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PCR detection of genes encoded antibiotic resistance in Stenotrophomonas maltophilia that isolated from clinical infections

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***Abstract*---**Background: *Stenotrophomonas maltophilia* is an aerobic, Gram-negative, no fermentative bacteria. It is an uncommon bacteria that is difficult to treat in people. The initial term was *Bacterium bookeri*, however it was eventually renamed to *Pseudomonas maltophilia*. It was resistant to multiple antibiotics, and its mechanisms also include acquired and intrinsic resistance. It is innately multi drug resistant (MDR) and found in watery and humid environments in the environment. Aim of study: The study amid to see if there were any antibiotic resistance genes in specimens that were found in *S. maltophilia*. Materials and methods: The *S. maltophilia* isolates were isolated and identified from 250 of clinical specimens, biochemically analyzed, susceptibility test by using Kirby-Bauer disk diffusion method and genetically screened for antibiotic resistance genes using a traditional (PCR). Results: The molecular profile revealed that (*L1Q* gene) was identified in 20 (100 %) of *S. maltophilia* isolates and (*L2Q* gene) was found in 20 (90 %) of *S. maltophilia* isolates for β- lactamase antibiotics, and (*Aph* gene) was found in 20 (15 %) of *S. maltophilia* isolates for aminoglycoside.

***Keywords*---**PCR detection, genes encoded antibiotic resistance, Stenotrophomonas maltophilia, clinical infections.

Introduction

Stenotrophomonas maltophilia is a aerobic, Gram-negative, glucose non-fermenting, motile bacilli. It was first isolated from pleural effusion in 1943 and initially named *Bacterium bookeri* by J. Edwards. The organism was reclassified as a member of the genus Pseudomonas in 1961 as *P. maltophilia , Xanthomonas* in 1983 as *X. maltophilia* , then, in 1993 after large study of DNA and RNA hybridization reclassified and named *S. maltophilia* by Palleroni and Bradbory (AlAnazi and Al-Jasser, 2014).

The *S. maltophilia* is a gram-negative bacillus that is innately multi drug resistant (MDR) and found in watery and humid environments in the environment, including plants, animals, and water sources (Gulcan *et al.,* 2004; Yu *et al.,* 2016; Han *et al.,* 2020). The *S. maltophilia* was resistant to multiple antibiotics, and its mechanisms also include intrinsic resistance and acquired resistance (Calvopina and Avison, 2018; Wang *et al.*, 2018). It is a nosocomial bacterium that causes health-care-associated infections (HCAIs) by direct contact, ingestion, aspiration, aerosolization of potable water, or healthcare workers' hands (Guyot *et al.*, 20

The non-fermentative, Gram negative, rod-shaped bacteria *S. maltophilia* is abundant in the environment and has a wide geographical spread. Both in and out of clinical situations, this bacterium species has been isolated from aquatic sources (Brooke, 2012; Flores-Trevino *et al.*, 2014) .The *S. maltophilia* infections have a significant attributable death rate (37.5 %), based on the patients' initial clinical state (Paez & Costa, 2008; Falagas *et al.*, 2009; Pedrosa-Silva *et al.*, 2022).

In immunocompromised individuals, this opportunistic bacterium can cause serious infections as bacteremia, sepsis, pneumonia, meningitis following neurosurgical operations, endocarditis, urinary tract infection, septic arthritis, and endophthalmitis (Botana-Rial *et al.*, 2016; Waite *et al.*, 2016). Intensive care units (ICUs), emergency departments, respiratory units, cancer units, and surgical wards have all been linked to outbreaks of *S. maltophilia* clinical strains (Brooke, 2012) .

Antibiotic resistance has been found in HCAI-associated *S. maltophilia* strains, including β-lactams and aminoglycosides (Wu *et al.*, 2006; Chang *et al.*, 2007; Cruz-Córdova *et al.*, 2020 .(Due to high levels of intrinsic and acquired resistance to a broad range of antibacterial agents, including aminoglycosides, and the most common of β-lactam antibiotics, *S. maltophilia* has been considered one of the leading multi-drug resistant (MDR) organisms in hospital settings over the last decade (Brooke, 2014).

Antimicrobial resistance mechanisms include the production of antibiotic hydrolyzing or modifying enzymes, as well as changes in membrane permeability, as well as multi-drug efflux systems in *S. maltophilia* have been discovered (Hu *et al.*, 2008; Huang *et al.*, 2014) .Low membrane permeability, efflux pumps, and the intrinsic betalactamases *L1Q* and *L2Q,* among other drug resistance factors, are related with *S. maltophilia* strains' intrinsic resistance (Sanchez *et al.*, 2002; Crossman *et al.*, 2008; Mojica *et al.*, 2019).

The *S. maltophilia* can also develop resistance by horizontal gene transfer, which involves the acquisition of mutations or resistance genes (Sanchez, 2015) .Isolates are also commonly resistant to aminoglycosides that are known to be substrates of aminoglycoside phosphotransferase (*Aph*) enzymes, such as kanamycin and neomycin, indicating that the *S. maltophilia* genome may include an *Aph* gene (Li *et al.*, 2003; Okazaki *et al.*, 2007).

Materials and Methods

Specimens Collection

During study period from November 2021 to April 2022 ,250 clinical specimens were collected from patients suffering from different infections were included by (90) of urine from UTI , (30) swab from wound infection , (65) from burn infection, (45) from foot ulcer infection and (20) from bed ulcer infections. From those (119) of specimens were collected from Al-Sadder Medical City, (59) Al-Hakeem General Hospital, (12) the Public Health Laboratory (Center Laboratory) , and (60) Burn center in Al-Najaf province.

Bacterial Isolation and Identification

All of the specimens were grown on appropriate media, such as MacConkey agar and blood agar, and incubated at 37°C for 24-48 hours. The primary identification of bacterial isolates was based on morphological aspects of the colonies and microscopically examined with Gram's stain, followed by preparation of pure cultures for biochemical tests and The susceptibility of *S .maltophilia* isolates to 11 of common antibiotics which used in treatment of bacterial infections ,using Kirby-Bauer disk diffusion method. The results were interpreted according to the diameter of inhibition zones and compared with standard zones of inhibition of CLSI (2021) Table (1) and ultimately identification with the Viteck-2 system.

Table (1):Antibiotics Discs Used in Current Study

Antibiotic Class	Antibiotic disk	Symbol	Disc Content µg / disk	Manufacturing company
B- lactam	Ceftriaxone	CRO	10	Bioanalyse (Turkey)
	Ceftazidime	CAZ	30	Bioanalyse (Turkey)
	Cefepime	FEP	10	Bioanalyse (Turkey)
	Cefotaxime	CTX	30	Bioanalyse (Turkey)
Fluoroquinolones	Ciprofloxacin	Cip	10	Bioanalyse (Turkey)
	Levofloxacin	Lev	5	Bioanalyse (Turkey)
sulfamethoxazole -	Trimethoprim/	STX	30	Bioanalyse (

Sulfonamides	Sulfamethoxazole			Turkey)
Aminoglycosides	Gentamicin	CN	10	Bioanalyse (Turkey)
	Amikacin	Ak	10	Bioanalyse (Turkey)
Macrolides	Erythromycin	E	60	Bioanalyse (Turkey)
	Azithromycin	AZM	15	Bioanalyse (Turkey)

Molecular identification
DNA extraction

The *S. maltophilia* isolate after cultured on MacConkey agar , inoculated individually into broth and incubated at 37˚C/ 24h. the Genomic DNA Extraction Kit (Favorgen /Taiwan) was used for DNA extraction by manufacturers protocol. The extracted DNA samples were stored at – 20 C°.

Primers Selection

The following primers were used to detected the presence of gene in bacterial isolates according to the Vu-thien *et al.*,(1999) that show in Table (2)

Table (2): The primers were used in this study

Type of Primer	Primer sequence	size product (bp)	Reference
L1Q	R/ (5′-CAG CAG CAC CGC CGT TTC-3′) F/ (5′-ACC CCT GGC AGA TCG GCA C-3′)	257	Huang *et al.*,2010
L2Q	R/ (5′-CGC CTG TCC AGC AAT GCC -3′) F/ (5′-AAC GCA CCC ACC GAT GCC -3′)	221	Huang *et al.*,2010
Aph	R/ (5′-TGC TGG CGT GGG ACA ACA -3′) F/ (5′- ATG GAA GCA CCC AAT CC -3′)	1102	Okazaki, and Avison, 2007

PCR Mixture

Optimization of polymerase chain reaction was accomplished after several trials, thus the following mixture was according to information of Promega company (USA) .The mixture of PCR consist from the following :

Table (3): The PCR Mixture

Mixture solution	
Distilled water (D. W)	2.5 µL
Gotaq master mix	12.5 µL
Forward primers	2.5µL of each primer

Reverse primers	2.5µL of each primer
DNA template	5 µL
Final volume 25 µL	

Polymerases chains reactions (PCR)

Initial denaturation at 95°C for 5 minutes was followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at (65°C - 60°C) for 30 seconds to *L1Q* and *L2Q* genes respectively, and at (55°C) for 30 seconds to *Aph* gene, extension at 72°C for 35 seconds to *L1Q* and for 30 seconds to each *L2Q*, *Aph* genes respectively , and a final extension at 72°C for 5 minutes.

Agarose Preparation

It was prepared by dissolved (1-2g) of agarose in100ml 10X TBE buffer after boiling , left to cool at 50 C^o , then 5 µl of ethidium bromide was added to agarose and poured on preparing tray, Comb was removed after hardening of agarose leaving wells.

Agarose Electrophoresis

TBE (10X) buffer was added to the electrophoresis tank, tray with agarose was immersed in electrophoresis tank. Each well is loaded with 6-12µl of DNA sample and standard molecular weight of DNA ladder (marker) is loaded in a first well, electrophoreses run at 70 volt/cm for 1hr, then gel was visualized with UV transilluminator and photographed by using digital Camera (Mishra *et.al.*, 2009).

Results and Discussion

From November 2021 to April 2022, 250 of clinical specimens were collected from patients suffering from different infection , which were included 60 (24%) specimen were collected from burn center , 119 (47.6%) from Al-Sadder Medical City , 59 (23.6%) specimen from Al-Hakeem teaching hospital , 12 (4.8%) specimen were obtained from the Central health laboratory (Central Laboratory)

Table (4): Occurrence of Different Clinical Specimens in Different Najaf Hospitals

Medical center	Types of Clinical specimens					
	Burn	Urine	foot ulcer	Bed ulcer	Wound	Total
Burn Center	60	-	-	-	-	60
Al-Sadder Medical City	-	50	45	10	14	119
Al-Hakeem Teaching Hospital	-	40	-	8	11	59
The central Health Laboratory (Central Laboratory)	5	-	-	2	5	12
Total	65	90	45	20	30	250

The specimens were cultured on MacConkey agar and Blood agar, and incubated for 18-24 h at 37 C° , after incubation period the results reveal that 200 (80%) of specimens were gave bacterial growth and 50 (20%) were appear no growth (Table 5) .

Table (5): Distribution of Bacterial Growth According to Infection Site

Results Specimens	Bacterial Growth	No Growth	Total
Burn swab	60	5	65
Urine	75	15	90
Foot ulcer swabs	30	15	45
Bed ulcer swabs	15	5	20
Wound swabs	20	10	30
Total	200	50	250

From 200 bacterial growth , 135 of bacterial isolates recorded as a Gram-negative bacteria, since they were grow on MacConkey agar media and 65 they were recorded Gram positive bacteria were grow on blood agar only .From bacterial growth on MacConkey agar (135) 43 of bacterial isolates were produce pink colony ,since the bacteria was lactose fermented growth ,when grow on MacConkey agar produce pink colony , while isolates 92 were produce yellow or colorless colony ,since they were lactose non fermented bacteria According to biochemical tests , 92 of lactose non –fermented isolates on which many of the biochemical tests available were conducted, including , catalase , oxidase , motility and kligllar reaction test to approximate the results for the diagnosis of *Stenotrophomonas maltophilia* .

Identification of *Stenotrophomonas maltophilia* Isolates

The primary Identification of *S. maltophilia* was depend on the bacteriological characteristics including colonial morphology since the *S maltophilia* produce pal yellow colony when grow on MacConky agar, in addition to biochemical tests ,that , 45 of bacterial isolate were gave oxidase negative, catalase positive , motile and produce alkaline / alkaline on kliglar , were suspected as *S maltophilia*. Thus, for final identification all suspected isolates were sent to confirmed by Vitek 2-automated system., the results appear . 20 of isolates identified as *Stenotrophomonas maltophilia*, 14 of isolates were appear *Acinetobacter baumannii* , 3 of isolates were *Providinicia spp,* and 8 isolates were identified *Pseudomonas aeruginosae* .

Phenotypic Detection of Antibiotic Susceptibility test

The susceptibility of *S. maltophilia* isolates to common antibiotics which were used in treatment of bacterial infection were tested using the Kirby-Bauer disk diffusion method , and the results were recorded according to CLSI (2021) guidelines, which included 11 antibiotic from four antimicrobial categories, Overall , the results of study appear that highly resistance of bacterial isolates to

β -lactamase represented by the resistance of isolates to the third generation cephalosporins were recorded in (95%), (90%) and (75%) of isolates were resistance to ceftriaxon , cefotaxim and ceftazidime respectively , while the resistance to fourth generation cephalosporins represented by cefepime were appear in (95 %) of isolates , The resistance of isolates to aminoglycoside was found (90 %) of isolates were resistance to Amikacin and (65 %) to Gentamicin, but , the resistance of bacterial isolates to macrolides was recorded in (10 %) of isolates were resist to Erythromycin and (5%) to Azithromycin ,while the resistance to Trimethoprim/Sulfamethoxazole was recorded in (5 %) of bacterial isolates. In contrast, no one of bacterial isolates (0%) were appear resistance to fluorinated quinolones represented by ciprofloxacin and levofloxacin table (6).

Table (6): Antibiotic Susceptibility pattern of *S. maltophilia* Isolates

Antibiotic		Results			Resistant(%)
		R	I	S	
β-lactamase	Ceftazidime	15	1	4	75 %
	Ceftriaxon	19	0	1	95%
	Cefotaxim	18	1	1	90%
	Cefepime	19	0	1	95 %
Aminoglycosid	Gentamicin	13	3	4	65 %
	Amikacin	18	2	0	90%
Macrolides	Erythromycin	2	0	18	10 %
	Azithromycin	1	0	19	5 %
Quinolons	Ciprofloxacin	0	1	19	0 %
	Levofloxacin	0	0	20	0 %
	Trimethoprim/ Sulfamethoxazole	1	0	19	5 %

R=Resistant , I=Intermediate , S=Sensitive

Molecular Detection of Genes encoding Antibiotic Resistance
Molecular Detection of β Lactamase Genes

The PCR technique were used to detected the predominance of β-lactamase genes among *S. maltophilia* isolates using specific primer , the results appear that all isolates (100%) carrying *L1Q* gene (Figure 1) , while ,18 (90%) of isolates were carrying *L2Q* genes (Figure 2)

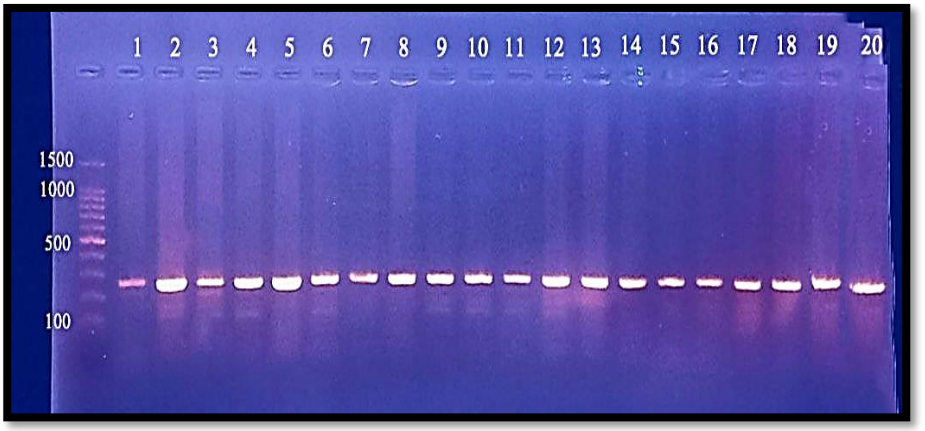

Figure: (1): Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of *S. maltophilia* using primer *L1Q* with product 257 bp, the electrophoresis was performed at 70 volt for 1- 1.5 hr. (L), DNA molecular size marker (100 bp ladder). all isolate show positive result.

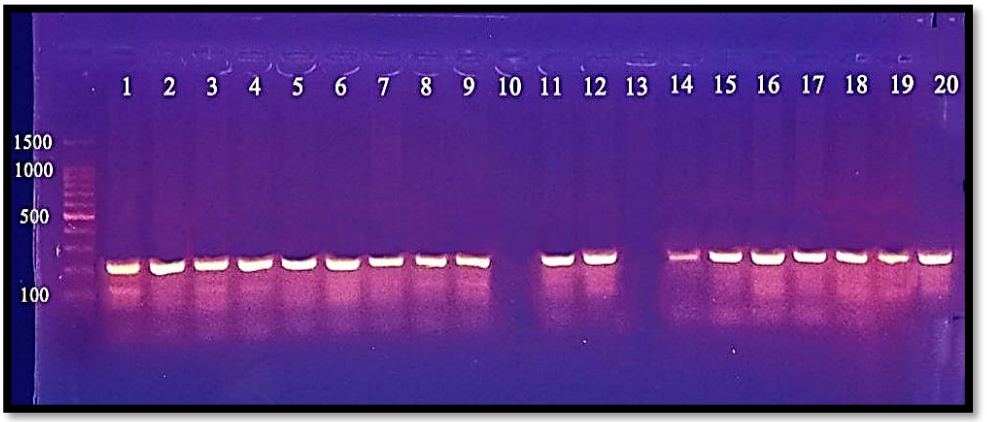

Figure:(2): Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of *S. maltophilia* using primer *L2Q* with product 221bp, the electrophoresis was performed at 70 volt for 1- 1.5 hr. (L), DNA molecular size marker (100 bp ladder). all isolates show positive result except 10 and 13 were negative results

Mojica *et al.*, (2019) reported that *S. maltophilia* is naturally resistant to many broad-spectrum antibiotics due to the production of two inducible chromosomal β-lactamases (*L1* and *L2*), this makes treatment of infected patients very difficult, *S. maltophilia* is ubiquitously present in the environment and impossible to eradicate, which makes prevention also extremely difficult.

Molecular Detection *Aph* gene

The results of PCR amplification of aminoglycosides gene ,appear that *Aph* genes were detected in 3(15%) of *S. maltophilia* isolates (Figure 3).

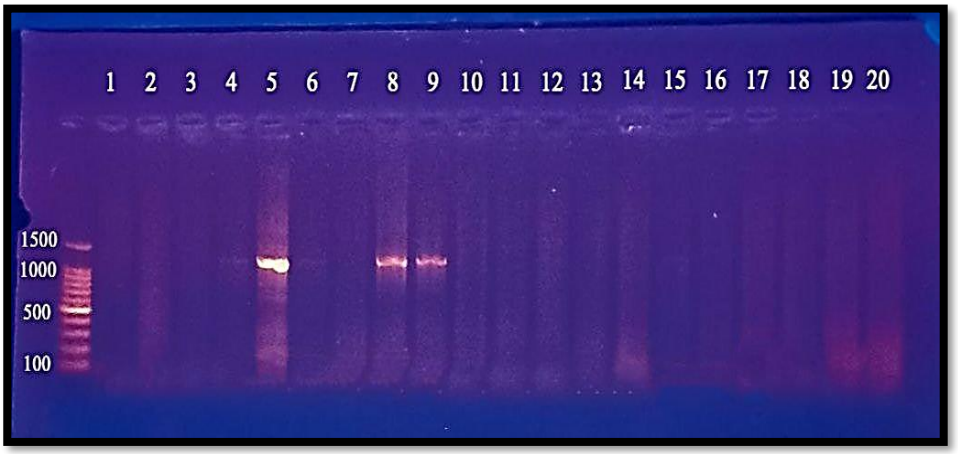

Figure:(3): Ethidium bromide-stained agarose gel electrophoresis of PCR products from extracted total DNA of *S. maltophilia* using primer *Aph* with product 1102 bp, the electrophoresis was performed at 70 volt for 1- 1.5 hr. (L), DNA molecular size marker (100 bp ladder).only the 5, 8, 9 isolates show positive result

Okazaki *et al.*,(2007) in Bristol, United Kingdom that noted *Aph* gene in *S. maltophilia* strains are (45%). In study of Mercier-Darty *et al.*,(2020) in France that found *Aph* gene reported in (94.7 %) of *S. maltophilia* strains.

Conclusions

Stenotrophomonas maltophilia was newly and emergenic multi drug resistance pathogen invade Iraqi patients causing different infections. And all the bacterial isolates appear highly resistance to B –lactamase antibiotic.

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