxitin and imipenem (100 %) followed by amoxicillin and clavulanic and cefoxitin in mediate resistance in *Acinetobacter* spp , while erthromycin did not show any resistance by *Acinetobacter* spp. the results indicated that the *Ampc* gene wasnot present in *Acinetobacter baumannii* and *Acinetobacter lwoffii* ,while the results indicated that the *MotA* gene was presented in *Acinetobacter lwoffii* as well as the *MotB* gene was presented in *Acinetobacter baumannii* and *Acinetobacter lwoffii*)

Keywords---*Acinetobacter* spp., *Ampc*, *MotA*, biofilm.

Introduction

These bacteria belong to the genus *Acinetobacter* which are characterized as coccobacilli, Gram-negative, prevalent in water and soil , isolated from the mucous membranes, skin, and hospital environment (Brooks, 2013), non-motile, obligate aerobic, all species were negative for oxidase test, positive for catalase

test (Bergogne-Bér  zin *et al.*, 1996; Medhat and Aljanabay, 2022) . This species was first isolated by Bowman in 1968. It is considered a pathogen, as it is a pathogen that causes hospital-acquired infections, especially in the intensive care unit (Munoz-Price *et al.*, 2010). And the infections they recorded among the Americans led the military to interest in this pathogen until it acquired the designation Iraqi bacteria (Shea, 2012; Hadi and Aljanaby, 2022), and it is clinically one of the most difficult pathogens to treat because it is the second negative pathogen after *Pseudomonas* in clinical isolates (Yang *et al.* , 2010). *A. baumannii* can be distinguished from the rest by the fact that it grows at a temperature of 44 C  . It is not necessary to extend to the middle of the solid blood (Dijkshoorn *et al.*, 2011). This species shows the ability to grow on dry surfaces and for long periods of time, which is one of the reasons for its spread, and is characterized by its ability to multi-resistance to antibiotics, and it is similar to *Staphylococcus aureus* (MRSA). Several multi-resistant infections have been recorded in the Middle East (Al-Hassan, 2012; Al-Hadraawy *et al.*, 2022) .

A. baumannii is an opportunistic pathogen that causes many of the infections that threaten the health, especially in immunocompromised patients, or their injuries pose a challenge to doctors, as it is difficult to treat them with antibiotics . Available, especially after the emergence of isolates with multiple resistance and Extensively Drug-Resistant (XDR). (Shields *et al.*, 2011) . The virulence factors that Pathogenicity: outer membrane proteins, biofilm formation, capsule and external membrane vacuoles and phospholipase enzyme . (Cerqueira and Peleg, 2011) . *A. baumannii* is responsible for many injuries, including wound infections, and burns (Zander *et al.*, 2012) , urinary tract infection , otitis media (Pachon-Ibanez *et al.*, 2010; Aljanaby *et al.*, 2022). It is also one of the causes of pneumonia associated with gastric ulcers. ventilator-associated pneumonia (Kosmidis *et al.*, 2012). In addition to bacteriuria (Zhong *et al.*, 2012), and meningitis and infect patients with infected syphilis and cystic fibrosis, and it also causes endocarditis , frozen and soft tissue injuries (Lee *et al.*, 2008; Alhasnawi and Aljanaby, 2022) . Infections of *A. baumannii* are often associated with injuries from war and natural disasters (Mihu and Martinez, 2011). The factors that contribute to the survival of *A. baumannii* in the hospital environment include multiple resistance antibiotics, resistance to drought and detergents, ability to colonize non-living surfaces and grow in the form of biofilm (Kurcik, 2009).

The aim of this study: Molecular detection of some virulence genes in pathogenic bacteria isolated from different samples of drinking water in Al-Najaf Governorate.

1. Samples of drinking water were collected randomly from different areas of Najaf Governorate.
2. Determination of bacterial isolation using biochemical properties and vitek2 technique.
3. Identification of common bacteria isolated
4. Detection of some virulence factors for common isolate bacteria and the presence of genetic coding for some virulence factors using PCR technique.

Methods and Materials

Sample

A total of 120 water sample were achieved during the period from October 2021 to March 2022. water sample collected from drinking water from a different region in Al-Najaf city. The samples were placed in separate sterile plastic bags before being immediately transported to a cool box filled with ice. All samples were transferred to the laboratory and cultured on MacConkey agar medium for 24 hours at 37° C. Isolates were purified several times until pure isolates were obtained, then subjected to microscopic and special biochemical tests before being transferred to VITEK 2 for identification.

Congo Red Agar (CRA)

It was made by mixing 52 grams of Brain heart infusion agar media with a liter of distilled water and autoclaving it for 15 minutes at 121°C/15 pressures. Congo red stain (0.8 g/L) was made as a concentrated aqueous solution and autoclaved for 15 minutes at 121°C, while sugar (50 g/L) was sterilized by filtering. After cooling to 55°C, both dye and sugar were added to the agar. After that, plates were infected and incubated aerobically at 37°C for 24 hours to detect biofilm formation (Freeman *et al.*,1989). Black colonies with a dry crystalline quality indicated a positive result. Weak slime producers stayed pink most of the time, however there was some darkening in the colonies' cores. An uncertain result was indicated by the darkening of the colonies in the absence of a dry crystalline colonial morphology.

Antibiotic Sensitivity Test: This test performed by Kirby-Bauer method

Extraction and Isolation of DNA

DNA of *Acinetobacter spp.* isolates was prepared by boiling method. In brief, colonies were suspended in 100 microliters of sterile distilled water, boiled at 100C in a water bath for 15 minutes, then rapidly cooled at -20C for one hour, centrifuged, and the supernatant was saved for use in the amplification processes (Shah *et al.*, 2017).

PCR amplification was used to identify the presence of genes. The primer used in this study was show in (Table 1).

Table 1
The sequence of Primer that were used in the present Study

Primer Type	Primer Sequence (5'-3')	Amplicon size(bp)	Reference
<i>MotA</i>	F:GATGGTGACGGGGAATATGAA R:CCATTTCCCCAGCAGGTCTA	215	Cusick <i>et al.</i> , (2012)

<i>MotB</i>	F:GCGTTACGTCCACATCTCAA R:ATGTCGCGCATATAGGGTTC	150	Maisuria <i>et al.</i> , (2015)
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Each 25 µl of PCR reaction mixture for PCR contained 2.5µl of upstream primer, 2.5µl of downstream primer, 2.5µl of free nuclease water, 5 µl of DNA and 12.5µl of master mix thin walled PCR tube. The Thermal cycler conditions were as follow in (Table 2).

Table 2
PCR Program that Apply in the Thermo-Cycler

Gene	Temperature (C°) / Time					Cycles Number
	Initial Denaturation	Condition of one cycle			Final Extension	
		Denaturation	Annealing	Extension		
MotA	94\3 min.	94 \30 sec.	52\30 sec.	72\1min.	72\5min.	30
MotB	94\3 min.	94 \30 sec.	52\30 sec.	72\1 min	72\5min	30

Results and Discussion

Identification of Bacterial Species that Isolated From Drinking Water

After collecting drinking water samples, they were cultured on differential media (blood agar, mannitol agar and MacConkey agar) to obtain pure isolates. Many bacterial isolates were identified using Gram stain test, bacterial culture, morphology and biochemical tests, where the bacteria were isolated and examined in the laboratory according to McFadden (2000). Total *Acinetobacter spp.* 9 isolates, they were grown on the MacConkey agar in small, circular colonies. Regular, smooth , pale, some of them appear sticky, and not fermented to the sugar lactose ,on blood agar, gray colonies ,non-beta-hemolytic because it do not produce hemolysin ,Table(3).

The results of the microscopy examination showed the isolated bacteria were small, with almost round, rod-shaped in pairs, or they may be single . The results of the biochemical examinations were revealed, as the isolates positive results for each of the oxidase ,catalase and citrate consumption test, while the result was negative for oxidase , indole , methyl red , and voges proskauer.

Table 3
Detecting of Number and Percentage of Bacterial Isolates from Different Samples

NO.	Type of bacteria	Number of Bacterial Isolates	Percent (%)
1-	<i>Acinetobacter baumannii</i>	5	55.5
2-	<i>Acinetobacter lwoffii</i>	4	44.4

3-	Total	9	100
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Biofilm Production by using Congo-Red Agar Method

Congo-Red Agar Method and Tube method was used to investigate the biofilm production, In this study showed that *Acinetobacter* spp. had ability to biofilm formation in medially, this results were disagreed with the result study of Almayali *et al.*, (2017) they showed that of all isolates were biofilm producers. Any microbe including primary and opportunistic pathogens present in water may attach or become enmeshed in the biofilm. However, the survival time for many pathogens in biofilms is uncertain and likely varies depending on the organism. Aquatic microbes are well-adapted to the low nutrient level and cool water temperature of the distribution system. In nature, bacteria can exist in planktonic and biofilm embedded state (Ghaima *et al.*, 2017). Biofilm formation an alternative lifestyle in which microorganisms adopt a multicellular behavior that facilitates and/or prolongs survival in diverse environmental niches. Biofilms form on biotic and abiotic surfaces both in the environment and in the healthcare setting. In hospital wards, the formation of biofilms on vents and medical equipment enables pathogens to persist as reservoirs that can readily spread to patients. Inside the host, biofilms allow pathogens to subvert innate immune defenses and are thus associated with long-term persistence (Kostakioti *et al.*, 2013)

Antimicrobial Susceptibility Test

The antimicrobial sensitivity test of *Acinetobacter* spp. showed a high resistance to amoxicillin, levofloxacin, cefoxitin and imipenem (100 %) followed by amoxicillin and clavulanic and cefoxitin in mediate resistance in *Acinetobacter baumannii*, while erythromycin did not show any resistance by *Acinetobacter baumannii* as in Table (4). This is disagreement with (Narten and Maïke, 2012) that *Acinetobacter baumannii* showing (13 %) multi-drug resistant strains..

Development of multidrug resistance by *Acinetobacter baumannii* isolates requires several different genetic events, including acquisition of different mutations and/or horizontal transfer of antibiotic resistance genes. Hypermutation favours the selection of mutation-driven antibiotic resistance in *Acinetobacter baumannii* strains producing chronic infections, whereas the clustering of several different antibiotic resistance genes in integrons favors the concerted acquisition of antibiotic resistance determinants. Some recent studies have shown phenotypic resistance associated to biofilm formation or to the emergence of small-colony variants may be important in the response of *Acinetobacter spp* populations to antibiotics treatment (Cornelis, 2008).

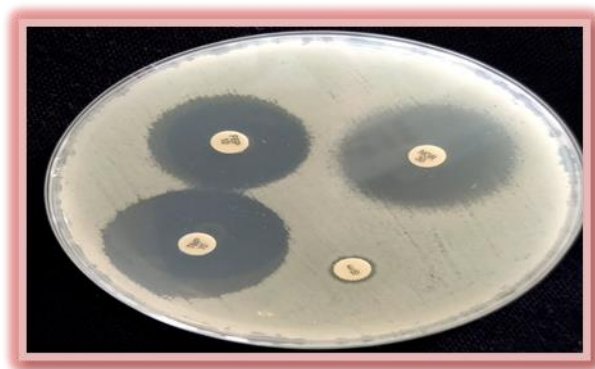
The susceptibility of bacterial isolates was tested against antibiotics that commonly used in the treatment of infections, based on the Kirby-Bauer disk diffusion method on Muller-Hinton agar. The results had been recorded based on measured the diameter of inhibition zones and then compared with (CLSI, 2021), The results showed different susceptibility towards tested antibiotics Table (4) fig(1). many studies reported that the development of drug-resistance bacteria is due not only to the presence of drugs in the aquatic environment, but also to the

density of resistance bacteria, antibiotic exposure time, and nutrient enriched environment . Long exposure time to sub therapeutic dose of antibiotics potentially leads to creating suitable conditions for resistance gene transfer. Furthermore, many studies documented the prevalence of amoxicillin and ciprofloxacin resistant bacteria in river water, waste water, and drinking water.

Table 4
Antibiotic resistance of bacteria isolates from drinking water sample using disc diffusion method

NO.	Type of Antibiotics	<i>Acinetobacter baumannii</i> n=5	<i>Acinetobacter lwoffii</i> n=4
1	Amoxicillin (AM)	5(100%)	4(100%)
2	Amoxicillin/clavulanic acid(AMC)	4(80%)	4(100%)
3	Imipenem (IPM)	5(100%)	3(75%)
4	Amikacin (AK)	1(20%)	2(50%)
5	Tobramycin (TOB)	3(60%)	3(75%)
6	Erythromycin (E)	0(0%)	3(75%)
7	Norfloxacin (NOR)	1(20%)	2(50%)
8	Levofloxacin (LEV)	5(100%)	1(25%)
9	Trimethoprim (TMP)	3(60%)	3(75%)
10	Cefepime (FEP)	4(80%)	0(0%)
11	Meropenem (MEM)	1(20%)	4(100%)
12	Cefoxitin (CTX)	5(100%)	3(75%)
13	Vancomycin (VAN)	4(80%)	4(100%)

Figure 1. Disk approximation test exhibiting positive on Muller Hinton agar surface after 24 hr. of incubation at 37°C.



Molecular Study of some Genes in isolates **Detection of *motA* and *motB* Gene**

The *motA* and *motB* genes play a role in the rotary motor of flagella (Che *et al.*, 2014). The two *motA* and *motB* genes are required for swimming and twitching motility of *Proteus mirabilis*. The stator complexes are not only encoded in *P.*

mirabilis but also in most pathogenic bacteria that need to swarm in over surfaces (Belas, 2014). 36% of isolates have *motA* gene, while *motB* was detected in about (80%) of isolates. These results were inconsistent with the result of Armbruster *et al.*, (2018) and Wasfi *et al.*, (2020) who reported that the three genes were encoded in chromosomal DNA of pathogenic bacteria strain.

The investigated correlations between genes of adhesion, swarming and biofilm formation was reported in many previous studies. Sharma *et al.*, (2013) demonstrated that the presence of abundant curli fimbriae on bacterial cell surfaces was correlated with increased biofilm formation, this phenotype indicates that the expression of genes is necessary for the biosynthesis of curli fimbriae. Interesting findings were showed by Verstraeten *et al.*, (2008), who demonstrated that *wosA* and *rscB* have a role in swarming and biofilm formation of pathogenic bacteria.

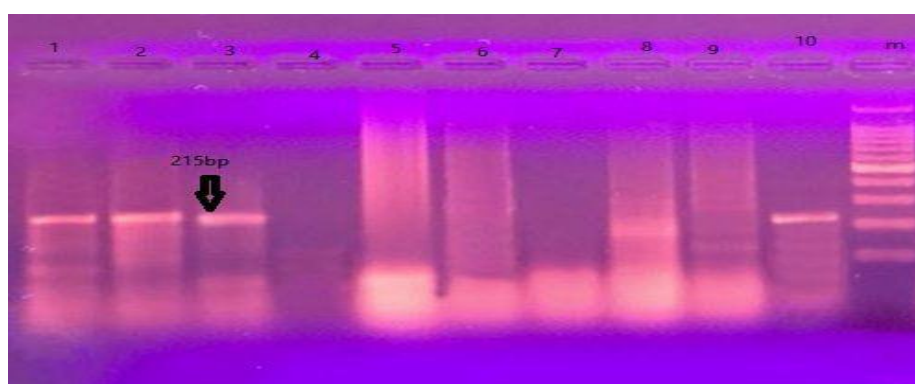


Figure 2. Ethidium Bromide-Stained Agarose Gel Electrophoresis of PCR Products from Extracted Total DNA of bacterial isolates Using Primer *motA* Gene with Product (215bp). The Electrophoresis was Performed at 70 volt for 90min. lane (L), DNA Molecular Size Marker (100 bp ladder). Lanes (10) (*MotA* gene was presented in *Acinetobacter lwoffii* Show Positive Results with Gene *motA*).

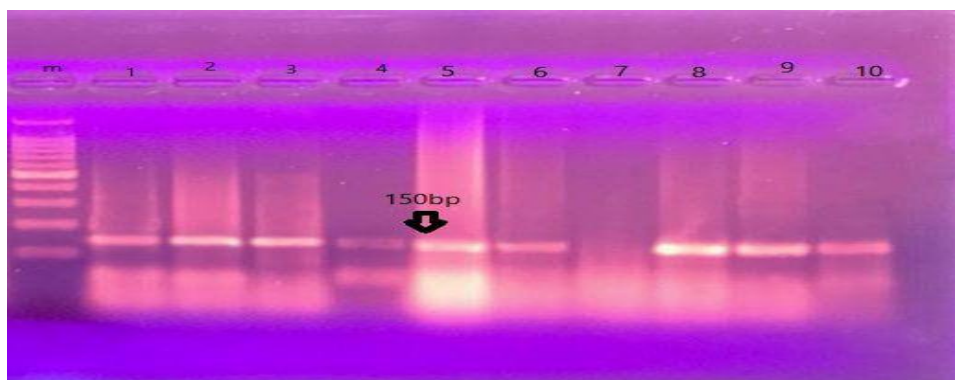


Figure 3. Ethidium Bromide-Stained Agarose Gel Electrophoresis of PCR Products from Extracted Total DNA of bacterial isolates Using Primer *motB* Gene with Product 150bp. The Electrophoresis was Performed at 70 volt for 90min. lane (L), DNA Molecular Size Marker (100 bp ladder). Lanes (4, 10) (*Acinetobacter baumannii*, *Acinetobacter lwoffii* Show Positive Results with Gene *motB*).

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