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Original Article

Study of Antibiotic Resistant Genes in *Pseudomonas aeroginosa* Isolated from Burns and Wounds

Rashid Mahmood, A[1] *, Mansour Hussein, N[1]

1. Microbiology Department, School of Medicine, University of Kirkuk, Kirkuk, Iraq

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Corresponding Author: ayhan39b@uokirkuk.edu.iq

Abstract

Pseudomonas aeruginosa (P. aeruginosa) is frequently associated with infections with high mortality rates. The intrinsically high resistance to many antibiotics and multidrug resistance in the hospital setting is considered to be among the reasons for high pathogenicity of *P. aeruginosa*. In this study, a total of 200 wound and burn swabs were collected from patients. The collected specimens were examined for *P. aeruginosa* through biochemical and antibacterial sensitivity tests performed in the Microbiology Laboratory in College of Medicine, University of Kirkuk, Kirkuk, Iraq. The polymerase chain reaction was then used to detect mexA, mexB, mexR, and oprD genes. In total, 31 isolates of *P. aeruginosa* were collected from 200 patients with wounds and burns. Most cases were isolated from 23 (74.19%) and 8 (25.80%) wound and burn swabs, respectively. Antibiotic sensitivity was tested on all isolates against 17 antimicrobial agents. The obtained results revealed a high resistance rate to gentamicin, trimethoprim, amikacin, and amoxicillin, and a low resistance rate was observed to ceftazidime, tobramycin, levofloxacin, cotrimoxazole, ciprofloxacin, and aztreonam. Regarding antibiotic resistance, mexB, mexR, and oprD genes were observed in three isolates, in which mexB and mexR were detected in two isolates, and only one isolate carried mexA gene.

Keywords: Molecular diagnosis, Multidrug resistance, *Pseudomonas aeruginosa*

1. Introduction

Nosocomial infections mainly occur in individuals during their hospital stay or in other clinical places. In the hospital environment, the increased prevalence of pathogenic microorganisms is correlated with an increase in different forms of nosocomial infections (1). Surgical site infection is the most common hospital-acquired infection in developing countries (2). The source of infection at the postoperative surgical site may be endogenous or exogenous. Following surgical manipulation, patients can be contaminated with their normal body flora, which in major circumstances overwhelms regular body resistance mechanisms. However, workers of hospital, other visitors and patients, fomites, water, and food are among external causes of infection (3). Multi-drug resistant bacterial infections are among the most important universal problems in hospitalized patients which elevate the rate of death and hospital costs. Increasing antimicrobial resistance is a serious threat to health, based on the World Health Organization and the Centers for Disease Control and Prevention (4).

Varieties of bacterial pathogens, including *P. aeruginosa*, *Klebsiella pneumonia, Enterococcus faecium*, *Acinetobacter baumannii*, *Enterobacter,* and *Staphylococcus aureus* are classified as highly drug-resistant pathogens that play a crucial role in the emergence of hospital-acquired infections. Additionally, *P. aeruginosa* is one of the most common bacteria and is the cause of nosocomial infections and acquired drug

resistance. Accordingly, certain strains of *P. aeruginosa* can effectively escape antibiotic therapy (5). Carbapenems, meropenem, and imipenem have been classified as active therapy against *P. aeruginosa* infections in the last era. Nevertheless, for the past few years, the bacteria have formed a resistance against such antibiotics, and most recently, colistin or polymyxin B has been recognized as a last option for handling extensive drug-resistant (XDR) *P. aeruginosa*; however, recently, colistin resistance has also been acknowledged (6, 7). Moreover, *P. aeruginosa* (a non-fermenting gram-negative organism) accounts for 11% of hospital-acquired infections (with great rates of death and illness) and diseases, particularly in immune-compromised individuals. The upper respiratory tract, urinary tract, and kidney are the sites of colonization, surgical wound infection, urinary tract infections, pneumonia, and bacteremia. Adhesions, hemolysin, exotoxins, proteases, and siderophores are some major virulence factors (8). Several mechanisms of antibiotic resistance recorded for *P. aeruginosa* include decreased manifestation or damage of OprD porin which leads to decreased antibiotic permeability and increased expression of the MexAB-OprM pump. This in turn raises the outflow of antibiotics, gyrase, and topoisomerase mutations cause resistance to fluoroquinolone, leading to the development of aminoglycosides and β-lactams deactivating enzymes. The described process can contribute to multiple drug resistance (9, 10). *Pseudomonas aeruginosa's* outer membrane is a major barrier that restricts the penetration of antibiotics. *P. aeruginosa* forms many porins, such as OprD and OprF. In this regard, imipenem resistance is associated with loss or reduced expression of OprD (11). The current study is focused on the multi-drug resistance of *P. aeruginosa* and its relationship with the efflux pump mexA, B, and R genes and oprD gene, involved in antibiotic resistance.

2. Materials and Methods

2.1. Patients

A total of 200 swab samples were collected from patients in the surgical wounds and burns unit of Azadi Teaching Hospital, Kirkuk, Iraq, from June 2018 to February 2019 by sterilized cotton swabs soaked with NaCl. The swaps were then transported to the Microbiological Laboratory of the College of Medicine, University of Kirkuk, Kirkuk, Iraq. Colony characteristics, gram stain, drug susceptibility test, and biochemical tests were conducted to distinguish and confirm *P. aeruginosa* (12).

2.2. Isolation of *P. aeruginosa*

The morphology of colonies and the development of fluorescence were determined for *P. aeruginosa* by cetrimide agar (Oxoid, Basingstoke, UK). The shape of bacteria and cell wall were detected by gram staining technique by biochemical examinations (i.e. catalase test, oxidase test, Simmons citrate test, Indol test, and gelatin liquefaction) (13).

2.3. Antibiotic Sensitivity

Antibiotic sensitivity was tested by disc diffusion using the Kirby-Bauer method, and commercially available antibiotics (MAST, UK) (14). Moreover, the antibiotic discs used included amoxicillin (AMX 30 µg), gentamicin (GEN 10 µg), ciprofloxacin (CIP 10 µg), ceftazidime (CAZ 30 µg), amikacin (AK 30 µg), trimethoprim-sulfamethoxazole (TMP-SXT 5 mg), cefoxitin (FOX 30 µg), tobramycin (TN 10 µg), piperacillin (PRL 100 µg), augmentin (AUG 30 µg), ceftriaxone (CTR 30 µg), cefotaxime/clavulanic acid (CEC 30 and 10 µg), cotrimoxazole (TS 25 µg), aztreonam (ATM 30 µg), levofloxacin (LEV 5µg), imipenem (IPM 10 µg), and amoxicillin/clavulanic acid (AMC 30 µg). Finally, the results were analyzed according to the CLSI 2018 guidelines (15).

2.4. Deoxyribonucleic Acid (DNA) Extraction

DNA extraction was conducted using solution-based Bacteria DNA Preparation Kit (Jena Bioscience GmbH, Germany), according to the manufacturer's guideline:

1. Cell lysis: 1 ml of cultured cells was transferred into a 1.5 ml microtube to harvest the cells. Centrifugation was performed at 15.000 g for 1 min. The supernatant was discarded, and the pellet was re-suspended in 300 µl of a solution of cell lysis.

2. Treatment with RNase solution: 1.5 µl of RNase A solution was added and mixed by turning upside down, and incubation was maintained at 37 °C for 15-30 min and ice-cooled for 1 min.

3. Protein deposition: 100 µl of protein deposition solution was added and vortexed for 20-30 s and then centrifuged at 15.000 g for 5 min.

4. DNA precipitation: The supernatant was moved to a disposable 1.5 ml microtube holding 300 µl Isopropanol >99%. The specimen was then mixed by flip-flopping slightly for 1 min and centrifuged at 15000 g for 1 min. The supernatant was discarded and the tube was drained temporarily on disposable porous paper. Subsequently, a washing buffer solution (500 µl) was added. The tube was inverted many times to wash off the DNA pellet and again centrifuged for 1 min at 15000 g to remove the ethanol. Air drying was conducted for 10-15 min at room temperature.

5. DNA hydration: 50-100 µl of a DNA hydration solution was added to the dried DNA pellet. Afterward, DNA was hydrated through incubation at 65 °C for 60 min and kept at -20 °C or -80 °C.

2.5. PCR for *mexA*, *mexB*, *mexR*, and *oprD* Genes

In the current study, the polymerase chain reaction (PCR) technique was applied to reveal mexA, mexB, mexR, and oprD genes in isolates of *P. aeruginosa*. Table 1 presents the primers (Macrogen, Korea) used in this study. The PCR reaction setups and thermal cycling protocol is tabulated in tables 2 and 3.

2.6. Electrophoresis Using Agarose Gel

After PCR amplification, electrophoresis by agarose gel (1.5%) and ethidium bromide (10 mg/ml) staining was conducted to confirm amplification. A 100 bp DNA ladder was used as a DNA molecular weight marker. The bands were stained by ethidium bromide that could be visualized in the gel using the gel-based imaging method.

Table 1. List of primers utilized in the present study

Primer Target	Primer Sequence (5`-3`)	Temperature of Annealing (˚C)	Size of Product (bp)	Reference
mexA-F	CGACCAGGCCGTGAGCAAGCAGC		275	(16)
mexA-R	GGAGACCTTCGCCGCGTTGTCGC			
mexB-R	AAGGTCACGGTGATGGT	55	280	(17)
mexB-F	TGTCGAAGTTTTTCATTGATAG			
mexR-F	GAACTACCCCGTGAATCC		411	(17)
mexR-R	CACTGGTCGAGGAGATGC			
oprD - F	ATGAAAGTGATGAAGTGGAGCG		949	(16)

Table 2. Polymerase chain reaction components

Constituents of the Master mix	Standard	Unit	Final	Unit	Size
Master mix solution	2	X	1	X	10
Forward primer	10	µM	1	µM	1
Reverse primer	10	µM	1	µM	1
Nuclease Free Water					2
DNA fragments	10	ng/µl	10	ng/µl	6
Total volume					20
Aliquot /single run	18 µl of master mix solution for each tube in addition to 2µl of template				

Table 3. Polymerase chain reaction program

Stages	° C	m: s	Cycle
Primary Denaturation	95	05:00	1
Denaturation	95	00:30	
Annealing	55,56,58 OR 60	00:30	30
Extension	72	01:00	
Ending extension	72	07:00	
Hold	10	10:00	1

3. Results

3.1. Identification and Estimation of Antibiotic Resistance

In this study, a total of 31(15.5%) *P. aeruginosa* isolates were collected from 200 patients with wounds (23, 74.19%) and burns (n=8, 25.80%). Antibiotic sensitivity was tested in all isolates against 17 antimicrobial agents. Based on the results, a high resistance rate was observed to gentamicin (n=26, 83.87%), trimethoprim (n=21, 67.74%), as well as amikacin and amoxicillin (n=17, 54.83%). The rate of resistance to other isolates, including ceftazidime and tobramycin (n=8, 25.80%) and levofloxacin and imipenem (n=7, 22.58%), was low. The low resistance rates were observed in 5 (16.12%) isolates to cotrimoxazole, ciprofloxacin, aztreonam, amoxicillin /clavulanic acid, and augmentin, in 5 (16.12%) isolates to cefoxitin, ceftriaxone, and piperacillin, and in 2 (6.45%) isolates to cefotaxime /clavulanic acid (Figure 1).

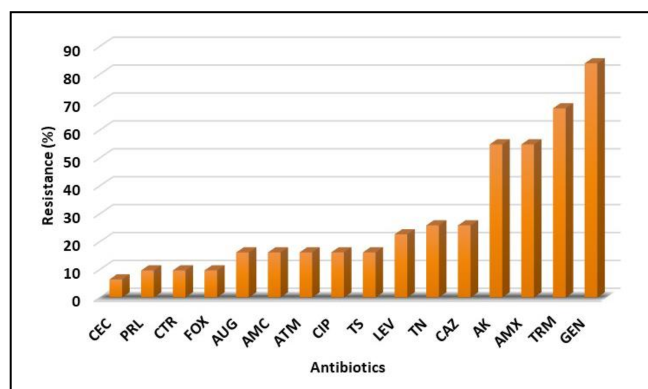

Figure 1. Antibiotic resistance of *Pseudomonas aeruginosa* clinical isolates

The highest resistance was observed in 14 (45.16%) isolates (against AK 30, AMX 30, GEN 10, TRM 1.25), 7 (22.58%) isolates (against GEN 10, LEV 5, TMP 10, and IMP 1), 5 (16.12%) against (against AMC 30, ATM 30, CAZ 30, CIP 5, TN 10, and TS 25), 2 (6.45%) isolates (against AUG 30, CEC 30/10, GEN 10), and 3 (9.67%) isolates (against AK 30, AMX 30, AUG 30, CAZ 30, CTR, FOX, GEN, PRL 100, and TN 10), as shown in table 4.

Table 4. Antibiotic resistance patterns of *Pseudomonas aeruginosa* isolates

Antibiotic resistance pattern	Number of isolates
AK 30, AMX 30, AUG 30, CAZ 30, CTR, FOX, GEN, PRL 100, TN 10	3
AMC 30, ATM 30, CAZ 30, CIP 5, TN 10, TS 25	5
AK 30, AMX 30, GEN 10, TRM 1.25	14
AUG 30, CEC 30/10, GEN 10	2
GEN 10, LEV 5,TMP 10, IMP	7

Molecular analysis of mexA, mexB, mexR, and oprD genes was conducted through PCR to multidrug resistance isolates (3). The mexA gene was detected at 275 bp in 2 (55%) multidrug resistance isolates, and mexB gene was detected in 2 (55%) isolates from wound swabs at 280 bp (Figure 2).

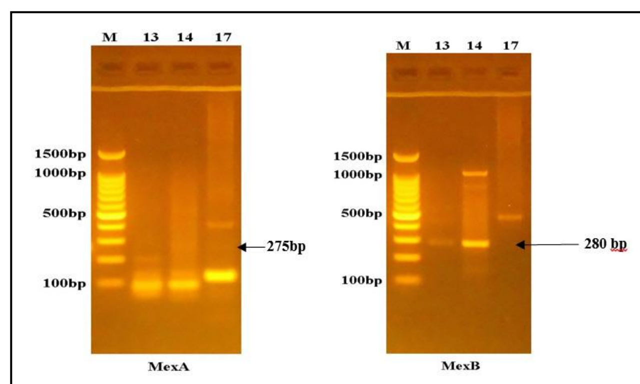

Figure 2. Gel electrophoresis of PCR products of mexA gene at 275 bp and mexB gene at 280 bp in multidrug resistance *P. aeruginosa* isolates. Electrical power was turned on at 100v/mAmp for 75 min

A total of 2 *P. aeruginsa* isolates were carrying mexR gene at 411 bp, and oprD gene at 949 bp was not detected in 3 isolates (Figure 3). Regarding antibiotic resistance, mexB, mexR, and oprA genes were observed in 3 isolates; however, both mexB and mexR were detected in 2 isolates, and only 2 isolates carried mexA gene.

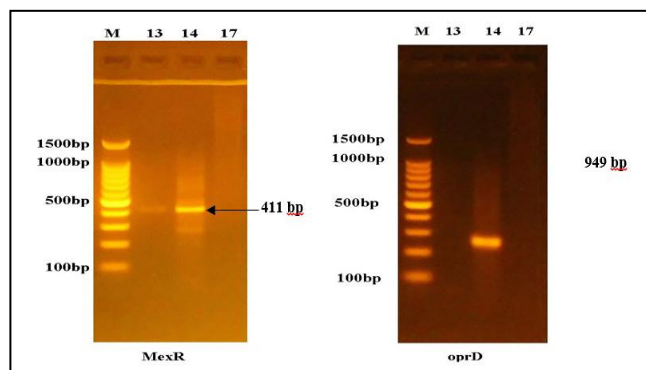

Figure 3. Gel electrophoresis of PCR products of mexR gene at 411 bp, and oprD gene at 949 bp in multidrug resistance *Pseudomonas aeruginosa* isolates. Electrical power was turned on at 100v/mAmp for 75 min

4. Discussion

P. aeruginosa is a gram-negative bacterium and an opportunistic human pathogen responsible for bacterial infections of some forms. In addition, it affects immunocompromised individuals in particular and is more commonly associated with burns, wounds, the pulmonary tract, the urinary tract, and blood infections (18).

In this study, 31 isolates of *P. aeruginosa* were collected from 200 patients with wounds and burns, and high resistance rates were observed to gentamicin, trimethoprim, amikacin, and amoxicillin. Other isolates showed fewer resistance rates to ceftazidime, tobramycin, and levofloxacin. The low resistance rates were observed to cotrimoxazole, ciprofloxacin, aztreonam, amoxicillin/clavulanic acid, augmentin, cefoxitin, ceftriaxone, piperacillin, and cefotaxime /Clavulanic acid. The results of the current study confirmed those reported by Shahzad, Ahmed (19). Furthermore, results of a study performed by Shahzad, Ahmed (19) showed varying rates of resistance to amikacin (35%), ceftriaxone (85%), ciprofloxacin (70%), and gentamicin (70%). In addition, the strains of *P. aeruginosa* had the greatest ratio of resistance to cefepime (56.67%) and gentamicin (57.97%), followed by carbapenems (55.02%) and fluoroquinolones (55.11%) (20). On the other hand, another study showed that the bacterium isolates were more liable to two aminoglycosides, including amikacin (98%) and gentamicin (100%) and two third-generation cephalosporins, including ceftazidime (88%), cefepime (94%), and ciprofloxacin (96%) (21).

P. aeruginosa decreases aminoglycoside intake by the bacteria and has also been recognized as a form of resistance. This has been considered to be a mechanism of resistance development during therapy in some situations. Aminoglycosides develop cross-resistance; however, the strength of resistance is lower than that formed by enzymatic modification. Some aminoglycoside-producing microorganisms transfer the 16S rRNA methylase gene (rmtA gene) to *P. aeruginosa* through intergeneric lateral gene transfer (22).

Aminoglycoside resistance in *P. aeruginosa* is due to enzymes that cause modification of aminoglycoside (called aminoglycoside-inactivating enzymes) encoded by the genes found on plasmids. Adenylation of a hydroxyl group by adenylyltransferase, phosphorylation of a hydroxyl group by phosphotyltransferases, or acetylation of an amino group by acetyltransferases can all inactivate aminoglycosides. *P. aeruginosa* can produce at least 14 different aminoglycoside modifying enzymes, the most common of which is acetyltransferase. The modification of aminoglycoside results in losing the ability to bind to the ribosome in this class of drugs (23).

The current study showed the highest antibiotic resistance against AK 30, AMX 30, GEN 10, and TRM 1.25. In total, 7 isolates were resistant to GEN 10, LEV 5, TMP 10, and IPM 10, 5 isolates to AMC 30, ATM 30, CAZ 30, CIP 5, TN 10, and TS 25, 2 isolates showed resistance to AUG 30, CEC 30/10, and GEN 10, and 3 isolates had multidrug resistance to AK 30, AMX 30, AUG 30, CAZ 30, CTR, FOX, GEN, PRL 100, and TN 10.

Nowadays, the discovery of an appropriate treatment for *P. aeruginosa* infections is the main public health issue considering the troubling trends in the emergence of antibiotic resistance to several antibiotics which can lead to a greater occurrence of pandrug-resistant (PDR), MDR, and XDR isolates of *P. aeruginosa*. Carbapenems (imipenem, meropenem) are

the most successful treatments of MDR and XDR isolates; however, bacteria have developed resistance to these drugs primarily due to their inadequate excessive use (24).

MDR bacteria are recognized as a source of hospital-acquired infection outbreaks in units of burn and as colonizers of wounds in burned patients (25). Patients with nosocomial infections are often infected with strains of *P. aeruginosa,* which are multi-drug resistant (i.e. has the resistance against at least these three antibiotics: ciprofloxacin, gentamicin, imipenem, and cefotaxime) (16). Many studies were conducted on multidrug resistance to antimicrobial agents. In one study, 3, 12, and 20 isolates out of 87 clinical isolates were PDR, XDR, and MDR, respectively. In addition, 100% of all isolates were resistant to antibiotic macrolides, while 80% of isolates were resistant to tetracycline and cephalosporins antibiotics. Resistance to fluoroquinolones and carbapenem was demonstrated in 33% and 22% of isolates, respectively, and resistance to carbapenem and fluoroquinolones was observed in 17% of isolates (17).

Aminoglycosides, third-generation cephalosporins, quinolones, carbapenem, and trimethoprim-sulfamethoxazole resistance were identified in MDR strains of some gram-negative bacteria (26). *P. aeruginosa* was infective in 37.5% of hospitalized patients in Iran, and their additional isolates showed 100% resistance to gentamicin amikacin, carbenicillin, tobramycin, ciprofloxacin, and other antibiotics (27). In a study coducd in Saudi Arabian, reduced susceptibility of all *P. aeruginosa* isolates was detected in the following proportions: gentamicin (85-60%), ciprofloxacin (96-64%), ceftazidime (68-47%), cefepime (78-64%), and piperacillin/tazobactam (83-77%) (28). *P. aeruginosa* was MDR in Iran, with 87%, 49%, 87%, 36%, 44%, 58%, and 10% resistance to ceftazidime, ciprofloxacin, trimethoprim-sulfamethoxazole, norfloxacin, amikacin, gentamicin, and imipenem, respectively (29).

The current study aimed at studying the frequency of multidrug resistance in *P. aeruginosa* and its relationship with the efflux pump mexA, B, and R genes and oprD gene involved in antibiotic resistance. The results of mexA, mexB, mexR, and oprD genes analysis by PCR revealed the presence of MexA, mexB, and mexR genes in multidrug resistance isolates (Figures 1 and 2), where oprD gene was not detected (Figure 3).

The development of β-lactamase is one of the most important sources of resistance. The expression of gene encoded β-lactamases enzyme in a *P. aeruginosa* increases innate resistance to β-lactams with the exception of ceftazidime, carbapenems, and cefepime. However, an excessive expression of AmpC with little penetrability or over-expression of efflux pumps can give an additional carbapenem resistance (30).

Antibiotic resistance is mediated by efflux pumps, which are active mechanisms of transportation and transfer antibiotics outside the bacterial cell. The resistance is aided by the greatest important efflux pump known as MexAB-OprM. Upregulation of the MexAB-OprM efflux pump system results in decreasing quinolone, antipseudomonal penicillin, and cephalosporin susceptibility (31). Efflux pump regulation correlates to the regulation of outer membrane proteins. A particular operon (mexA, mexB, and OprK) stimulates resistance to beta-lactams, tetracycline quinolones, and chloramphenicol by drug efflux. This energy-dependent process could be supported by outer membrane proteins (OprK) (32).

The MexAB-OprM efflux mechanism is accountable for increased resistance to numeral antimicrobial agents, including β-lactams, quinolones, macrolides, tetracyclines, lincomycin, and novobiocin (33). Recent studies have shown that *P. aeruginosa* isolates exhibit low levels of MexAB-OprM, an efflux pump involved in the out-of-cell export of antimicrobials, and enhanced resistance to broad-spectrum antibiotics, such as penicillin, cephalosporins of the third generation, monobactams, macrolides, tetracyclines, and fluoroquinolones (Ciprofloxacin, Levofloxacin, and Ofloxacin) (34). The expression of the mexAB-oprM operon is regulated by mexR (35).

Some chromosomally encrypted efflux structures and external membrane porins play vital roles in MDR phenotype resistance. In the wild type of *P. aeruginosa* isolates, the MexAB-OprM pump is conveyed at a high sufficient level to activate inherent MDR. Overexpression of the MexAB-OprM efflux pump is caused by mutations in the mexR gene (36).

Pseudomonas aeruginosa must allow nutrients transported into the cell to survive. This exchange is facilitated by a series of barrel proteins that produce water-filled diffusion channels known as porins. *P. aeruginosa* genomes contain up to 163 recognized or anticipated outer membrane proteins, 64 of which belong to one of three porin families, (i.e., the OprD-specific porin, the TonB-dependent gated porin, and the OprM efflux/secretion). The majority of these porins have small molecular masses, with the largest OprF (37.6 kDa). Further, porins are involved in the carrying of amino acids, sugars, phosphates, siderophores, and divalent cations (37) and are also linked to the transportation of certain hydrophilic antibiotics, including lactams, aminoglycosides, tetracyclines, and fluoroquinolones (38).

5. Conclusion

Most *P.aeruginosa* isolates are multi-resistant to gentamicin, trimethoprim, amikacin, and amoxicillin antibiotics. Regarding antibiotic resistance, mexB, mexR, and oprD genes were in 3 isolates, in which both mexB and mexR were detected in two isolates. Only one isolate carried mexA gene.

Authors' Contribution

Study concept and design: A. R. M.
Acquisition of data: A. R. M.
Analysis and interpretation of data: N. M. H.
Drafting of the manuscript: N. M. H.
Critical revision of the manuscript for important intellectual content: A. R. M.
Statistical analysis: A. R. M.
Administrative, technical, and material support: A. R. M.

Ethics

Ethical approval for this Study was obtained from the Regional Ethical Review Board University of Kirkuk, Kirkuk, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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