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RESEARCH ARTICLE
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Biofilm Formation, Antimicrobial Resistance in Serratia marcescens Isolated from Different Clinical Cases

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ABSTRACT

The current study aimed to detect the ability of Serratia marcescens to biofilms formation and antibiotics-resistance isolated from different clinical cases in Iraq. Two hundred Samples were collected from the UTI, burn, and conjunctive. Samples were cultured on MacConkey and blood agar. Isolate was identified according to colony morphology, Gram staining, conventional biochemical interactions, and analysis profile Vitek® 2 compact system. The culture results revealed an overall 20 positive isolates. Targeting the 16S rRNA gene, all positive culture isolates were confirmed as S. marcescens by PCR assay. Biofilm production is one of the virulence factors of S. marcescens using Congo red agar, it was found that 18 (90%) were producing biofilm while 2 (10%) only were not producing biofilm. But at molecular detection targeting the biofilm gene, all positive 20 (100%) isolates were confirmed to have this gene by conventional PCR assay. The detection of an antimicrobial susceptibility test by the Disc diffusion method and the isolates showed that they were susceptible to (Ciprofloxacin, Levofloxacin, Tobramycin, Amikacin, and Gentamicin). In contrast, absolute resistance against (Amoxicillin, Amoxicillin Clavulanic acid, Cephalexin,, Cfazolin, and Loracarbef). The results showed that the bacteria that made up the strong biofilm were more resistant to the antibiotics used in the present study.

Keywords: *S. marcescens; Biofilm formation; Antibiotic resistance; 16S rRNA gene*

INTRODUCTION

The most dangerous species in this genus, Serratia marcescens, is an opportunistic pathogen that can cause a number of illnesses, including pneumonia, meningitis, wound infections, lung infections, urinary tract infections (UTIs), and septicemia keratitis. When the digestive and respiratory systems are damaged, it is believed that the bloodstream is where infections are spread most frequently. S. marcescens produces virulence factors, including the ability to form biofilms on abiotic or biotic surfaces and enzymes like phospholipases, lipases, nucleases, chitinases, and proteases, which help bacteria colonize and persist in medical devices like catheters and prostheses while also boosting antibiotic resistance. (Abbas and Hegazy, 2020) A self-secreted compound of bacterial cells and macromolecules such polysaccharides, nucleic acids, lipids, and proteins has been referred to as a biofilm. The adhesion and proliferation of cells on different surfaces, such as contact lenses and epithelial cells, is mediated via biofilm formation by S. marcescens. Additionally, because of adherence to medical equipment, it is connected to the emergence of nosocomial infections.

Even with the development of tailored materials and the identification of compounds intended to prevent bacterial biofilm formation, biofilms still play a role in the pathogenicity of up to 80% of all human bacterial illnesses. (Jamal et al., 2018). An important characteristic of S. marcescens is its ability to produce β-lactamase. The β-lactamases confer resistance to the large spectrum of bacteriocidal β-lactam antibiotics including penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems, various types of Metallo β-lactamase have been identified in many strains of S. marcescens, including those responsible for epidemics (Iguchi et al., 2014). Our study aims to isolate and identify S. marcescens, and evaluate biofilm production and antibiotic resistance in clinical isolates of S. marcescens obtained from hospitals Al-Qadisiyah province, Iraq.

METHODS

Bacterial isolates

Totally, 200 clinical samples collected from hospitals Al-Qadisiyah province, Iraq. were included in this study. They were clinical samples (n = 200) from different specimens including urine (n = 80), conjunctivitis (n =60), and, burns (n = 60). All samples were collected by using sterile swabs, placed in sterile tubes containing 10 ml of buffer peptone water, and placed in a cool box for transport to the laboratory. All isolates had been identified at the species level using biochemical and molecular tests.

Identification of S. marcescens

All isolates were cultured on MacConkey agar and blood agar (Himedia, India). Culture characteristics and colony morphology were observed macroscopically. The genus S. marcescens was identified using gram staining, cultural characteristics, and biochemical tests, including estimating glucose and lactose fermentation via triple sugar iron test (TSI), urease, catalase, oxidase and IMViC test involved (indol, methyl red, voges proskauer, citrate utilization (Quinn et al., 2011).

Antimicrobial

At first, used the disk diffusion method on Muller-Hinton agar, according to CLSI guidelines (Kassim et al., 2016). the susceptibility pattern of isolates was determined against Amoxicillin (30 µg), Amoxicillin/Clavulanic acid (30 µg), Cefalexin (30 µg), Cefazolin (30 µg), Loracarbed (10 µg), Ciprofloxacin (5 µg), Levofloxacin (10 µg), Tobramycin (10 µg), Amikacin (10 µg), Gentamicin (10 µg), (Oxoid, England). Then confirmed the results by using VITEK® 2.

Biofilm production test

A specially prepared medium known as Congo Red Agar (CRA) is used for this test. The S. marcescens isolates were inoculated onto CRA and incubated at 37°C for 24 hours. Readings were taken after 24 hours and again after 48 hours. A positive result was indicated by black colonies with black crystalline morphology. Non-biofilm producers mostly produced pink- or red-colored colonies (Tahmourespour and Kermanshahi, 2011).

Molecular detection by PCR

DNA was extracted using a genomic DNA Extraction Kit (Scientific Research Company, Iraq) according to the manufacturer's instructions. Using specific primers for molecular detection of the 16S rRNA gene and biofilm gene for S. marcescens (Table 1) The PCR-Master mix for each sample was prepared at a total volume of 25µl. The tubes of the master mix were mixed well using the vortex and subjected to thermocycler conditions (Table 2).

TABLE 1: The primers used in PCR assay.

Genes		Primer Sequences (5′-3′)	Product Size (bp)	Reference
16S rRNA	F	AGAGTTTGATCCTGGCTCAG	1500bp	(Embaby et al., 2014)
	R	GGTTACCTTGTTACGACTT		
BssS – biofilm	F	ATGGACAGAAATGATGAAGTAATTC	255bp	Primer design depend on NCBI
	R	TCAATGTTTTCTGCGATCAAGAT		

TABLE 2: PCR programs of biofilm gene.

Gene	Step	Temperature	Time	Cycle
BssS-biofilm	Initial denaturation	95.0 C°	5 min	1
	Denaturation	95.0 C°	1 min	35
	Annealing	52.0 C°	1 min	
	Extension	72.0 C°	1 min	
	Final Extension	72.0 C°	10 min	1
	Hold	4.0 C°	forever	-

RESULTS

The findings showed that S. marcescens was present in 10% (20/200) of samples. Among these isolates, 10/80 (12.5%) were obtained from UTIs; 5/60 (8.3%) from the conjunctivitis; and 5/60 (8.3%) from the burns. according to culture methods and biochemical tests. The bacterial isolates cultured on MacConkey and blood agar plates in aerobic conditions. On MacConkey agar is characterized by the production of small pale pink smooth round colonies (late lactose fermenter) with a production of red pigment when grown at 25°C. While in blood agar, S. marcescens colonies showed hemolytic activity visible as clear zones on human and sheep blood agar plates and the zones were larger for bacteria grown at 30°C than at 37°C.

Biochemical testing of S. marcescens isolates showed positive results for motility of the catalase, voges proskier, Simmon citrate, while giving negative results for indole and oxidase. The urease test and methyl red are variable, some isolates were positive and others were negative for the test. TSI agar gives alkaline - acid (pink-yellow) on the slant and the bottom does not appear gas bubbles and no H_2S production. These results confirmed that these isolates belong to S. marcescens by Identification of isolates was dependent on the VITEK 2 compact system the result of this study was 99% probability S. marcescens as shown in Fig. 1. And depended on PCR using 16S RNA gene amplified a product size of about 1500bp as shown in Fig 2.

bioMérieux Customer:
System #:
Patient Name:

Laboratory Report

Printed by: Labadmin
Patient ID:

Isolate: 21231a-1 (Qualified)
Card Type: GN Bar Code: 2411993403177442 Testing Instrument: 000014EEB97D (VITEK2C)
Setup Technologist: Laboratory Administrator(Labadmin)

Bionumber: 6521711455104230
Organism Quantity:

Selected Organism: Serratia marcescens

Comments:	

Identification Information	**Card:**	GN	**Lot Number:**	2411993403	**Expires:**	May 12, 2023 13:00 CDT
	Status:	Final	**Analysis Time:**	3.87 hours	**Completed:**	Jan 2, 2023 22:28 CST
Organism Origin	VITEK 2					
Selected Organism	99% Probability **Serratia marcescens** Bionumber: 6521711455104230 **Confidence: Excellent identification**					
Analysis Organisms and Tests to Separate:						
Analysis Messages:						
Contraindicating Typical Biopattern(s)						

Biochemical Details

2	APPA	-	3	ADO	+	4	PyrA	+	5	lARL	+	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	+	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	-
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	-	39	5KG	+
40	lLATk	+	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	+	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

FIGURE 1: Results sheet of VITEK-2 compact system for S. marcescens.

FIGURE 2: Gel electrophoresis for PCR products targeting 16S rRNA gene in S. marcescens at size 1500bp in 1.5% agarose M: Marker ladder (100 -1500 bp)

Biofilm produce in S. marcescens

S. marcescens has recently become a source of rising concern due to its tendency to form biofilms. Biofilm formation of the S. marcescens isolates (n = 20) was assessed for its ability to produce biofilms using a qualitative method using Congo red agar (CRA). In the CRA method for detection of biofilm production, Observations were made by assessing the colors exhibited by bacterial colonies growing on the surface of the Congo Red Agar plate after (24) hrs. The results showed that 18 (90%) were black isolates of S. marcescens strong producer biofilm and 2 (10%) were pink isolates of S. marcescens non-producers' biofilm.

Antimicrobial susceptibility pattern

In the present study, the S. marcescens isolates showed the highest rate of resistance in the case of Amoxicillin, Amoxicillin/Clavulanic acid, Cefalexin, Cefazolin, and Loracarbed were 100%, 95%, 90%, 90%, and, 90% respectively. Regarding antibiotic sensitivity, all the S. marcescens were (100%) sensitive to Gentamicin, Amikacin, and Tobramycin and (90%) to Ciprofloxacin and Levofloxacin respectively. (Table 3).

TABLE 3: Prevalence of antibiotic resistance among S. marcescens isolates.

Antibiotics Resistant [n (%)]	Antibiotics Resistant [n (%)]	Susceptible [n (%)]
Amoxicillin	20 (100)	0 (0)
Amoxicillin/Clavulanic acid	19 (95)	1 (5)
Cephalexin	18 (90)	2 (10)
Cfazolin	18 (90)	2 (10)
Loracarbef	18 (90)	2 (10)
Ciprofioxacin	2 (10)	18 (90)
Levofloxacin	2 (10)	18 (90)
Tobramycin	0 (0)	20 (100)
Amikacin	0 (0)	20 (100)
Gentamicin	0 (0)	20 (100)

Detection of biofilm gene

Molecular studies of biofilm gene were done for all 20 S. marcescens isolates by using specific PCR markers. It was found that biofilm gene marker was observed in 20 (100%) isolates of these bacteria, which include isolates from urine, and burns. It was amplified products produced a band at the level of (255bp) when compared with the allelic ladder as shown in Fig 3. The high presence of the biofilm gene in the UTI confirms the important role of biofilms as a colonization factor in UTIs.

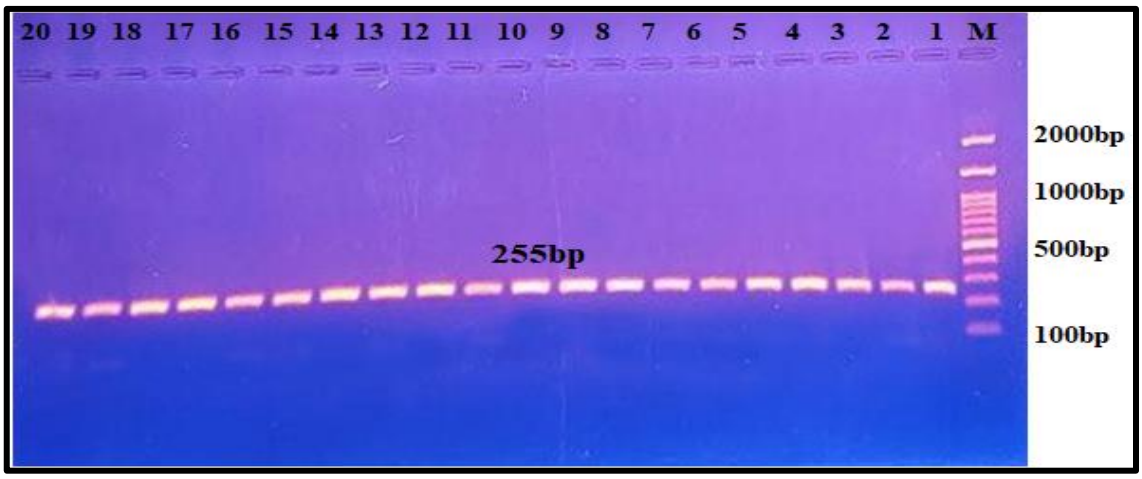

FIGURE 3: Gel electrophoresis for PCR products detecting of biofilm gene in S. marcescens. M: 100 bp molecular weight marker, lane 1-20: positive strains (255 bp).

DISCUSSION

Serratia marcescens was once considered a harmless saprophytic organism that appeared to grow on decaying organic matter, plants, animals, food, water, and soil. Now it is considered an opportunistic pathogen that has been recognized to cause nosocomial infections, especially in intensive care units of pediatric hospitals, where it is associated with a wide range of hospital-acquired infections such as respiratory and urinary tract infections, bacteremia, septicemia, conjunctivitis, after-surgery wound infection, pneumonia, otitis media, meningitis, endocarditis, and osteomyelitis (Mohajerani et al., 2019). this research assessed biofilm formation, and antibiotic resistance in 20 clinical S. marcescens isolates. Based on the results, the isolation of S. marcescens was higher from UTI than from conjunctivitis and burns. This is in accordance with Kashash and Abdul-Kareemet (2022). and Montagnani et al.(2015) who isolated S. marcescens from clinical samples in Baghdad. Iraq. But it was different from the results of Al-Mosawi (2018). He shows the prevalence of S. marcescens in urine and burns (73.52%, and 37.5%).

A major worry in human medicine is the rise of multidrug resistance, particularly resistance to extended-spectrum beta-lactamase among Serratia species. Consequently, studies investigated the epidemiology of these resistance enzymes. (Harada et al., 2019). In now study, The highest resistance among all isolates was to amoxicillin, Amoxicillin/Clavulanic acid, Cefalexin, Cefazolin, and Loracarbed. but higher sensitivity in all isolates was to Gentamicin, Amikacin, Tobramycin, Ciprofloxacin, and, Levofloxacin A similar study by Zaric et al.(2023).

In the past, third-generation cephalosporins, fluoroquinolones, and aminoglycosides were the predominant treatments for S. marcescens infections. But certain S. marcescens clinical isolates now exhibit multidrug resistance to these medications (Merkier et al., 2013). ESBLs are the result of mutations in classical plasmid-encoded β-lactamases that broaden the enzymes' hydrolytic spectrum to include broad-spectrum agents such as cefotaxime, ceftazidime, and cefepime (Lynch et al., 2013).

This study shows Serratia marcescens ability on produced biofilms on Congo red agar with 90%. While showing PCR results the biofilm gene was the most important virulence factor in S. marcescens with 100%. This study is in accordance with Ray et al.(2017) and Ramanathan et al.(2018) who detected the phenotype and genotype of biofilm in S. marcescens isolated from clinical samples.

Clinical isolates of S. marcescens have been demonstrated to create biofilms that are resistant to commonly prescribed antibiotics, and the generation of biofilms by clinically significant bacterial pathogens is the main contributor to device-associated chronic infections (Ray et al., 2017). Because biofilm cells exhibit a markedly higher level of antimicrobial resistance than planktonic or free-floating cells, the ability of bacteria to form biofilms complicates the issue of antimicrobial resistance. S. marcescens has

evolved an adaptive approach, as evidenced by the ability of bacteria buried in an extracellular matrix to develop resistance to environmental pressures necessary for ecological survival and competition. Biofilm production, however, poses a risk to human health and bio-industrial settings. The mechanism of S. marcescens' attachment to biotic and abiotic surfaces is unknown, despite the fact that its adherence to a variety of substrates is well understood (Koo and Yamada, 2016).

CONCLUSION

The significant levels of antibiotic resistance found in this study presumably point to S. marcescens production of biofilms. The transmission of transposable elements containing resistant genes in clinical isolates, as well as the indiscriminate and uncontrolled use of antibiotics, may be to blame for the increasing antibiotic resistance among patient isolates.

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