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Detection of some Virulence Factors and *aac*(6)Ib Resistance Gene in Clinical *Proteus mirabilis* Isolates

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Abstract

Proteus mirabilis are opportunistic members of Enterobacteriaceae, considered the reason behind 10% of urinary tract infections and other primary clinical infections. They produce beta-lactamases that can confer resistance to beta-lactam antibiotics. This study aimed to investigate the prevalence of some virulence factors with antimicrobial resistance and molecular characteristics of *aac-Ib* gene The current study have been included 250 specimens isolates were collected from urine sample, wound, burn, diabetic foot, and vaginal swabs conducted in Baqubaa Teaching Hospital, Al-Batool Teaching Hospital, and AlKhalis hospital in Diyala province during the period extended from August 2023 to January 2024. A total of 250 specimens Isolates were identified by morphological form on blood agar and macConkey agar, traditional biochemical tests. The results showed that 75 isolates were identified as *P. mirabilis* (30%) consisting of 35 isolates (46.7%) obtained from urine, 8 isolates (10.7%) obtained from wound, 10 isolates (13.3%) obtained from burns and diabetic foot and 12 isolates (16%) obtained from vaginal swabs. A higher rate of positive isolates was recorded in urine samples. The female isolation rate was much higher than in males 60% and 40% respectively and patients aged group 21-30 years were more prevalent than other groups. It was found that the more effective antibiotic against the isolates was norfloxacin followed by levofloxacin, where all the isolates were sensitive to it. On the other hand, the less effective

antibiotics were amikacin and oxacillin when they were resisted by all isolates. The results showed that 63% of isolates were moderately produced biofilm, while 37% were strongly biofilm producers. Results revealed that 30% of *P. mirabilis* isolates were β-Hemolysin producers and 70% of them were γ- Hemolysin producers .All isolates showed swarming motility ,urease production75(100%),protease production43 (57%) and β-lactamase28 (37%) .The results showed different categories of dines lines production, where 63% of them intensive producers, 30% moderate and 7% were weak producers. PCR analysis of *Proteus mirabilis* genes among positive patients recorded *aacIb* (33.33) of 12 MDR and high virulence factors isolates.

Keywords: *P. mirabilis*, biofilm, amikacin, virulent factors, PCR, *aacIb*.

Introduction

Proteus mirabilis is an opportunistic pathogen, which causes infections in people with reduced natural immunity [1]. This organism can be defined as a gram-negative rod which is a member of the Enterobacteriaceae family, motile, urease-positive, lactose-negative, indole-negative and produces hydrogen sulfide [2]. It possess several virulence factors, which are necessary for the colonization of surfaces, involving host tissues and organs [3]. The virulence factors expressed by *P. mirabilis* including Flagella, quorum sensing molecules, efflux pumps, adhesion proteins, hemolysins, lipopolysaccharides, and IgA proteases, ability to acquire antibiotic resistance and urease enzyme are a few examples of such elements [4]. In addition to urinary tract infections, *P. mirabilis* has been associated with opportunistic pulmonary system infections, burns, wound, skin, eyes, nose, ears, and gastroenteritis, this also causes an autoimmune disease in human who is genetically susceptibility to develop rheumatoid arthritis [5]. *P. mirabilis* is naturally resistant to several antibiotics and has reduced susceptibility to Imipenem. Emergence and spread of multidrug-resistant *P. mirabilis* isolates, including those producing extended-spectrum b-lactamases (ESBLs), AmpC cephalosporinases, and carbapenemases, are being more frequently reported. Through acquisition of genes for resistance to multiple antibiotic classes, *P. mirabilis* is turning into a Pandrug resistant bacterium, and infections are more difficult to treat [6]. This study aims to isolate *P. mirabilis* from different sources and detecting virulence factors and antibiotics resistance.

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Materials and Methods

Bacteria Isolation and Identification

This is a cross-sectional study was conducted in Baquba Teaching Hospital, Al-Batool Teaching Hospital, and AlKhalis hospital in Diyala province during the period extended from August 2023 to January 2024. A total of 250 specimens were collected from urine sample, wound, burn, diabetic foot, and vaginal swabs. Identification of these samples depended on microscopic examination (Gram's stain), morphological and biochemical tests through appearance swarming, non-lactose fermentation on selective and differential MacConkey and enriched condition on blood agar to another step that included confirmation of *Proteus mirabilis* diagnosis and standard biochemical tests according to [7].

The biochemical tests were done to identify the bacterial isolates, namely oxidase, indole, citrate utilization, catalase, urease production, H_2S formation, lactose fermented, Voges- proskauer reaction, Methyl red, Trible sugar iron test (TSI) and Simmon citrate [8].

Antibiotic sensitivity test (Kirby-bauer method)

Sixteen antibiotic disks (Amoxicilline-Clavalante 20/10mg, Oxacillin, Azetronam, Cefepime Ceftazidime, Cefoxitin, Imipenem, Gentamicin 10mg, Amikacin 10mg, Kanamycin, Azithromycin, Ciprofioxacin 10mg, Levofioxacine, Nalidixic acid, Ofloxacin and Amoxicilline) were used to detect the sensitivity of 75 isolates of *P. mirabilis* by using Kirby-bauer method according to (MacFaddin, 2000) [9]. The inhibition zone was assessed and interpreted by the percentage of susceptible, intermediate, or resistant isolates as defined by CLSI [10].

DNA Extraction

Genomic DNA was extracted from bacterial isolates using extraction Kits of Genomic DNA and purification depending on the instruction of manufacturing company (Promega USA). The DNA concentration was misheard by using a Quantus Fluorometer (Promega, USA).

Table 1: The primers used for virulence genes detection aacIb

Primer Name	Seq	Annealing Temp. (C°)	Product Size (bp)

aacIb-F	TTGCGATGCTCTATGAGTGGCTA	54	**482**
aacIb-R	CTCGAATGCCTGGCGTGTTT		

PCR Program

Table 2: The PCR thermocycler program for P. mirabilis target gene

Steps	**°C**	**m:s**	**Cycle**
initial denaturation	**95**	**05:00**	**1**
Denaturations	**95**	**00:30**	**30**
Annealings	**54**	**00:30**	**30**
Extensions	**72**	**01:00**	**30**
Final extensions	**72**	**07:00**	**1**
Holds	**10**	**10:00**	**1**

Detection of some virulence factors

Swarming Motility and Hemolysin Production

The β-hemolysis properties and swarming phenomenon of *P. mirabilis* strains were determined by observing clear zones around bacterial colonies on blood agar [11].

Urease production

The isolates of *P. mirabilis* were cultured on urea agar medium by stabbing and incubated at 37ºC for 24-48 hrs. The change of color from yellow to pink is a sign of a positive result [9].

Protease production

Skim milk agar has been inoculated with the bacterial suspension using the drilling method and incubated at 37ºC for 24 hours. The result is positive when the appearance of clear zones around the colonies [12].

Dienes Test

The Dienes test used for identifying and differentiating strains of the bacteria Proteus mirabilis in microbiology. Dienes test was performed according to the protocol described by [13].

β-Lactamase production

β -Lactamase production in *P. mirabilis* was identified by the double disk synergy test (DDST) according to [14].

Biofilm Formation

The microtiter plate is the best test for identifying biofilm formation, it was described by [15].

Results and Discussion

Isolation and Identification of *Proteus mirabilis*

The isolates were first identified by the swarming phenomenon on blood agar, the cultures' smell, and the pale appearance of bacteria (non-lactose fermenting) on the MacConkey agar. Also by microscopic examination, it appeared as straight rods and Gram-negative when it stained with Gram stain. Conventional biochemical tests were done to characterize the suspected isolates and the results showed positive results for the catalase, urease, methyl red and motility, but were negative to citrate and oxidase test (table 3).

Out of 250 clinical samples, 75 isolates were identified as P. mirabilis (29%) consisting of 35 isolates (46.7%) obtained from urine, 8 isolates (10.7%) obtained from wounds, 10 isolates (13.3%) obtained from burns and diabetic foot and 12 isolates (16%) obtained from vaginal swabs. The near result was recorded by Abdullah [16]. who indicated that the total isolation percentage of *P. mirabilis* indicated that the total isolation percentage from different clinical specimens was 23.75%. The reason for the difference in isolation percentages may be due to the differences in the size of samples, isolation sources, and number of hospitals surveyed.

Table 3: Biochemical tests of *Proteus mirabilis*

Tests	Results
Catalase	+
Oxidase	-
Indol	-
Methyl Red	+
Voges-Proskauer	-
Citrate Utilization	+
Lactose fermenting	-
Motility	+

In this study, a higher rate of positive isolates was recorded in urine samples, because the number of urine samples was higher than in other sources(Table 4). Similar findings were reported by [17], who showed that urine had the highest rate of *P. mirabilis* among other clinical sources. This is usually linked to the possession of many virulence factors which are significant

for causing urinary tract infections, these virulence factors include adherence capability, urease production, and flagella [18].

Table 4: Distribution of *Proteus mirabilis* isolates among clinical sources

Type of samples	No. of isolates (%)
Urine	35 (46.7)
Wounds	8 (10.7)
Burns	10 (13.3)
Diabetic foot	10 (13,3)
Vaginal	12 (16)
Total	75 (100)

For age groups, the results showed that patients age group 21- 30 years were more prevalent than other groups, while those from (11-20) years had the lowest infection rates (Table 5). Results revealed that females' isolation rate was much higher than in males 60% and 40% respectively fig (1). This result is in agreement with Mirzaei [19], and [20], who found that females' rate of infection is more than males' 72.5% and 27.5% respectively. This result is in disagreement with Al-Nabhani and Shami's [21], and Abdullah [16]. results, where the infection rate was higher in males than females.

Table 5: Percentages of *Proteus mirabilis* isolates by age groups

Age groups	No. of the isolates	Percentage %
11-20	2	3
21-30	32	44
31-40	8	10
41-50	15	20
51-60	10	13
61-70	8	10
Total	75	100

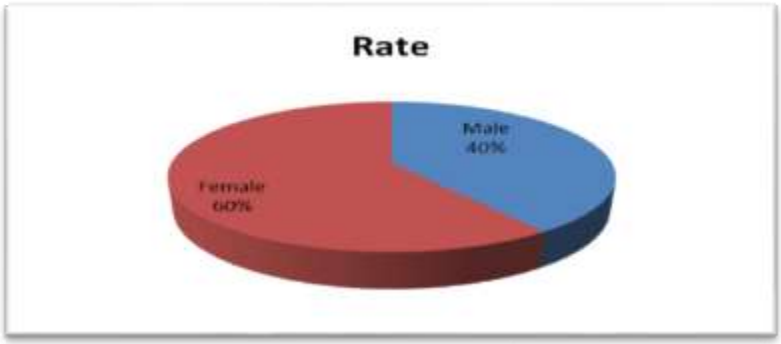

Figure 1: Percentages of *Proteus mirabilis* infection by sex

Determination of Antibiotic Susceptibility

The susceptibility patterns of the isolates are presented in Figure (2). It was found that the more effective antibiotic against isolates was Norfloxacin (100%), followed by Levofloxacin (93%). On the other hand, the less effective antibiotics were Amikacin and Oxacillin, they were resisted by all isolates, followed by Amoxicillin (97%). However, the effect of other antibiotics was variable among the isolates. Results showed 100%, 97%, 47% and 27% resistance for Oxacillin, Amoxicillin, Azetronam and Augmentin respectively. The resistance rate of Amoxicillin was in agreement with the results of a study [22], as the resistance ratio was (93.7%). For Azetronam, the result agree with Al-Nabhani and Shami [21], which was 44.5%.As for Augmentin the result agree with Mirzaei [19], about 22.5%. The resistance to beta lactams due to the production of beta lactamase enzymes. As for the carbapenems represented by Impenem, the percentage of resistance of the isolates against this antibiotic in the current study was (17%). This result was agree with Al-Nabhani and Shami [21], study that found (15.3%) showed resistance to imipenem, as well as agree with Mirzaei [23], results that found 11.8% resistant to Impenem. The resistance to carbapenems by Gram-negative bacteria has become increasing, and this resistance is linked to the presence of many factors, including MBL mineral beta-lactam enzymes, OXA enzymes and mobile genetic elements, and have been indicated by many previous studies in Iraq [24]. Resistance to Aminoglycosides represented by Amikacin, Kanamycin and Gentamicin were 100%, 63% and 50% respectively, this result agrees with (Attalah et al., 2020) were the isolates show high resistance to Amikacin (80%). Abdullah [25], mentioned that the rate of resistance to gentamicin was (40%) which agree with the result. Isolates showed resistance to quinolones antibiotics represented by Naldixic acid (33%), this close to Hussein [26], results which was 46%. For Ofloxacin the resistant was 10%. Ciprofloxacin and Levofloxacin that belongs to the fluroquinolone showed only 3% resistance. While all isolates were sensitive for Norfloxacin. The result for Levofloxacin agreed with Al-Kaim and Al-Dahmoshi [27] that found 4.20% of isolates resisted to that antibiotic. For Ciprofloxacin the result was close to Jamel [28], which found all isolates were resistance.The resistance to Ceftazidime and Cefoxitin were 60% and 3% respectively. Development of antibiotic resistance is often related to the overuse, and misuse of the antibiotics prescribed.

Resistance of *P. mirabilis* continues to be an important clinical therapeutic problem, such that which can be found in an increasing multidrug resistance in these bacteria. The emergence of high-level resistance to antimicrobials is an increasing threat to global health [29], it has been found that the majority of the isolates were multidrug-resistant since they were resistant to three antibiotics or more. In this study the prevalence of MDR P. mirabilis was high. 73 (97%) of all isolates were found to be MDR, while only 2 isolate was tend to be XDR (3%) while no PDR was detected (figure 2). The results agree with Al-Kaim and Al-Dahmoshi [27], who recorded that 96% of isolates were MDR.

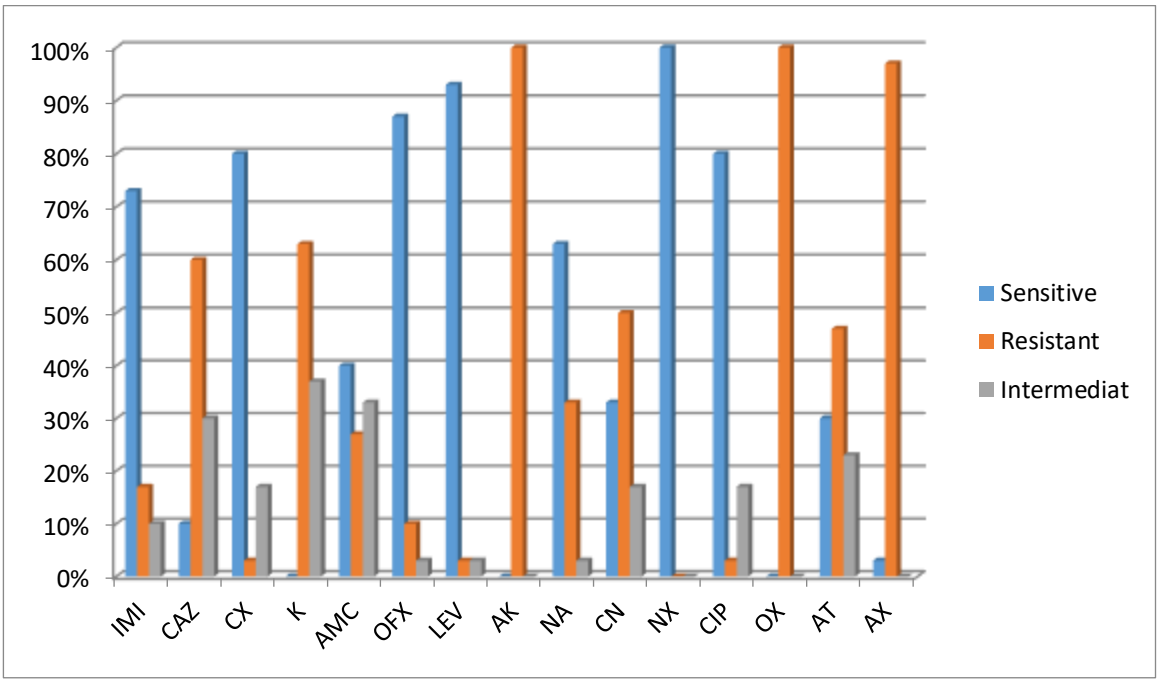

Figure 2: Antimicrobial resistant pattern of *P. mirabilis*

It is also clear that regional habit equally affect the exponential growths of resistance since the majority of people resort to patronizing patent medicines stores without proper prescriptions, shelf life and dosage in communities [30].

Virulence factors

Results revealed that 30% of *P. mirabilis* isolates were β-Hemolysin producers and 70% of them were γ- Hemolysin as shown in table 6. All the isolates show swarming motility, urease

production. Protease production and β-lactamase, Dienes test and the results of biofilm formation showed in table 8.

Table 6: Detection of virulence factors for *Proteus mirabilis* isolates

Virulence factors	Positive results (%)
β-Hemolysis	23 (30%)
γ- Hemolysis	53 (70%)
Swarming	75 (100%)
Urease	75 (100%)
Protease	43 (57%)
β-lactamase	28 (37%)

In vitro (on basic urea agar), urease hydrolyzing urea to alkaline ammonia and carbon dioxide, thereby increasing the pH and will be changing the color of phenol red indicator to pink [31] However, *in vivo* (human body) this enzyme catalyzes the formation of kidney and bladder stones or to encrust or obstruct indwelling urinary [32].

Protease

The result revealed that 43 (57%) of isolates are producers of protease enzymes. These results disagreed with Elhoshi [33], who revealed that all proteus mirabilis isolates produced by protease enzyme were 100%. The proteus mirabilis is a gram-negative enteric bacterium that occurs as vegetative swimmer cells and hyperflagellated swarmer cells [34]. Moreover, Proteus mirabilis is one of the most common causes of UTIs in individuals with long-term indwelling catheters, complicated UTIs, and bacteremia among the elderly. As the ageing population expands, more individuals will be at risk for Proteus mirabilis UTIs and stone formation [35].

***β*-lactamase**

Results showed 37% Beta-lactamase production between P. mirabilis isolates and this is in agreement with Hussein and AL-Shwaikh [36], which was 36.7%, β-Lactam antibiotics are the most common therapeutic agents in animals and humans and are used to treat various illnesses ranging from gastrointestinal to life-threatening infections. β-Lactamase production is the major resistance mechanism of bacteria to these antibiotics. The β-lactamases include ESBLs, ACBLs, and carbapenemases [36]. ESBLs confer bacterial resistance to penicillins and broad-spectrum cephalosporins, among others. However, β-lactamases can be blocked by β-lactamase inhibitors such as clavulanic acid, tazobactam, and sulbactam. ACBLs also confer resistance to

a wide variety of β-lactam antibiotics and β-lactamase inhibitors but are susceptible to fourth-generation cephalosporins and carbapenems [37].

dines test

when two identical Proteus cultures are inoculated at different points on the same plate of a non-inhibitory medium, the resulting swarming of growth coalesce without signs of demarcation .When, however, two different strains of Proteus are inoculated, the spreading films of growth fail to coalesce and remain separated by a narrow easily visible area .The observation of this appearance the Dienes phenomenon has been used to determine the identity or non-identity of strains in epidemiological studies [38]. For dines test results the expansiveness/rate of swarming motility was evaluated for each strain by its proportional coverage of the plate after overnight incubation compared to other strains cultured on the same plate. The expansiveness was classified into three categories: category 1, weak swarming (coverage < 5%); category 2, medium swarming (5– ≤ 25%); and category 3, intensive swarming (25– ≤ 50%). The bacterial behavior in each combination of strains was tested in triplicate. The results are nearly in agreement with Filipiak [39], who expressed 20% weak, 36% medium and 44% invasive production dines lines production, fig (4).

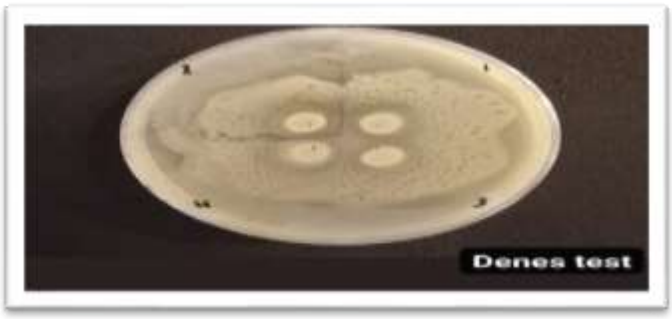

Figure 4: Dienes test intensive producer's results

Biofilm formation

The results showed that 63% of isolates were moderately produced biofilm, while 37% were strongly biofilm producers. However, no isolates (0%) were weak to form the biofilm. The results agree with Mirzaei [23], that all isolates were biofilm producers. The ability to generate biofilm, bacteria inside biofilms are shielded from various stresses, including immune

responses and antimicrobial agents. The biofilm-forming capacity of bacteria has been associated with increased antibiotic resistance and chronic recurrent infections, in a matrix of extracellular polymeric substances called biofilm, numerous microorganisms may exist independently or join together to form micro-communities. A significant virulence factor is the ability of a microorganism to form biofilm as it provides a defensive environment to survive and evade antibiotics for the species [40]. Many Gram-negative bacteria are classified as biofilms that confer resistance to environmental stress and bactericides in environmental stress and bactericides in microbial classes [41].

Detection of *aac*(6) gene in *Proteus mirabilis* isolates by PCR

The genetic study enhances the conventional investigation and detection methods along with Polymerase Chain Reaction (PCR) which plays an essential role as a powerful tool in clinical microbiology studies and has been widely used to detect the genes of interest [42]. Determination of DNA concentration and purity were done using the Quantus Fluorometer and Nano drop spectrophotometer by using genomic DNA purification kit (promega,USA). The results indicated that DNA concentration of the extracts were variable ranging from (30-70 ng/ µl). It was also observed that the purity of DNA extracts was satisfactory ranging from (1.55-1.98) according to the value rate of 260/280 nm. The output depended on culturing methods, bacterial category, amount of pellet and type of extraction kit. All these have an effect on the quality and properties of nucleic acids. The aac(6') gene was amplified and showed presence of aac(6')gene in the (12) isolates and confirmed by using uniplex PCR as a gold standard method. Single band was not observed at a given molecular weight (482bp). The results of the current study illustrated in figure (5) demonstrated that (33.33%) of studied isolates have aac(6') gene. These findings disagreed with other studies which reported before by Al-Saadi [43], showed that aac(6)was not found in their studied isolates and the results also dis agreement with Abed [44], who found ten multidrug resistance Proteus mirabilis contain the *aac(6') Ib* gene in (80%). The current study Partially agreement with Alsherees [45], Who found that the percentage of aac(6')-Ib (22.2%) .The isolates of Proteus mirabilis contain aac(6')-Ib gene that encodes to an aminoglycoside-modifying enzyme that acts inhibition effect against antibiotics

in this group like amikacin and gentamycin, then isolates of these pathogenic bacteria become resistant against antibiotic of aminoglycoside group [45].

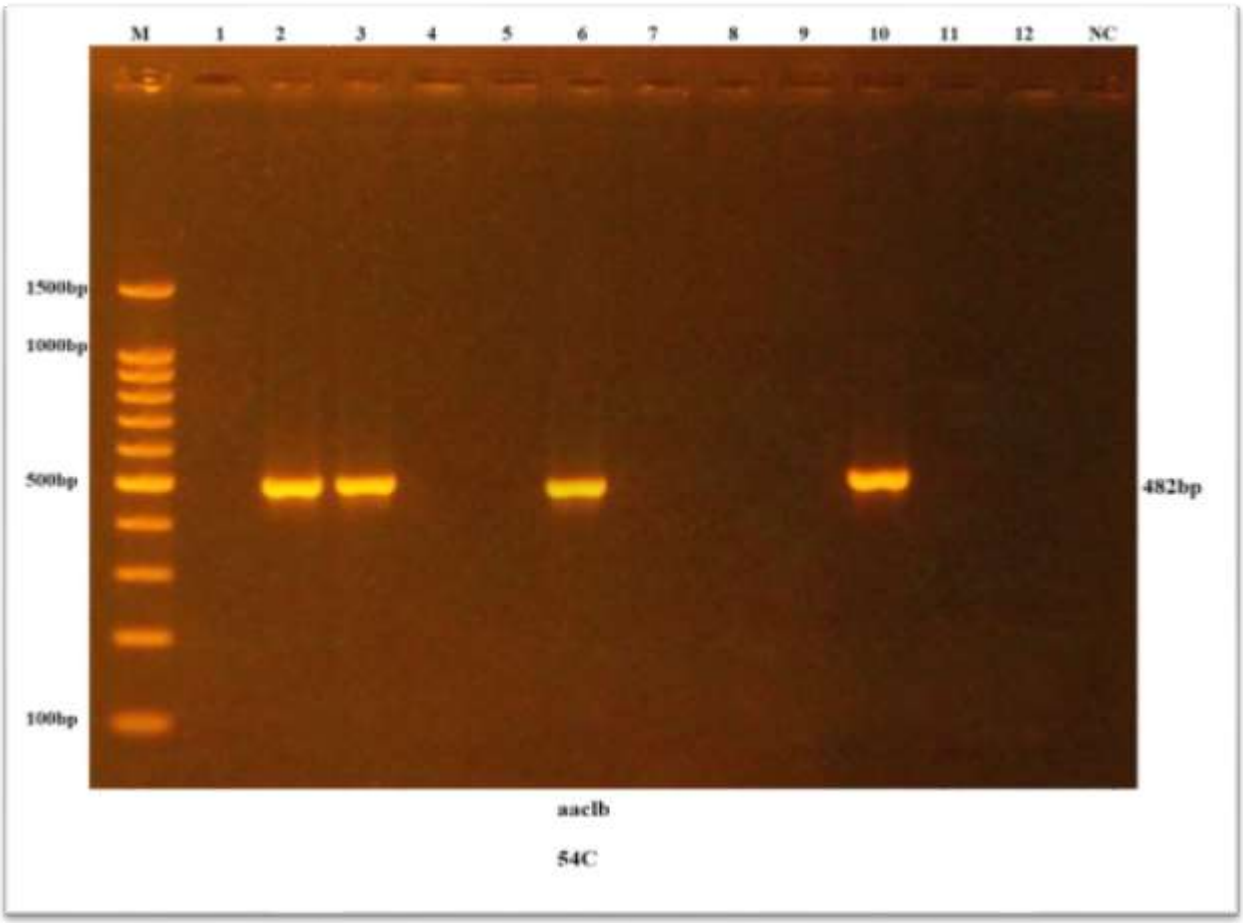

Figure 5: PCR amplification of the aaclb gene from *P.mirabilis*, with the amplicon size 482bp.. DNA amplification products were separated by electrophoresis in (2%) agarose gel. The electrophoresis was performed at 70 volt for 1.5 hour. The symbol "M" refers to ladder marker.

Conclusions

A high prevalence of MDR Proteus mirabilis isolates was observed in the present study towards commonly used drugs. This highlights the alarming levels of antimicrobial resistance and the need for applying effective antibiotic therapy. Therefore, MDR bacteria should be identified along with their antibiogram by every hospital and clinic to help clinicians choose the most

suitable antimicrobial therapy for patient benefit as well as to reduce the emergence of drug-resistant bacteria.

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Ethical clearance: The samples were obtained according to Local Research Ethics Committee Approval in the College of Science, University of Diyala, No. 2775 in 6/8/2023

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