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Shewanella putrefaciens from Clinical Specimens and Environmental Samples. Biofilm Formation & Eradication

Hadeel T. AL-Hadithi[1,2*] and Saad A. Attia[3]

[1]Department of Biology, College of Science, University of Basrah, Iraq.
[2#]Faculty of Pharmacy, Isra University, Amman, Jordan.
[3]Faculty of Medicine, University of Thi-Qar, Iraq.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate occurrence of *Shewanella putrefaciens* in clinical and environmental sources, to determine antimicrobial susceptibility profiles of isolates and to determine the efficacy of minimum inhibitory concentration (MIC) and lesser concentrations of the most effective antibiotic and the most commonly used biocides in Iraqi hospitals: Povidone–iodine and Sapton, to inhibit or eradicate biofilms produced by recovering isolates.
Methodology: Three hundred and twenty samples collected from clinical specimens (n=173) and environmental sources (n=320) at Thi Qar General Hospital / Iraq were processed by standard techniques to isolate and identify *S. putrefaciens*. Antimicrobial sensitivity toward eight antibiotics was determined. Adhesion and biofilm formation assays were performed by recovered strains grown artificially in 96-well microtiter plates and measured spectrophotometrically. Minimum inhibition concentration and lesser concentrations of ciprofloxacin and the biocides: Povidone – iodine and Sapton for inhibition or eradication of biofilm were investigated.
Results: Eleven *S. putrefaciens* isolates (3.4%) were identified from clinical (5 isolates, 2.9%) and environmental (6 isolates, 4%) sources. Multiresistant isolates were evident, though ciprofloxacin was the only antibiotic effective against all isolates. Ear isolate demonstrated the highest degree of

**Corresponding author: E-mail: hadeelalhadithi@yahoo.com;*
[#]Present address of the first author

attachment followed by that of sewage isolate. Bacterial ability to produce biofilm was reduced up to 42.7%, 56.9% and 64.4% of the total biomass when exposed to 1/4MIC, 1/2MIC and MIC respectively. Biofilm formation was reduced up to 65.8 % and 48.7% of total biomass by exposing to Povidone-Iodine and Sapton respectively.

Conclusion: The penetration ability and the removal and killing efficacies of ciprofloxacin and the two biocides against biofilms formed in microtiter plates by *S. putrefaciens* although evident, but none of the above antimicrobial agents led to the total biofilm removal and/or killing. Hence, multidrug resistant *S. putrefaciens* have a tremendous challenge accompanied with the formation and persistence of bacterial biofilms particularly when adhere to medical devices or damaged tissue can become the cause of persistent infections.

Keywords: Shewanella putrefaciens; Multidrug resistant; Biofilm formation; Biofilm eradication.

1. INTRODUCTION

Shewanella putrefaciens is a non fermenting gram negative rods, have their natural habitat in several environments and were considered to be non pathogenic and commensals of little significance. Lately, there has been a tremendous interest in these organisms as they serve as potential reservoirs for human infections and were recovered with increasing frequency from clinical specimens [1-4]. Chronic infections and prolonged antibiotic therapy are the predisposing factors for infection with nonfermenters including *S. putrefaciens* which are becoming increasingly resistant to commonly used antimicrobial agents [6-9]. Besides, pathogenic potentials of these organisms might also related to their capability to grow as surface-attached microbial communities or biofilms that are considered as a serious health and medically related problem as being an important source of nosocomial infections [10-16].

The purpose of the present study is to investigate occurrence of *S. putrefaciens* in clinical and environmental sources at Thi-Qar Teaching Hospital / Iraq. To determine antimicrobial susceptibility profiles of isolates. Also, to determine the efficacy of minimum inhibitory concentration (MIC) and lesser concentrations of the most effective antibiotic (examined against isolates) in addition to the efficacy of the most commonly used biocides in Iraqi hospitals: Povidone – iodine and Sapton, to inhibit or eradicate biofilms produced by isolated strains of *S. putrefaciens* when grown artificially in 96-well microtiter plates.

2. MATERIALS AND METHODS

2.1 Samples

One hundred and seventy three clinical swab samples were collected from wounds (n= 108) and ears (n= 65) from in and outpatients attending Thi-Qar Teaching Hospital / Iraq. Besides, one hundred and forty seven samples were obtained from the hospital environment by swabbing hospital beds (n=71) and floor (n=51), water from garden (n=15), and sewage (n=10).

2.2 Isolation and Identification of *S. putrefaciens*

Clinical swabs and environmental samples were processed by standard techniques. They were cultured on MacConkey's agar and incubated at 35℃ for 24-48hrs. Convex, mucoid pink - salmon colonies were subjected to the following characterization tests: Gram staining, motility, oxidase, catalase, indole, citrate tests, production of H_2S on Kligher Iron Agar (KIA) slants, OF reactions (Hugh-Leifsons method), growth at 42℃, growth on Salmonella – Shigella agar (S.S agar) and in 10% NaCl [7, 17- 20]. Identification of *S. putrefaciens* was confirmed using API 20E system (bioMérieux Industries, Hazelwood, MO).

2.3 Antimicrobial Sensitivity

Antimicrobial sensitivity of each isolate was determined adopting Bauer Kirby disc diffusion method [21]. Inocula were prepared in Mueller-Hinton broth at a density adjusted to a 0.5 McFarland turbidity; standard antibiotic disks containing: Chloramphenicol (30µg), Augmentin {Amoxycillin (20µg) & Clavulanic acid (10µg)}, Ampicillin (10µg), Erythromycin (15µg), Co-trimoxazole (Trimethoprim (1.25µg) & Sulfamethoxazole (23.75µg), Tetracyclin (30µg),

Ciprofloxacin (5µg) and Cefotaxime (30µg) were placed on each inoculated plate at equal distances. Plates were incubated for 24 hrs. at 35℃, followed by measuring diameters of inhibition zones (IZDs).

2.4 Adhesion on Plastic Surface

The method of Christensen [22] was adopted. Twenty colonies of each isolate were transferred into sterile plastic tubes containing 5 ml trypticase broth and incubated for 24h at 35℃. Contents of the tubes were poured out, empty tubes were stained with safranin, then safranin was decanted and tubes were left to dry. Biofilm production was reported to occur when inner sides of the tubes were stained. Formation of a circle at liquid interface was reported as a negative result.

2.5 Biofilm Formation Assay

The method of Bonaventura, et al. [23] to measure total biofilm biomass was undertaken. Briefly, 3ml of overnight grown cultures in Tryptic Soy Broth (TSB) of *S. putrefaciens* were washed three times with sterile diluted (1:3) TSB using a centrifuge at a speed of 3000 rpm. Aliquots (200 µl) of standardized inoculums ($5x10^5$ to $1x10^6$ CFU/ml) were added to the wells of 96-well microtitre plates and incubated at 35℃ over a series of intervals (1, 4, 8, 12, and 24 hrs) in a closed humidified plastic container. The medium was then discarded, and non adherent cells were removed by washing three times with sterile phosphate-buffered saline. Biofilms were fixed by incubating plates at 60℃ for 1hr, then stained with crystal violet for 5 min., washed with running water then incubated at 37℃ for 30mins. Total biomass of biofilms was measured spectrophotometrically at a wave length 492nm. TSB without bacteria was used as a control.

2.6 Determination of MICs

MICs of ciprofloxacin (The most effective antibiotic against all isolates of *S. putrefaciens* as examined in the present study), were determined by the CLSI broth microdilution technique [24]. Aliquots of 0.1ml TSB 18hr. cultures of isolates were spread on Muller Hinton agar and 0.01ml of ciprofloxacin concentrations(0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4 and 4.5 µg/ml) were added at equal spaces. MIC was defined as the lowest drug concentration which inhibited visible growth after incubation at 35℃ for 24 h.

2.7 Reducing Biofilm Formation Assay

The role of MIC, 1/2 MIC and 1/4 MIC of ciprofloxacin to reduce capabilities of *S. putrefaciens* isolates to produce biofilm was investigated. The degree of reducing total biomass of biofilms was estimated by spectrophotometer at a wavelength of 492 nm. Aliquots of 100 µl cultures grown in TSB were placed in wells of 96-microtitre plates containing 100 µl of MIC, 1/2 MIC or 1/4 MIC of ciprofloxacin respectively as determined against each of four selected isolates. Plates were incubated at 35℃ for the periods: 1, 4, 8, 12 and 24hrs. [23].

Rate of biofilm reduction was calculated as following:

$$\text{Rate of Reducing Biofilm Formation} = \frac{\text{Rate of Optical Density Readings with Ciprofloxacin}}{\text{Rate of Optical Density Readings of Control}} \times 100$$

Besides, capabilities of the antiseptic and disinfectant: Povidone – iodine and Sapton for inhibition or eradication of biofilm were also determined by adding of either 100ul of 10% Povidone-Iodine and Sapton into wells of microtitre plates containing 100ul TSB 18hrs. Cultures of isolates then incubated at 35℃ for 24h.

2.8 Analytical Statistics

ANOVA test was applied.

3. RESULTS AND DISCUSSION

Eleven *S. putrefaciens* isolates (3.4%) were identified from clinical (5 isolates, 2.9%) and environmental (6 isolates, 4%) sources. Table (1) illustrates percentage distribution of these isolates. Isolates from sewage, hospital's garden water and ear infection reported the highest frequency (10%, 6.6% and 6.1%) respectively.

Holt, et al. [20] indicated that *Shewanella* infections occur during especially warm summers in temperate climates which matches up with the warm to hot climate of Thi-Qar Province which is located at southern of Iraq. In the present study, the highest percentage recovery of *S. putrefaciens* was reported from ear infection (6.1%), which might become chronic [25]. However, although it is accepted as

saprophytic, different clinical syndromes, most commonly skin or soft tissue infections, have been associated with *S. putrefaciens*, mainly in immunocompromised cases and patients with underlying diseases [4, 5**,** 26 – 31].

Zone diameter interpretative standards for *Pseudomonas aeruginosa* and other nonfermenting gram-negative rods [32] were adopted in the present study to reveal susceptibility or resistance of *S. putrefaciens*, because, the disk diffusion method for other non-Enterobacteriaceae has not been systematically studied by the subcommittee nor have clinical data been collected for review [33]. Table (2) demonstrates antibiotic susceptibilities of *S. putrefaciens* isolates and clarified resistance against augmentin, ampicillin and erythromycin. Ciprofloxacin was the only antibiotic effective against all isolates followed by tetracycline and chloramphenicol where 47% of isolates were sensitivite.

Isolates from ear infection, hospital bed (No.2), hospital floor (No.1) and sewage showed similar antibiogram being resistant to all antibiotics under study apart from ciprofloxacin. Multidrug resistance is common and increasing among gram-negative nonfermenters, including *S. putrefaciens* strains and have now been identified to exhibit resistance to essentially all commonly used antibiotics, including penicillins and cephalosporins, aminoglycosides, tetracyclines, fluoroquinolones, trimethoprim-sulfamethoxazole, and carbapenem [6–9].

Nevertheless, when Durdu, et al. [34] identified *S. putrefaciens* from sputum culture of a 43-year-old female patient with pneumonia they found it was susceptible to ceftriaxone, ceftazidime, cefepime, ciprofloxacin, though resistant to ampicillin, amoxycillin-clavulanate, cefazolin and cefuroxime. A comprehensive review carried out by Vignier, et al. (25) who confirmed 16 cases of *Shewanella* spp. infections in Martinique since 1997 and reviewed another 239 cases reported in the literature since 1973, found that most *Shewanella* spp. isolates are susceptible to cefotaxime (95%), piperacillin and tazobactam (98%), gentamicin (99%) and ciprofloxacin (94%).

All isolates were capable to grow as biofilms, though varied in degrees of binding to plastic surfaces. Ear isolate demonstrated the strongest degree of attachment (Table 3).

Initial adhesion of isolates has started within one hour as compared to control (optical density of control without bacteria=0.012nm). Table (4) illustrates the optical density of biofilms formed by selected isolates over 24 hrs. Apart from the first hour, attachments of isolates were significantly different ($P<0.05$) throughout time intervals under study. Also, isolates from different sources demonstrated various degrees of biofilm formation. Ear isolate demonstrated the highest degree of biofilm total biomass followed by that of sewage isolate. The ability to form a biofilm in microtiter dishes has been strongly correlated with the ability of particular isolate to cause disease in a clinical setting [10-12,14,35]. Consequently, formation and persistence of bacterial biofilms is a tremendous challenge in overcoming diseases that faces medical community.

3.1 Minimum inhibition concentration (MIC)

Values of MIC of Ciprofloxacin affecting against selected isolates of *S. putrefaciens* were 3 µg/ml for ear infection and hospital floor (No.1&2) isolates while MIC for sewage isolate was only 1 µg / ml. These results are in accordance with the strong abilities of isolates for attachment (15)

3.2 Effect of Ciprofloxacin (CIP) in Reducing Biofilms

Table (5) illustrates results of exposing isolates of *S. putrefaciens* to: MIC, 1/4 MIC and 1/2 MIC of Ciprofloxacin over 1, 4, 8, 12 and 24 hrs. periods.

No significant reduction in optical density values of biofilms was observed after exposing for 1hr.; Nevertheless, a significant reduction was recorded ($P<0.05$ and $P<0.01$, $P<0.05$) after exposing biofilms for 4, 8 and 12 hrs. respectively, as compared to control. Moreover, although optical density values of biofilms were considerably increased after 24hrs.but reduction in density of the total biomass due to exposure to CIP was remarkable.

A significant difference ($P<0.01$) in reducing capabilities of CIP was also reported among concentrations. Biofilm produced by ear infection isolate was the most resistant to reduction by the

three concentrations of CIP, while that formed by hospital floor (2) was the least resistant.

It should be reported that bacterial ability to form biofilm was reduced up to only 42.7%, 56.9% and 64.4% of total biomass by 1/4MIC, 1/2MIC and MIC respectively.

3.3 Effect of Povidone-Iodine and Sapton in Reducing Biofilm

Both biocides were effective in reducing biofilms formed by *S. putrefaciens* isolates (Table 6). Biofilm formation was reduced up to 65.8 % and 48.7% of total biomass by exposing to Povidone-Iodine and Sapton respectively.

The penetration ability and the removal and killing efficacies of ciproflaxocin and the two biocides as demonstrated in Tables: 5 and 6, determined against biofilms formed in microtiter plates by *S. putrefacience*, clarified that none of the above antimicrobial agents led to total biofilm removal and/or killing. This result is accordance with that reported by Araújo, et al. [36].

Increasing antimicrobial resistance and multidrug-resistant nonfermenters in addition to the tremendous challenge accompanied with the formation and persistence of bacterial biofilms particularly when bacteria adhere to medical devices or damaged tissue can become the cause of persistent infections [11,15,36,37].

Table 1. Percentage frequency of *Shewanella putrefaciens* in clinical and environmental samples

Source of Isolates	No. of Samples	No. of Isolates	Percentage Frequencey (%)
Clinical Specimens:			
Ear Infection	65	4	6.1
Wounds	108	1	0.9
Hospital Environment:			
Beds	71	2	2.8
Floor	51	2	3.9
Gardens Water	15	1	6.6
Sewage	10	1	10
Total	320	11	3.4

Table 2. Antibiotic susceptibility of *Shewanella putrefaciens* isolates

Source of Isolates	C	AMC	AMP	E	CTX	SXT	TE	CTP
Ear infection	R	R	R	R	R	R	R	S
Hospital bed (1)	S	R	R	R	R	S	S	S
Hospital bed (2)	R	R	R	R	R	R	R	S
Hospital floor (1)	R	R	R	R	R	R	R	S
Hospital floor (2)	S	R	R	R	S	R	S	S
Garden's water	S	S	R	R	S	S	S	S
Sewage	R	R	R	R	R	R	R	S

C: Chloramphenicol AMC: Augmentin AMP: Ampicillin E: Erythromycin; CTX: Cefataxime SXT: Cortimoxazole TE: Tetreacyclin CTP: Ciprofloxacin

Table 3. Degree of adhesion and biofilm formation by isolates of *Shewanella putrefaciens* on smooth surfaces

Source of isolates	Adhesion
Ear infection	+++
Hospital bed (1)	++
Hospital bed (2)	+
Hospital floor (1)	++
Hospital floor (2)	+
Garden's water	+
Sewage	+

Table 4. Optical density of biofilms formed by selected isolates of *Shewanella putrefaciens* at the indicated time

Source of Isolate	After 1hour	After 4 hours	After 8 hours	After 12hours	After 24hours
Ear infection	0.049	0.103	0.208	0.216	0.230
Hospital floor (1)	0.050	0.095	0.104	0.119	0.151
Hospital floor (2)	0.033	0.055	0.071	0.097	0.103
Sewage	0.067	0.079	0.098	0.109	0.173

Table 5. Effect of 1/4 MIC*, 1/2 MIC and MIC of ciproflaxin in reducing biofilm formed by *Shewanella putrefaciens* after exposure for 1, 4, 8, 12 and 24 hours

Source of isolate	1 Hour				4 Hours				8 Hours				12 Hours				24 Hours			
	Cont.† O.D.**	MIC O.D.	1/2 MIC O.D	1/4 MIC O.D.	Cont.† O.D.	MIC O.D.	1/2 MIC O.D.	1/4 MIC O.D.	Cont.† O.D	MIC O.D.	1/2 MIC O.D.	1/4 MIC O.D.	Cont.† O.D	MIC O.D.	1/2 MIC O.D.	1/4 MIC O.D.	Cont.† O.D	MIC O.D.	1/2 MIC O.D.	1/4 MIC O.D.
Ear infection	0.049	0.034	0.031	0.049	0.103	0.065	0.76	0.084	0.208	0.115	0.119	0.134	0.216	0.122	0.139	0.157	0.230	0.134	0.194	0.139
Hosp. floor 1)	0.050	0.050	0.038	0.049	0.095	0.067	0.056	0.067	0.104	0.033	0.083	0.110	0.119	0.058	0.087	0.103	0.103	0.061	0.078	0.097
Hosp. floor(2)	0.033	0.033	0.023	0.031	0.055	0.035	0.038	0.044	0.071	0.034	0.038	0.041	0.097	0.034	0.044	0.051	0.173	0.047	0.055	0.059
Sewage	0.067	0.067	0.039	0.047	0.079	0.033	0.047	0.035	0.098	0.027	0.031	0.043	0.109	0.016	0.024	0.041	0.151	0.034	0.086	0.108

**MIC: Minimum Inhibition Concentration ** O.D. Optical Density; † Cont.: Control*

Table 6. Effect of Povidone-Iodine and Sapton in reducing biofilm formed by *Shewanella putrefaciens* after exposure for 24 hrs*

Source of isolate	Control O.D.**	Povidone-Iodine O.D.	Sapton O.D.
Ear infection	0.230	0.070	0.140
Hosp. floor (1)	0.151	0.095	0.112
Hosp. floor (2)	0.103	0.086	0.082
Sewage	0.173	0.069	0.101

** hrs.: Hours ** O.D. Optical Density*

The choice of disinfectant or cleaning agent along with the optimum concentration and the time of action is very important when destroying microbes [38]. Besides, it is also important to stress on the fact that antibiotics and biocides effective against suspended bacteria, are not valuable to control microbial biofilm which has become difficult to eradicate due to bacterial colonization and attachment on surfaces.

4. CONCLUSIONS

This study will throw more light on the prevalence and pathogenic role of these organisms that pose a particular difficulty for the healthcare community.

Finally, it is quite obvious that penetration ability and the removal and killing efficacies of ciprofloxacin and the two biocides against biofilms produced in microtiter plates by *S. putrefaciens* clarified that none of the above antimicrobial agents led to total biofilm removal and/or killing.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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