



Research Article

Occurrence of Biofilm Formation in *Serratia fonticola* and *Pantoea* sp. Isolates among Urinary Catheterized Patients

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Abstract

Serratia fonticola and *Pantoea* sp. are gram negative bacteria belonging Enterobacteriaceae, which were considered opportunistic pathogens and resulted in a great number of cases of nosocomial infections with serious problems of multi-drug resistance, leading to increasing morbidity and mortality rate. Recently, they were recorded as biofilm producers. There were only a few studies about the capability of these bacteria of forming biofilm. So our aim was evaluate the occurrence of *Serratia fonticola* and *Pantoea* sp. biofilm former phenotypically and genetically with the determination of their abilities to multi-drug resistance. *Serratia fonticola* and *Pantoea* sp. isolated from urine catheterized patients who were hospitalized in Iraqi hospitals. They were then examined for detection of biofilm formation phenotypically by congo red and tissue culture plate methods and genetically by detecting *SmaI* and *EsaI* genes (quorum sensing genes) in *Serratia fonticola* and *Pantoea* sp. respectively by using polymerase chain reaction method and tested for antimicrobial susceptibility by disc diffusion and VITEK2 system according to Clinical and Laboratory Standards Institute (CLSI). *Serratia fonticola* at 3 and *Pantoea* sp. at 4 isolates revealed to possess the ability of forming biofilm which contained *SmaI* and *EsaI* genes with 100% resistance to most tested antibiotics except imipenem and azithromycin. *SmaI* and *EsaI* genes are present in *Serratia fonticola* and *Pantoea* sp. respectively, and are responsible for biofilm formation and considered as indicator; biofilm formation is a strong cause of multidrug resistance in bacteria.

Keywords: Biofilm; *Serratia fonticola*; *Pantoea* sp.; Urinary catheter; Multidrug resistance

Introduction

Biofilm is an aggregate of microorganisms such as bacteria and the attachment to biotic surface which is protected by an extracellular polymer matrix composed of polysaccharides and extracellular DNA; it has widespread implications in the medical field [1]. According to the Center for Disease Control (CDC),

the rate of bacterial infection associate with biofilm formation was estimated to be 65% of all infections, while the National Institutes of Health (NIH) estimated 80%. Many microorganisms form biofilms, including fungi and bacteria. The most common bacterial species as causative organisms of biofilm in urinary catheter are Enterobacteriaceae, such as (*Serratia* and *Pantoea* sp.). Recently some bacterial strains have entered the

world of biofilm, including *Pantoea* sp.; the first report concerning this bacterium was published in 1972, and it was previously named *Enterobacter* and *Erwinia*, belonging to the Enterobacteriaceae family [2]. It is an opportunistic pathogen that can cause human diseases by two ways; wound contaminated with plant materials or nosocomial infections [3]. Hospitalized patients, especially those with immunocompromised infections, may be exposed to contaminated equipment or fluids with these bacteria to cause infection [4, 5].

Serratia sp. is considered an opportunistic human pathogen, as having achieved a great number of cases of nosocomial infections with the serious problem of multi-drug resistance (MDR) [6]. A large number of *Serratia* spp. are found to be isolated from urinary tract clinical samples [7, 8]. Manikandan et al. reported that *Serratia* sp. were the least dominant uropathogen causing urinary tract infection (UTI) [9].

Experimental

Materials

Collection of specimens

Twenty-eight biofilm bacterial isolates were isolated from urine samples collected from catheterized patients from four Iraqi hospitals (Al Diwaniya Educational, Al Hilla Educational, Al Qassim and Al Hashimiya Hospitals) during the period from February to April 2017 in sterile tubes of 10 mL and then transferred to laboratory immediately.

Bacterial isolate

S. fonticola and *Pantoea* sp. were isolated from urine of catheterized patients which were identified and diagnosed by the automated system VITEK 2 (bioMérieux, Marcy l'Etoile, France) to achieve final diagnostics and identification of the species level.

Reference bacterial strain:

Serratia marcescens and *E. coli* were diagnosed by biochemical tests as reference strains to *Serratia fonticola* and *Pantoea* sp., respectively.

Biofilm diagnosis

To detect the biofilm forming bacteria, three methods were employed as follows.

Congo red agar method (quality method)

According to Freeman, et al. [10], black crystalline colonies were considered as strong biofilm producers,

dark colonies without dry crystalline colonies as moderate biofilm producers and dark pink colonies as non-biofilm producers.

Tissue culture plate method

The test was carried out according to Christensen, et al. [11]. The mean of the absorbance value from replicate wells was read; the biofilm degree was calculated according to the following equation:

$$\text{Biofilm degree} = \text{Mean OD}_{630} \text{ of tested bacteria} - \text{Mean OD}_{630} \text{ of control}, \quad (1)$$

where OD_{630} means optical density at 630 nm.

The results were interpreted in Table 1.

Table 1 Classification based on OD values

Mean OD value	Adherence	Biofilm formation
< 0.120	Non	Non/weak
0.120 ~ 0.240	Moderate	Moderate
> 0.240	Strong	Strong

Note: OD = optical density

Genetic diagnosis by polymerase chain reaction (PCR) method

PCR technique was performed for biofilm formation genes, i.e. *smal* and *Esal* genes in *Serratia* sp. and *Pantoea* sp., respectively. The primers were designed by using National Center for Biotechnology Information (NCBI) gene sequence data base and primer 3 plus design. This primer was provided by Bioneer, South Korea (Table 2).

DNA extraction

DNA extraction was carried out according to manufactured instructions of commercial DNA extraction kit (Presto Mini-DNA Bacteria Kit. Geneaid Biotech Ltd. USA). Then, extracted DNA was estimated by the nanodrop device at 260/280 nm.

PCR master mix preparation

PCR master mix was prepared from Accu-Power® PCR-PreMix-Kit master mix reagent according to the company directions (Table 3).

The PCR mixture revealed in Table 3 was placed in AccuPower PCR-PreMix that contained all PCR components (Taq DNA polymerase, dNTPs, and 10 PCR buffers). Then, all the PCR tubes were transferred into vortex vibration for 3 min and transferred into thermocycler apparatus (MyGene, Bioneer, Korea).

Table 2 Polymerase chain reaction (PCR) primers and their sequence with GenBank codes

Primer		Sequence 5'-3'	Size of PCR amplicon (bp)	Bacterium
EsaI gene	F	TTTGGCCACCGCTCAAAAAC	127	<i>Pantoea</i> sp.
	R	TGGCGTATCGTTGCTGAATC		
SmaI gene	F	TCACGTCATTTGCAGCTTGC	73	<i>Serratia</i> sp.
	R	ATTGTTGAACACGCCATCGC		

Table 3 Company instructions for polymerase chain reaction (PCR) master mix

PCR master mix	Volume (μ L)
DNA template	5
Forward primer (10 pmol)	1.5
Reverse primer (10 pmol)	1.5
PCR water	12
Total volume	20

PCR thermocycler conditions

Conditions of the PCR thermocycler are listed in Table 4.

PCR product analysis

The products of PCR were analyzed by electrophoresis in a 1% agarose gel then stained with ethidium bromide and observed under a ultra-violet (UV) transilluminator.

Antimicrobial susceptibility testing

The test for antimicrobial susceptibility was done by the following two methods.

Disc diffusion method

According to Clinical and Laboratory Science Institute (CLSI), 2015 [12], the test was carried out on Müller-Hinton agar with antibiotics disc listed in Table 5.

Minimum inhibitory concentration (MIC)

The testing was done by using VITEK 2 AST system for antibiotics including ampicillin/clavulanic,

cefazolin, ceftazidime, ceftriaxone, cefepime, ertapenem, imipenem, gentamicin, tobramycin, ciprofloxacin, levofloxacin, nitrofurantoin, trimethoprim/sulfamethoxazole.

The results in both methods were regarded as sensitive, intermediate or resistant based on the Clinical and Laboratory Science Institute (CLSI) standard guidelines, M100, 2017 [13].

Statistical analysis

All experiments were carried out in triplicate to validate the reproducibility of experiments. Statistical analysis was done by using one way ANOVA at p-value 0.05 by SPSS Statistics 24.0 software.

Results and Discussion

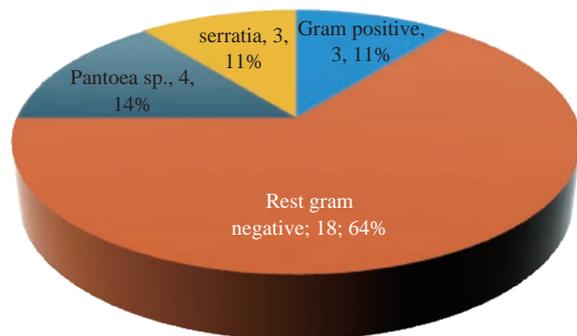
The results revealed that *Pantoea* sp. were the second predominant biofilm forming bacterium at 4 isolates (14%) followed by *S. fonticola* at 3 isolates (10.7%) (Fig. 1) which formed biofilm from 65 urine sample. *Pantoea* sp. belong to the Enterobacteriaceae; it was previously named *Enterobacter* and *Erwinia* [14]. It is an opportunistic pathogen that can cause diseases to hospitalized patients, especially those with immune compromised infections when exposed to contaminated equipment or fluids with these bacteria [3]. Recently, *Pantoea* sp. isolated from humans were considered as an opportunistic pathogen associated with contaminated catheters [15, 16]. Some species drive different gene expressions by quorum sensing

Table 4 Conditions of (polymerase chain reaction) PCR thermocycler

PCR step	Temperature ($^{\circ}$ C)	Time (min)	Repeat cycle
Initial denaturation	95	3	1
Denaturation	95	0.5	
Annealing	58	0.5	30
Extension	72	0.5	
Final extension	72	5	1
Melting	4		Hold

Table 5 Antibiotics disc with potency and the manufacturer company

Antibiotics	Symbol	Potency	Manufacturer
Amikacin	AK	30 µg	
Azithromycin	AZM	15 µg	
Carbenicillin	PY	100 µg	
Clindamycin	DA	2 µg	Bioanalysis, India.
Imipenem	IPM	10 µg	
Penicillin G	P	10 IU	
Tobramycin	TOB	10 µg	

**Fig. 1** Biofilm forming bacterial isolates.

ability which hence controls physiological activities [17] involving biofilm formation. Many studies managed to isolate *Pantoea* sp. from UTI patients at the rate of 10% to 21.4% [18, 19]. Meanwhile, a large number of *Serratia* sp. were isolated from urinary tract clinical samples [7, 8]. Manikandan and coworkers [9] reported that *Serratia* sp. were the least dominant uropathogen causing UTI, although the ability to form biofilm may contribute to its pathogenicity to make them clinically important [20]. It has flagella-mediated swimming and swarming motility which are associated with biofilm formation and facilitate the contact between bacterial cells and solid materials [21]. That may explain the ability of *Serratia* sp. to form biofilm phenomenon on urinary catheter. Biofilm production has been reported for several *Serratia* sp. [22].

The results revealed most bacterial isolates that showed biofilm formation belonged to the Enterobacteriaceae family. Biofilm formation associated with urinary catheter infections are mostly caused by Enterobacteriaceae microflora, gram negative rods (*Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter* sp., *Pseudomonas aeruginosa*, *Serratia* sp., *Proteus mirabilis*, etc.) [23], who are thought to be derived largely from the patient's own gut microbiota. The most common routes of urinary tract infection mainly involve fecal flora ascending to the bladder and kidneys via the urethra. The bacteria that colonize the

distal urethra attach to the external surface or lumen of the catheter, as it is inserted into the bladder [24, 25].

The main bacterial virulence factors involve in biofilm formation are urease production and different types of motility. Most Enterobacteriaceae members are urease positive, the most significantly, *Proteus* sp., *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Serratia marcescens* [26]. Also, the type of motility can facilitate the movement of bacteria and attach to catheter surface to form biofilm.

Biofilm detection

Pantoea sp. and *S. fonticola* isolates that produced biofilm appeared on Congo red media (Fig. 2). The black color was a result of polysaccharide matrix formation during biofilm forming process which was stained by congo red to black color [27]

**Fig. 2** Congo red agar indicating the biofilm of *Pantoea* sp. and *S. fonticola*.

In tissue culture plate (TCP) method, the bacterial isolates showed a strong, moderate and weak or non-biofilm production. *Pantoea* sp. and *S. fonticola* isolates showed strong and moderate biofilm degree, respectively, with significant difference at p value = 0.005 (Table 6), after data calculation of OD values obtained for individual biofilm bacterial isolates.

Molecular assay

The molecular assay was done for *Pantoea* sp. and *Serratia fonticola* which were isolated at the first time locally as biofilm forming bacteria from urinary catheterized patients.

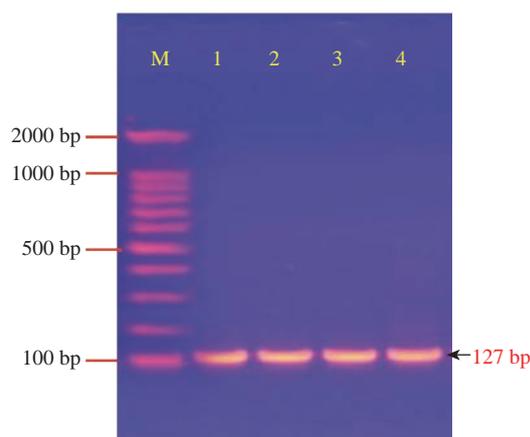
Pantoea sp.

PCR analysis of *Pantoea* sp. revealed that the four *Pantoea* isolates had Esal gene (Fig. 3) with the

Table 6 Statistical analysis of optical density (OD) of *Pantoea* sp. and *S. fonticola* in tissue culture plate (TCP)

Biofilm bacteria	OD mean \pm SE
<i>Pantoea</i> sp.	0.6597 \pm 0.1462 A
<i>S. fonticola</i>	0.2520 \pm 0.0450 B

Note: SE = stander error; Different capital letters (A, B) mean significant differences ($p \leq 0.05$) between different optical densities.

**Fig. 3** Agarose gel electrophoresis of PCR assay showing some positive results of biofilm formation by EsaI gene in *Pantoea* sp. positive isolates: **Lane (M)** DNA marker (2000 ~ 100 bp); **Lane (1-4)** Positive EsaI at 127 bp PCR product size.

Amplicon size of 127 bp, meaning they had the ability to synthesize quorum sensing (QS) signaling molecules N-acylhomoserine lactones (AHLs) and biofilm formation [28, 29]. It was pointed out that *Pantoea* members had been used to synthesize QS which regulated many phenotypes, such as the production of virulence factor, the aggregation of cells and biofilm formation [30]

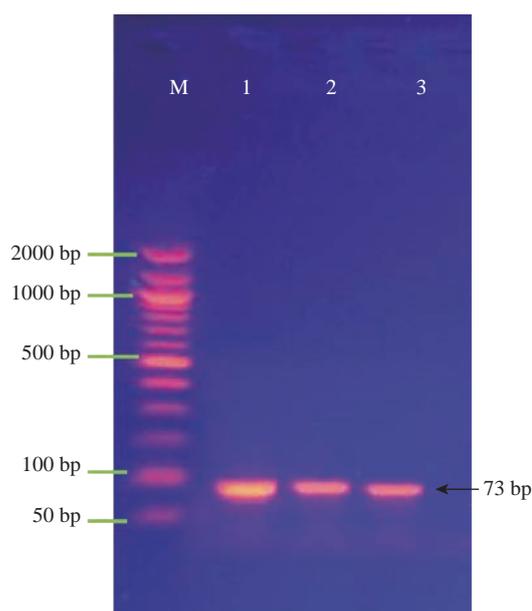
Pantoea sp. showed QS activity by increasing cell density-dependent exopolysaccharide substance (EPS) synthesis [17, 31]. Exopolysaccharide (EPS) was a major component of biofilm bacterial matrix and a powerful virulence factor which protected the bacterial cell from antibiotics action and hosted immune defense [32]. EsaI quorum sensing gene governed the synthesis of EPS in *Pantoea* sp. to appropriate bacterial adhesion and biofilm formation [33]. EsaI gene was considered a typical N-acyl-L homoserine lactone (AHL) synthase which catalyzed synthesis of N-3-oxo-hexanoyl homoserine lactone [34].

Other studies suggested that the occurrence of EsaI gene mutation led to the lack of adhesion ability and biofilm formation by inhibiting AHL synthesis [33]. Also, the degradation of QS by chemical or biological inhibitors might inhibit biofilm formation [35].

Most of the studies about *Pantoea* sp. EsaI gene as cited were those associated with plant diseases only; there was no study about *Pantoea* EsaI gene in human diseases.

Serratia fonticola:

Fig. 4 shows the amplification of the SmaI gene (quorum sensing gene) in *S. fonticola* isolates. The results revealed that bacterial isolates were positive to SmaI gene, with the Amplicon size as of 73 bp.

**Fig. 4** Agarose gel electrophoresis of PCR assay showing some positive results of biofilm formation by SmaI gene in *Serratia* sp. positive isolates: **Lane (M)** DNA marker (2000 ~ 50 bp); **Lane (1-3)** Positive SmaI at 73 bp PCR product size.

Biofilm formation and swarming motility of *Serratia* sp. were regulated by quorum sensing [36]. SmaI was a quorum sensing-regulated gene that was involved in biofilm development [37], which regulated the activity of hemolysis, swarming motility and biofilm formation [38, 39]. QS Signaling molecules are also called autoinducers; they are small chemical molecules having a major role in attaching and inducing bacterial cells to aggregate with each other to form biofilm [40, 41].

Coulthurst et al. showed that biofilm formation in *Serratia* sp. was dependent on SmaI gene, detected by TCP as a biofilm formation indicator [38]. The Biofilm form was controlled by AHL-dependent quorum sensing in this bacteria [37]. In 2014, it was provided evidence for the first time that quorum sensing activity was present in *Serratia fonticola* by confirming a three short chains of AHLs [8].

Serratia sp. expressed QS gene (SmaI) by utilizing C4-homoserine lactone (C4-HSL) as signal molecules and directing the production of various virulence factors like prodigiosin, protease, hemolysin production, and most significantly, biofilm formation [42]. Quorum sensing system (SmaI) appeared to play a role in regulating biofilm production for *Serratia* sp., as described above.

Antimicrobial susceptibility testing

The current study focused on determining and evaluating antibiotic resistance in biofilm forming

urobacteria (*Serratia fonticola* and *Pantoea* sp.) by two methods: disc diffusion and MIC in VITEK2.

Disc diffusion and VITEK AST method

These methods were applied to test the biofilm forming bacteria *Staphylococcus lentus*, *Serratia fonticola* and *Pantoea* sp.

S. fonticola and *Pantoea* sp. were revealed as multi-drug resistant. Antimicrobial susceptibility testing was carried out by two methods: disc diffusion and MIC by VITEK AST. In disc diffusion method, all isolates of three *Serratia* and four *Pantoea* sp. (Fig. 5, 7 and Fig. 6, 8) respectively displayed resistance rate of 100%

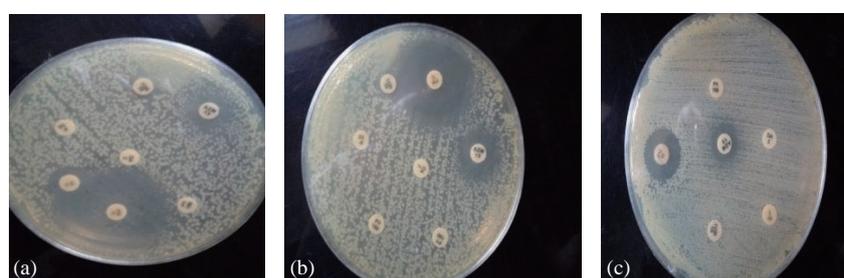


Fig. 5 Testing of antibiotic resistance to *S. fonticola* isolates by disc diffusion method. (a) *S. fonticola* 1; (b) *S. fonticola*; (c) *S. fonticola* 3.

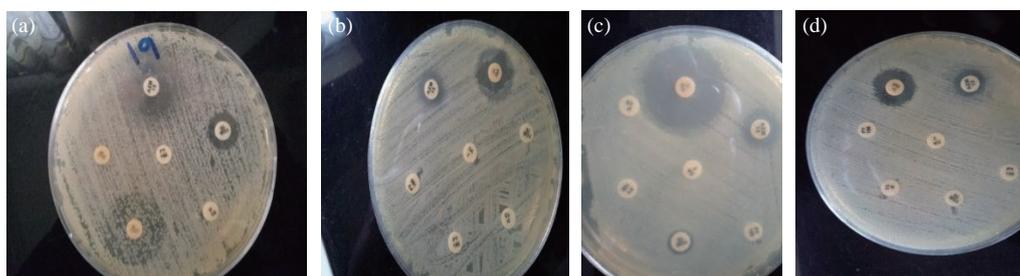


Fig. 6 Testing of antibiotic resistance to *Pantoea* sp. isolates by disc diffusion method. (a) *Pantoea* 1; (b) *Pantoea* 2; (c) *Pantoea* 3; (d) *Pantoea* 4.

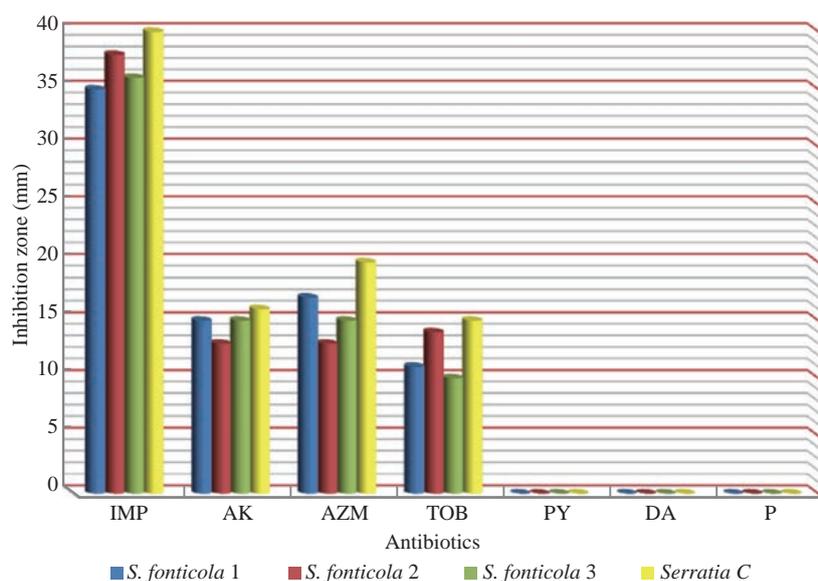


Fig. 7 Zone of inhibition (mm) of different antibiotics in disc diffusion method against *S. fonticola* and *S. marcescens* as control (IMP = imipenem; AK = amikacin; AZM = azithromycin; TOB = tobramycin; PY = carbenicillin; DA = clindamycin; P= penicillin).

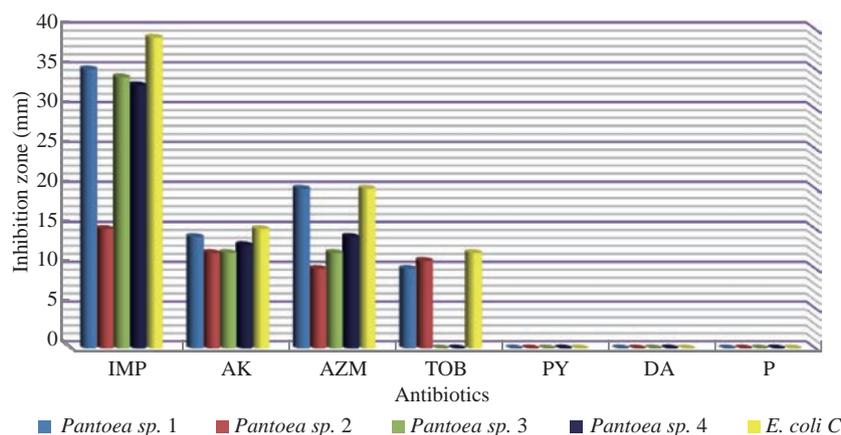


Fig. 8 Zone of inhibition (mm) of different antibiotics in disc diffusion method against *pantoea* sp. and *E. coli* as control (IMP = imipenem; AK= amikacin; AZM = azithromycin; TOB = tobramycin; PY = carbenicillin; DA = clindamycin; P = penicillin).

to amikacin, tobramycin, carbenicillin, clindamycin and penicillin. *S. fonticola* revealed sensitivity rate at 100% to imipenem and azithromycin, while *Pantoea* sp. revealed sensitivity rate of 100% to imipenem and 50% to azithromycin. On the other hand, the control non-biofilm bacteria (*S. marcescens* and *E. coli*) showed high sensitivity to imipenem, amikacin, azithromycin and tobramycin at the sensitivity rate of 100% to each one in comparison to *S. fonticola* and *Pantoea* sp. sensitivity results respectively.

Statistical analysis of susceptibility to imipenem revealed there was significant difference between *Pantoea* sp. isolates as well as between the isolates and control at $p < 0.05$ (Table 7). Meanwhile, *S. fonticola* showed a significant difference between its isolates and bacterial control (Table 8) at $p < 0.05$.

Some studies pointed out that non-biofilm *Pantoea* sp. were uniformly susceptible to most antibiotics tested in their studies [14, 43], but biofilm *Pantoea* sp. were less susceptible to antibiotics than free planktonic bacteria [44].

Table 7 Statistical analysis of susceptibility of biofilm forming of *Pantoea* sp. isolates and control to imipenem according to inhibition zone

Bacteria	Inhibition zone + SE
<i>Pantoea</i> sp. 1	35 ± 0.577 A
<i>Pantoea</i> sp. 2	15 ± 0.577 B
<i>Pantoea</i> sp. 3	34 ± 0.577 A
<i>Pantoea</i> sp. 4	33 ± 0.577 C
Control: <i>E. coli</i>	39 ± 5.77 D

Note: Values represent mean ± standard deviation (SE); Different capital letters (A-D) mean significant differences ($p \leq 0.05$) between different inhibition zones.

Table 8 Statistical analysis of susceptibility of biofilm forming *S. fonticola* isolates and control to imipenem according to inhibition zone

Bacteria	Inhibition zone ± SE
<i>S. fonticola</i> 1	38 ± 0.577 A
<i>S. fonticola</i> 2	36 ± 1.15 A
<i>S. fonticola</i> 3	35 ± 1.15 A
<i>S. marcescens</i>	40 ± 1.15 B

Note: Values represent mean ± standard deviation (SE); Different capital letters (A, B) refer to significant differences ($p \leq 0.05$) between different inhibition zones.

Imipenem was the most effective antibiotic against biofilm forming gram negative bacteria at 100% sensitivity. It was used to treat complicated urinary tract infections according to CLSI, 2017 [13]. Imipenem was a β-lactam antibiotic, resistant to β-lactamase producer bacteria which remained very stable against penicillinase and cephalosporinase bacteria; it had broad spectrum action on gram positive and gram negative bacteria according to CLSI, 2012 [45]. It acted as cell wall synthesis inhibitor by binding to penicillin binding proteins (PBPs) to cause loss of cell wall integrity and cell lysis, leading to rapid bacterial cell death [46].

In VITEK AST analysis, *S. fonticola* and *Pantoea* sp. revealed resistance rate at 100% to most antibiotics in the test as shown in Table 9.

In addition to the reasons above, many factors lie beneath the ability of bacteria to resist antibiotics, one of which is the biofilm. In this study, the biofilm forming bacteria displayed significantly high antibiotic resistance, which was correspondent to other studies [47, 48]. In the biofilm state, it was more difficult for the antibiotics

Table 9 VITEK2 antibiotic sensitivity test (AST) system results

Antibiotics	<i>S. fonticola</i>		<i>Pantoea</i> sp.	
	MIC ($\mu\text{g/mL}$)	Interp.	MIC	Interp.
Ampicillin/clavulanic	≥ 32	R	≥ 32	R
Cefazolin	≥ 64	R	≥ 64	R
Ceftazidime	≥ 64	R	≥ 64	R
Ceftriaxone	≥ 64	R	≥ 64	R
Cefepime	≥ 64	R	16	R
Ertapenem	≥ 0.5	S	≥ 8	R
Imipenem	≥ 2	I	≥ 16	R
Gentamicin	≥ 16	R	≥ 16	R
Tobramycin	≥ 16	R	≥ 16	R
Ciprofloxacin	≥ 4	R	2	I
Levofloxacin	≥ 8	R	4	I
Nitrofurantoin	128	R	128	R
Trimethoprim/sulfamithaxzol	≥ 320	R	≥ 320	R

Note: R = resistant; I = intermediate; MIC = minimum inhibitory concentration; Interp. = interpreter.

to diffuse into the bacteria, and the compounds of the matrix bound to the antibiotics also increased the difficulty [49]. The extracellular DNA displayed antibiotic chelating activity [50]. In addition to different metabolic states and as a result of grades of nutrients, oxygen viability depending on their depth inside the biofilm layers also affected bacterial susceptibility to antibiotics [47]. Gene transfer played a major role in antibiotic resistance by sharing genetic information via horizontal gene transfer among bacterial cells forming biofilm including antibiotic resistant ability [51].

Conclusions

In the present study, it was found that the *EsaI* and *SmaI* genes were present in *Pantoea* sp. and *S. fonticola* respectively, and were responsible for biofilm formation in these bacteria as biofilm regulatory genes. These genes could be considered as the indicator of biofilm formation. And the biofilm formation was a strong cause of multi-drug resistance in bacteria.

Conflict of Interests

The authors declare that no competing interest exists.

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