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Battling Biofilm Forming Nosocomial Pathogens Using Chitosan and Pluronic F127

Doaa Eid¹ , Ossama M. Sayed² , Walaa G. Hozayen³  and Ahmed F. Azmy^{4*} 

¹Biotechnology and Life Sciences Department, Faculty of Postgraduate Studies for Advanced Sciences (PSAS), Beni-Suef University, Egypt. ²Pharmaceutics and Industrial Pharmacy Department, Faculty of Pharmacy, Beni-Suef University, 62511, Beni-Suef, Egypt. ³Biochemistry Division, Chemistry Department, Faculty of Science, Beni-Suef University, Egypt. ⁴Microbiology and Immunology Department, Faculty of Pharmacy, Beni-Suef University, 62511, Beni-Suef, Egypt.

Abstract

Biofilm represents a potential strut in bacterial treatment failure. It has a dual action; it affords microbial resistance against antibiotics and facilitate transmission of pathogenic bacteria. Nosocomial bacteria pose a serious problem in healthcare units; it prolongs patient hospital stay and increases the mortality rates beside other awful economical effect. This study was planned for targeting nosocomial bacterial biofilm using natural and biologically safe compounds like Chitosan and/or Pluronic F127. Ninety-five isolates were recovered from 107 nosocomial clinical samples. Different bacterial and fungal species were detected, from which *Klebsiella pneumonia* (23%), *Pseudomonas aeruginosa* (19%), *Acinetobacter baumannii* (18%) and *E.coli* (17%) were the predominate organisms. *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumonia* were the abundant antibiotic resistant strains with multi-resistance pattern of 72%, 65% and 59%, respectively. A significant percentage of these isolates were strong biofilm forming. Herein, we investigate the effect of Chitosan and Pluronic F127 alone and in combination with each other against biofilm production. Chitosan show variable degree of biofilm inhibition, while Pluronic F127 was able to retard biofilm formation by 80% to 90% in most strain. There is no significant difference ($P < 0.05$) between Pluronic F127 alone and its effect in combination with Chitosan.

Keywords: Nosocomial infection, Biofilm, Chitosan, Pluronic F127

*Correspondence: ahmed.abdelaziz@pharm.bsu.edu.eg; +2-01004457502

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INTRODUCTION

Nosocomial infection is a major health care problem. It causes a large percent of morbidity and mortality, one from ten patients is affected by this type of infection¹. Nosocomial infection result in prolonged hospital stay with increased healthcare costs². These infections are mainly caused by multi or extensive drug resistant organisms like Methicillin-resistant *Staphylococcus aureus* (MRSA) and extended spectrum β -lactamases (ESBL). Scarcity in discovery of a new antibiotic generation worsen the problem, so the prevention is a suitable approach for decreasing this shocking infection².

According to National Health Institute (NIH) 65-80% of chronic microbial infections are related to biofilm forming microorganisms³. The biofilm infection may be device associated or non-device associated, upon detachment from the surface the microorganism releases hydrolytic enzymes enabling them to colonize a new area⁴. Colonization may occur in a sensitive and vital places in human body like endocardium, heart valves, lungs and joints resulting in dangerous and life-threatening infections⁵. The biofilm may be performed on or within medical devices such as venous catheter, contact lenses and prosthetic heart valves by one microorganism or mixed infections according to device type and duration. Also, biofilm down side infection treatment as it decreases antibiotic penetration and allow exchange of resistance plasmids between stains in biofilm matrix⁶. Resistance in biofilm forming bacteria is significantly higher than the planktonic cells⁷. Therefore, it is so difficult to be eradicated and removed⁸.

For management of these infections a new strategy should be evolved. Use of antibiofilm compounds is an interesting one, it will decrease rate of transmission by decreasing attachment and colonization or used in combination with other antibiotics to enhance its activity⁹. Different types of compounds are used as antibiofilm agents like sub MIC concentration of antibiotics¹⁰, antimicrobial peptides¹¹, enzymes^{12,13}, or quorum sensing inhibitors like N-acetyl homoserine¹⁴.

Chitosan is a natural biodegradable and biocompatible compound, has antibacterial activity in high concentration and potent antibiofilm

activity at lower concentration. Molecular weight and acetylation degree affect its potency as antimicrobial agent¹⁵. Being as positively charged cations, chitosan can act in 3 different ways: by interaction with negatively charged microbial cells; interact with microbial DNA; or chelating important metals required for metalloprotein enzymes¹⁶.

Pluronic F127 is a synthetic non-ionic surfactant with amphiphilic properties. It is a copolymer of hydrophilic poly(ethylene oxide) and hydrophobic poly(propylene oxide). Due to their amphiphilic characters, Pluronic has an excellent surfactant property¹⁷. Combination of chitosan and pluronic acid were mixed together in a nanoparticle form for delivery of anticancer drugs with less side effect^{18,19}. The combination is also used for preparation of different pharmaceutical dosage forms^{20,21}

The aim of this study is to evaluate efficacy of chitosan and pluronic F127 alone and in combination with each other against biofilm forming nosocomial pathogens as a new tactic to retard their transmission and resistance.

MATERIAL AND METHOD

Sample Isolation

Different biological samples (urine, sputum, endotracheal secretion and blood) were collected from 107 patients between May 2017 and June 2018. All patients admitted to hospital for at least 3 days without previous signs or symptoms of previous infection. All samples were isolated from patients in ICU and neonatal ICU following ethical consideration.

Strains were isolated and purified using different types of media (Blood agar and MacConkey agar). All isolates were stored in glycerol broth at -80°C till further analysis.

Bacterial Identification and Antibiotic Sensitivity

Bacterial isolates were primarily identified by Gram stain and biochemical reactions (catalase, oxidase, motility and triple sugar iron agar). Full identification was performed by VITEK2 system (BioMérieux, USA). Antibiotic sensitivity test was done by disc diffusion method according to CLSI guidelines²² using Muller-Hinton agar and Brain Heart infusion agar. The antibiotic discs used were ampicillin/sulbactam (SAM, 20 μ g), amoxicillin/

clavulanic acid (AMC, 30µg), ceftriaxone (CRO, 30µg), piperacillin/tazobactam (TZP, 110µg), cefepime (FEP, 30µg), Ceftazidime (CAZ, 30µg), imipenem (IPM, 10µg), Colistin (CT, 10µg), gentamicin (CN, 10µg), Doxycycline (DO, 30µg), ciprofloxacin (CIP, 10µg), Chloramphenicol (C, 5µg) and Vancomycin (VA, 5µg). Sensitivity pattern of *Candida* isolates is not determined.

Biofilm Formation Assay of Isolated Pathogens

A previously isolated pure colony was resuspended in 5 mL of tryptone soya broth supplemented with 1% glucose, incubated at 37°C or 30°C for 48 hrs for bacteria and *Candida*, respectively. Twenty microliters of overnight culture were diluted in 180 µL of the above media in 96 well sterile microplate and incubated at 37°C or 30°C for 48 hrs. After incubation the growth was discarded, and the plates were washed three times with phosphate buffered saline pH 7.5 to remove non-adherent cells. The plates were dried in the oven at 65°C and stained with 200 µL of 1% crystal violet solution for 15 min. the plates were washed gently under running water and dried. A solution of 1% acetic acid is used to retain adsorbed C.V stain for 15 min. and measured spectrophotometrically at 600 nm by microplate reader (Tecan SunRise/USA). The strains were classified as weak, moderate or strong biofilm forming bacteria according to classification of Stepanovic et al.²³ The experiments were done with six replicates in three independent experiments.

Effect of Chitosan and Pluronic Acid on Biofilm Formation

Chitosan was dissolved in 1% acetic acid solution to a final concentration of 10 mg/mL. The solution was stirred overnight at 50°C to ensure complete dissolution, pH was raised to 5.8-6.0 by 1 N NaOH and sterilized by 0.2µm microbial filter (Sartorius, Germany). Pluronic F127 was dissolved at a concentration of 10 mg/mL in distilled water at pH of 7±0.2. Bacterial overnight culture was diluted in TSB amended with 1% glucose to a concentration of 10⁸ CFU/mL containing different concentrations of Chitosan and/or Pluronic F127 (5, 2.5, 1.25, 0.625 mg/ml). In a flat bottom microtiter plate, a 200 µL of the above solution was added and incubated at 37°C or 30°C for 48 hr. Extent of biofilm inhibition occurred by Chitosan and/or Pluronic F127 is detected by C.V

method. Percentage of inhibition is calculated by the following equation by Pierce et al.²⁴

$$\text{Inhibition \%} = 100 - \left(\frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \right) \times 100$$

Scanning Electron Microscope for Biofilm Inhibition Assay

Ten microliter of 0.5 MacFarland solution of diluted overnight bacterial culture was placed in the center of Sterile Millipore membrane filter (0.22 µm, 47 mm diameter, mixed cellulose esters (MCE) membrane) (Merck, USA). The filter was loaded on tryptone soya agar plates (TSA) amended with 2.5 mg/mL of Chitosan, Pluronic F127 and combination of Chitosan and Pluronic F127. The plates were incubated at 37°C for 24 hr. The membrane filters were gently removed, fixed in 3% glutaraldehyde for 30 min. The filters were washed 3 times with PBS each for 10 min. The membranes were gradually dehydrated with gradient conc of ethyl alcohol (50%, 60%, 70%, 80%, 90%, 95%, 100%), then final chemical dehydration with hexamethyldisilazane. The coupons were coated with gold, and then examined with JSM-6510 (JEOL, Japan) at a voltage of 30 kV and magnifications at x5000 to x15000²⁵.

Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using statistical package for social sciences (SPSS) computer software (version 22), IBM software, USA. One-way ANOVA test was used to evaluate significance between groups followed by tukey posthoc analysis for pairwise analysis. Differences were considered statistically significant at p<0.05.

RESULTS

Bacterial Isolation and Identification

Out of 107 clinical samples taken, 95 samples were positive for bacterial or fungal culture. Large number of isolated strains were found in sputum (30 strains) and endotracheal intubation (28 strains). Urine samples were positive for 27 specimens while blood gives only 10 isolates (Table 1).

The top four species were *Klebsiella pneumoniae* (23%), *Pseudomonas aeruginosa* (19%), *Acinetobacter baumannii* (18%) and *E.coli* (17%). Gram +ve bacteria also present

in a significant percentage represented by *Staphylococcus aureus* and *Staphylococcus haemolyticus* with 8% and 4% respectively. Other Gram-negative bacteria like *Proteus spp.* and *Enterobacter spp.* were found in a small frequency (3% and 2% consequently). Fungal infection with *Candida albicans* and *Candida tropicalis* were also reported with a lower incidence at 3 and 2% respectively. (Table 1)

Antibiotic Sensitivity Testing

All Gram-positive strains were sensitive to Vancomycin, while all Gram-negative strains were sensitive to Colistin. Ten isolates (45%) of *Klebsiella*, seven isolates (41%) of *Acinetobacter* and six isolates (33%) of *Pseudomonas* show resistance against all tested antibiotics. Four strains of *E.coli* and one isolate of *Staphylococcus haemolyticus*, *Enterobacter spp.*, and *Proteus spp.* were resistant to nine of tested antibiotics. Overall, resistance to the Piperacillin/tazobactam and Imipenem were found to be much lower. (Table 2)

Biofilm Formation Assay of Isolated Pathogen

Thirty-five isolates show no biofilm activity, while 26 and 24 isolates show weak and moderate biofilm forming ability, respectively. Only 10 isolates were strong biofilm producer and they were distributed as one strain of *St. haemolyticus*, one strain *Candid tropicalis*, three strains of *Klebsiella pneumonia*, two strains of *Acinetobacter baumannii* and three strains of *Pseudomonas aeruginosa*. (Fig. 1).

Effect of Chitosan and Pluronic F127 on Biofilm Formation

All isolated strains were significantly reduced to different levels by chitosan at the concentration used 2.5 mg/mL. The most affected strain was the extensively drug resistant *Pseudomonas aeruginosa* (XDR) strain, it was decreased by 81% of initial biofilm formed in planktonic cells. Multi- and extensive drug resistant *Klebsiella pneumonia* strains (MDR and XDR) and *Acinetobacter baumannii* were inhibited by 25-35%. Also, chitosan diminished biofilm of *Pseudomonas aeruginosa* (MDR), *Staphylococcus haemolyticus* and *Candida albicans* by 33%, 51% and 58% respectively. (Fig. 2)

Pluronic F127 has a more potent effect than Chitosan in prevention of biofilm creation at concentration used 1.25 mg/mL. It inhibits all *Pseudomonas* and *Klebsiella spp.* by more than 80%. It also prohibits biofilm production in other strains of *Staphylococcus haemolyticus*, *Acinetobacter baumannii* and *Candida albicans* by 69%, 43% and 59% respectively. Statistical analysis shows significant difference ($P < 0.05$) in biofilm inhibition between Pluronic F127 and Chitosan in *Klebsiella pneumonia* (XDR, MDR) and *Pseudomonas aeruginosa* (MDR). Other strains show no significant difference between Pluronic F127 and Chitosan. overall, all strains show no significant difference between Pluronic F127 and Mixture of both materials. (Fig. 2)

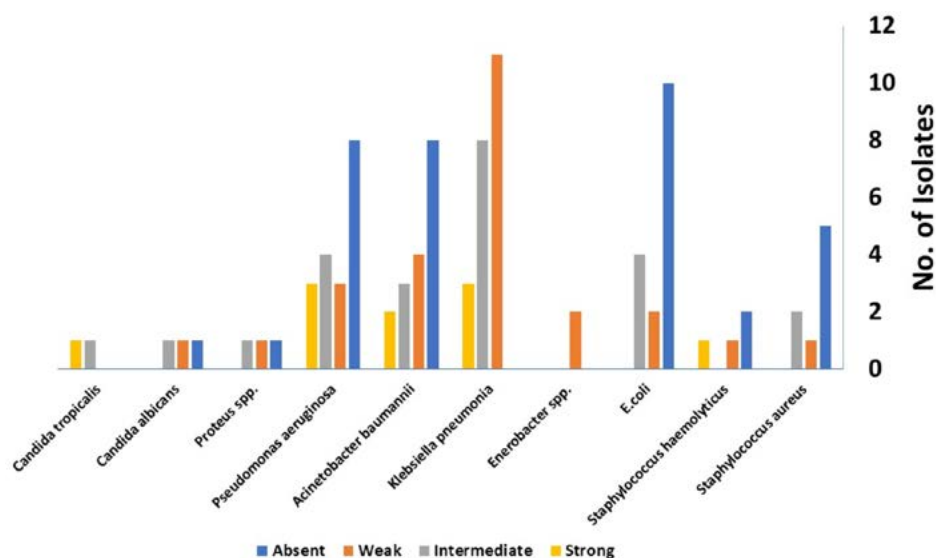


Fig. 1. Biofilm forming ability of clinical isolates recovered from different nosocomial clinical samples.

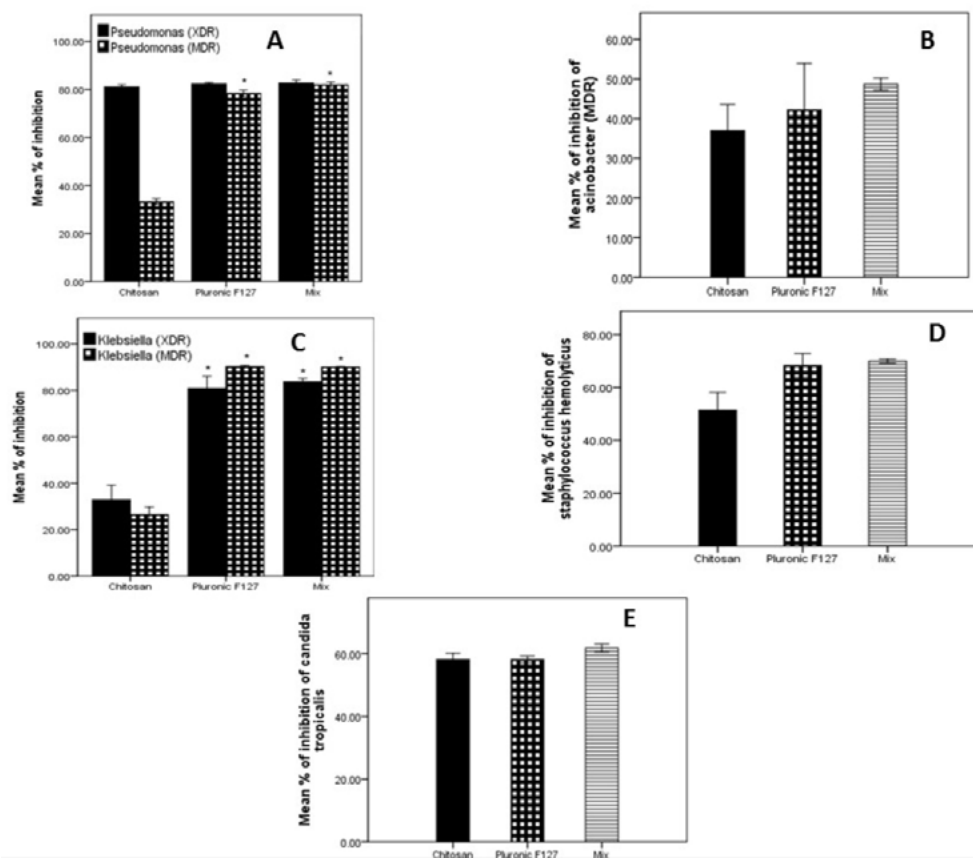


Fig. 2. Effect of Chitosan and Pluronic F127 on biofilm formation on (a) multi drug resistant *Pseudomonas aeruginosa* (MDR) and extensive drug resistant *Pseudomonas aeruginosa* (XDR) (b) *Staphylococcus haemolyticus* (c) multi drug resistant *Klebsiella pneumoniae* (MDR) and extensive drug resistant *Klebsiella pneumoniae* (XDR) (d) extensive drug resistant *Acinetobacter baumannii* (e) *Candida tropicalis*.

Table 1. Distribution of Nosocomial bacteria isolated from different clinical samples

Organism	Number	Isolation specimen			
		Urine	Sputum	endotracheal secretion	Blood
<i>Staphylococcus aureus</i>	8	1	3	3	1
<i>Staphylococcus haemolyticus</i>	4	1	2	1	-
<i>E.coli</i>	16	13	1	1	1
<i>Enerobacter spp.</i>	2	2	-	-	-
<i>Klebsiella pneumoniae</i>	22	2	10	7	3
<i>Acinetobacter baumannii</i>	17	-	6	9	2
<i>Pseudomonas aeruginosa</i>	18	4	6	7	1
<i>Proteus spp.</i>	3	2	1	-	-
<i>Candida albicans</i>	3	1	1	-	1
<i>Candida tropicalis</i>	2	1	-	-	1
Total No.	95	27	30	28	10

Table 2. Sensitivity testing of recovered clinical isolates: Number and Percentage of resistance of each pathogen to different antibiotics

Organism	Resistance pattern												
	SAM	AMC	CRO	CAZ	FEP	DO	C	VA	CIP	IPM	CN	CT	TZP
<i>Staphylococcus aureus</i>	2 (25%)	3 (37.5%)	1 (12.5%)	4 (50%)	0 (0%)	2 (25%)	1 (12.5%)	0 (0%)	1 (12.5%)	0 (0%)	2 (25%)	N.T	0 (0%)
<i>Staphylococcus haemolyticus</i>	2 (50%)	4 (100%)	3 (75%)	4 (100%)	2 (50%)	3 (75%)	3 (75%)	0 (0%)	2 (50%)	0 (0%)	2 (50%)	N.T	0 (0%)
<i>E.coli</i>	12 (75%)	14 (87.5%)	5 (31.25%)	4 (25%)	2 (12.5%)	7 (43.75%)	7 (43.75%)	N.T	4 (25%)	2 (12.50%)	5 (31.25%)	0 (0%)	0 (0%)
<i>Enerobacter spp.</i>	2 (100%)	2 (100%)	1 (50%)	1 (50%)	0 (0%)	1 (50%)	1 (50%)	N.T	0 (0%)	0 (0%)	1 (50%)	0 (0%)	0 (0%)
<i>Klebsiella pneumoniae</i>	19 (86%)	22 (100%)	12 (54%)	14 (63%)	10 (45%)	15 (68%)	13 (59%)	N.T	10 (45%)	10 (45%)	14 (63%)	0 (0%)	10 (45%)
<i>Acinetobacter baumannii</i>	17 (100%)	17 (100%)	13 (76%)	14 (82%)	11 (64%)	12 (70.5%)	14 (82%)	N.T	11 (64%)	9 (53%)	13 (76%)	0 (0%)	7 (41%)
<i>Pseudomonas aeruginosa</i>	16 (88.8%)	15 (83%)	13 (72%)	11 (61%)	9 (50%)	14 (77.7%)	15 (83%)	N.T	9 (50%)	8 (44%)	11 (61%)	0 (0%)	6 (33%)
<i>Proteus spp.</i>	3 (100%)	3 (100%)	3 (100%)	1 (33%)	1 (33%)	1 (33%)	2 (66%)	N.T	1 (33%)	0 (0%)	1 (33%)	N.T	0 (0%)

*N.T: not tested

SAM: Ampicillin/Sulbactam, AMC: Amoxicillin/Clavulanic, CRO: Ceftriaxone, CAZ: Cefazidime, FEP: Cefepime, DO: Doxycycline, C: Chloramphenicol, VA: Vancomycin, CIP: Ciprofloxacin, IPM: Imipenem, CN: Gentamicin, CT: Colistin, TZP: Piperacillin/Tazobactam

Table 3. Multi-drug resistance pattern of isolated nosocomial bacterial strains

Strain	Degree of resistance (No of antibiotic)				
	≥ 11	8-10	5-7	2-4	≤ 1
<i>Staphylococcus aureus</i> (n=8)	0 (0%)	1 (12.5%)	1 (12.5%)	2 (25%)	4 (50%)
<i>Staphylococcus haemolyticus</i> (n=4)	0 (0%)	2 (50%)	0 (0%)	2 (50%)	0 (0%)
<i>E. coli</i> (n=16)	0 (0%)	2 (12.5%)	3 (19%)	9 (56%)	2 (12.5%)
<i>Enterobacter spp.</i> (n=2)	0 (0%)	0 (0%)	1 (50%)	1 (50%)	0 (0%)
<i>Klebsiella pneumonia</i> (n=22)	10 (45%)	0 (0%)	3 (14%)	6 (27%)	3 (14%)
<i>Acinetobacter baumannii</i> (n=17)	7 (41%)	3 (18%)	1 (6%)	1 (6%)	5 (29%)
<i>Pseudomonas aeruginosa</i> (n=18)	6 (33%)	3 (17%)	4 (22%)	1 (6%)	4 (22%)
<i>Proteus spp.</i> (n=3)	0 (0%)	1 (33%)	0 (0%)	2 (66%)	0 (0%)

Electron Microscopic Image of Bacterial Strains

Biofilm inhibition by chitosan and/or pluronic acid were also confirmed by imaging with scanning electron microscope (SEM). Untreated strains show a high percentage of exopolysaccharide matrix with increased aggregation of cells in thick multicellular pattern. While, treated cells with chitosan and/or pluronic acid show a well isolated microcolonies with limited or no exopolysaccharide materials formed. (Fig. 3,4)

DISCUSSION

Most nosocomial infections are attributed to organisms with considerable degree of antibiotic resistance. This leads to increased demand on discovering new types of antibiotics with decreased resistance. Targeting bacterial biofilm is another strategy to reduce their transmission and increase efficacy of antibiotics.

The present study shows the high incidence of nosocomial infection among clinical samples, it is approximately 88%. This remarkable higher level may be attributed to

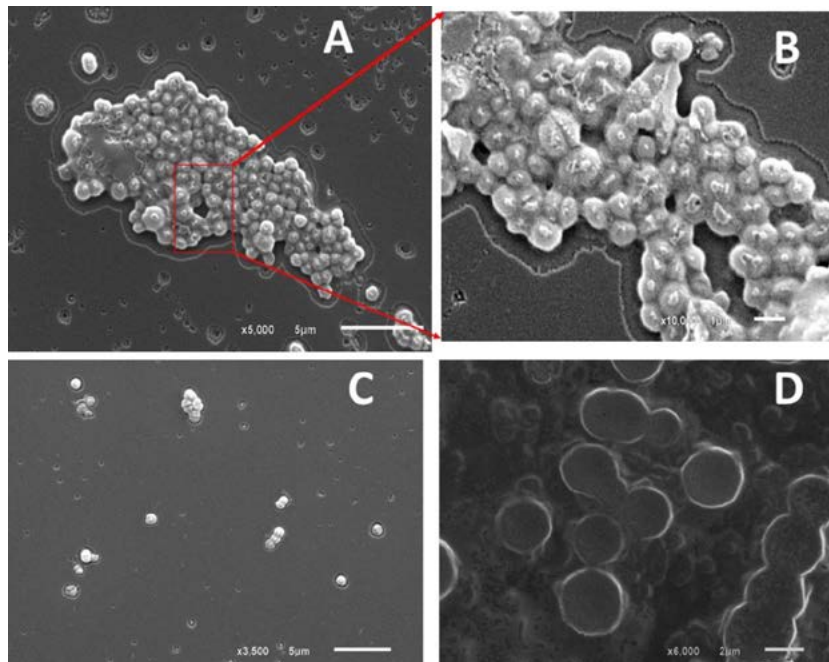


Fig. 3. Biofilm formation in *Staphylococcus haemolyticus* (a) Planktonic cell (b) enlargement of red box view of planktonic cell (c) in presence of Pluronic F127 (d) in presence of Chitosan.

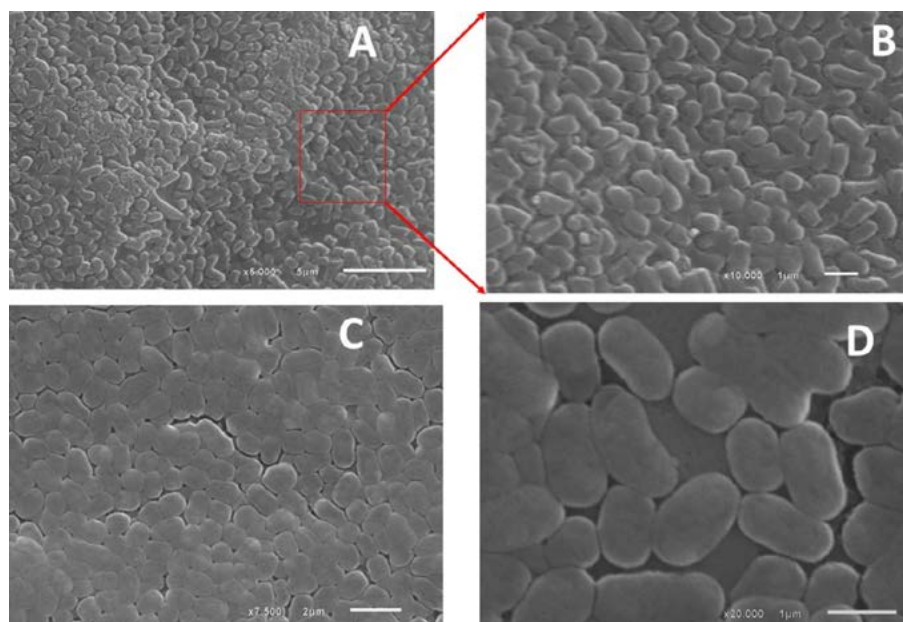


Fig. 4. Biofilm formation in *Klebsiella pneumoniae* (a) Planktonic cell (b) enlargement of red box view of planktonic cell (c) in presence of Pluronic F127 (d) in presence of Chitosan.

misuse of prescribed antibiotics and absence of disinfection policy in these hospitals. This finding is in accordance with Mama et al. who found a similar result (87%) at Jimma hospital²⁶. Also, Dessie and his colleagues reported a high incidence level (84%) in Addis Ababa hospital²⁷. Other researchers recorded a lower level 66%²⁸ at Gondor University hospital and 70% at Ethiopia²⁹.

Klebsiella pneumoniae, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *E. coli* were the predominant isolates with a low incidence of *Staphylococcus aureus* infection. This result is in agreement with Matta et al. who reported that *Pseudomonas aeruginosa* (12%), *Klebsiella pneumoniae* (6.2%) and *Acinetobacter baumannii* (3.1%) were the paramount isolates recovered from 258 patients in Lebanon³⁰. Another study by Peters et al. accounted for eighty-five percent of hospital-acquired infection were *Acinetobacter baumannii* (28%), *Klebsiella pneumoniae* (25%), *Pseudomonas aeruginosa* (21%), *Escherichia coli* (9%) and *Serratia marcescens* (3%)³¹. On contrary, Sserwadda and his colleagues reported that *Klebsiella pneumoniae* and *Staphylococcus aureus* were the most frequently isolated strains in Kawolo general hospital, Uganda³². Other researchers in a

Tertiary Hospital of northern Tanzania mentioned that *Staphylococcus aureus* was the most common isolated microorganism followed by *Enterococcus* and other coliform³³.

Vancomycin was the most sensitive antibiotic against all isolated *Staphylococcus* strains. Also, there is no detectable resistance to Colistin in isolated Gram-negative bacteria. The antibiotic with least resistance was found to be against Piperacillin/Tazobactam, Imipenem and Meropenem. Large percentage of *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were extensive drug resistant strains, they were resistant to twelve antibiotics from different classes. A similar finding was presented by Mauldin et al. who reported that fifty percent of hospital acquired infections were multi-drug resistant (MDR)³⁴.

In our study *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were potent biofilm producer while other types like *Staphylococcus* and *Candida spp* show weak to moderate biofilm forming ability. These results coincide with Mulla et al. who demonstrated that *Acinetobacter*, *Pseudomonas*, *Klebsiella*, *Staphylococcus spp.* were the main cause of infection in indwelling devices due to

biofilm formation³⁵. Also, Singhai et al. show that *Klebsiella pneumonia* represents the most abundant biofilm forming nosocomial microbe with multi-resistant pattern and extended β -lactamase producer³⁶. Other researchers show relationship between microbial resistance and biofilm formation^{37,38}.

For this work, Chitosan has a variable inhibitory effect on biofilm formation regarding different bacterial species. These findings are in agreement with Polyudova et al. who found that Chitosan has a fourfold inhibition of *Mycobacterium smegmatis* biofilm while has a minimal effect on *E.coli* strains, he refer his foundation to the increase in the hydrophobicity of attachment surfaces that will decrease the effect of chitosan as a biofilm inhibitor³⁹. Puga and his colleagues suggest that environmental stress leads to increased tolerance to Chitosan effect⁴⁰. Also, molecular weight and acetylation degree may control the effectiveness of Chitosan⁴¹.

Pluronic F127 shows potent antibiofilm activity at low concentration (1.25 mg/mL), its activity reaches up to 80% inhibition for strong biofilm producing strains. Treter et al. show similar effect of Pluronic F127 on *Staphylococcus epidermidis*, it inhibits 90% of biofilm formation⁴². Although, combination of Pluronic with Chitosan has a similar effect like Pluronic alone, but Pluronic can increase release, solubility and bioavailability of Chitosan. Alvarado-Gomez et al. reported synergistic activity of Pluronic F127 and silver nanoparticles in hydrogel form against biofilm forming *Pseudomonas spp* and *Staphylococcus spp*⁴³. Another study by Manaspon et al. reported increased cytotoxic activity of doxorubicin against breast cancer cells using folate-conjugated pluronic F127/chitosan core-shell nanoparticles¹⁸. From our work and previous studies, we conclude that Pluronic F 127 can increase effect of Chitosan suggesting their use in combination at low concentration level with high efficacy against biofilm formation.

CONCLUSION

Nosocomial infection represents a substantial health problem. A significant number of nosocomial isolates were moderate to strong biofilm producers. Despite of finding a new effective antibiotic for treatment of highly

resistant organisms, inhibition of transmission can represent a new effective approach. Chitosan and Pluronic F127 are safe biocompatible agents used in different medical formulation. They show a potential degree of biofilm inhibition. They could be used as an alternative source for inhibition of bacterial resistance and transmission.

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AUTHORS' CONTRIBUTION

AFA and WGH design the experiment; DE, AFA performed the experiments; WGH, AFA, OMS analyzed the data; AFA, OMS write the original draft. All authors read and approved the manuscript.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS STATEMENT

The study was approved by Ethics Committee, Faculty of Medicine, Beni-Suef University.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

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