

Full Length Research Paper

***In vitro* efficacies and durabilities of antibiotic/vitamin C coated hemodialysis catheters**

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Catheter associated bloodstream infections, exit-site infections, and tunnel infections are common complications related to hemodialysis central venous catheter use. Effective antimicrobial coating of catheters that can inhibit device colonization has the potential of preventing clinical infection. The study investigated *in vitro* the antimicrobial efficacies of hemodialysis catheters impregnated with an antibiofilm agent (ascorbic acid) (vitamin C) and a broad-spectrum antibiotics (Gentamicin and levofloxacin) against five clinical strains recently isolated from patients undergoing hemodialysis catheter removal. Impregnated catheters were also evaluated for their anti-adherence activities and durabilities. Levofloxacin/ascorbic acid (LEV/AS) catheters produced the most active and durable antimicrobial effect against both Gram-positive and Gram-negative isolates and significantly reduced adherence ($P<0.05$) by all tested pathogens compared to control catheters while, gentamicin/ascorbic acid (GENT/AS) catheters had lost activity against methicillin resistant *Staphylococcus aureus* and *Escherichia coli* but still produced zones of inhibition only for methicillin-resistant *Staphylococcus epidermidis* (MRSE), *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Effects of levofloxacin, gentamicin and vitamin C coated catheters on microbial adherence were found to be dose dependent. In conclusion, catheters impregnated with LEV/AS were shown to have broad spectrum, sustained antimicrobial durability and high efficacy. These *in vitro* results suggest that this antimicrobial combination can be used to protect against hemodialysis catheter colonization and catheter-associated infection.

Key words: Gentamicin-levofloxacin-ascorbic acid (vitamin C), biofilm, hemodialysis catheters, colonization.

INTRODUCTION

The prevalence of end-stage renal disease patients demanding renal replacement therapy has increased in the last decade and it is predictable that this increase will

last over the next 10 years (Bohlke et al., 2015).

Hemodialysis catheter is a commonly used device in renal replacement therapy. The main challenge to the

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use of these devices is the increased risk of a catheter-related bloodstream infection (CRBSI) (Sahli et al., 2016). Hemodialysis patients with a catheter have a 2 to 3 fold increased risk of hospitalization for infection and death compared with patients with an arteriovenous fistula or graft (Dhingra et al., 2001). CRBSI alone have a reported incidence of 1.1 to 5.5 episodes per 1000 catheter days and are associated with increased morbidity, hospitalization and death (Weijmer et al., 2005). The extent of the cost depends on the type and severity of infection. The total direct and indirect costs associated with hospitalizations due to hemodialysis catheter related infections range from 17,000 to 32,000 USD per episode (Kosa and Lok, 2013).

The process of catheter insertion disturbs the integrity of the skin facilitating an infection with bacteria or fungi. These infections are often preceded by colonization of the catheter with microorganisms. Scanning electron microscopy proved that most catheters become colonized with microorganisms during their dwell time inside blood vessels, whether catheter-related infection occurred or not (Raad et al., 1993). The presence of a biofilm in the catheter lumen is one of the factors that complicate the infection treatment. The biofilm is defined as a structured community of bacterial units covered by adherent material which makes this community 100 to 1,000 times less sensitive to antibiotics less than its free form (Fitzgibbons et al., 2011).

Impregnation or coating of catheters with antimicrobial agents has commonly been used to prevent bacterial colonization of hemodialysis catheters (Danese, 2002). However, some existing antimicrobial coated catheters designed to prevent catheter colonization may have partial clinical efficacy mostly against drug-resistant organisms and inadequate durability of antimicrobial activity (Fraenkel et al., 2006, Hockenull et al., 2008) relatively due to their inability to control biofilm forming microorganisms which can have minimum inhibitory concentrations (MIC) of up to 1,000 times higher than their MICs against their planktonic counterparts (Zhanel et al., 2006). This *in vitro* study investigated the comparative activities and antimicrobial durability of gentamicin/ascorbic acid and levofloxacin/ascorbic acid coated catheters against the tested microorganisms and the effect of catheters impregnated with gentamicin, levofloxacin, and ascorbic acid each alone and combinations of the previous antibiotics and ascorbic acid on microbial adherence.

MATERIALS AND METHODS

Bacterial strains

Five clinical strains recently isolated from patients undergoing hemodialysis catheter removal at the Department of Urology, Minia University Hospital, Egypt were used for the *in vitro* studies. Organisms used were methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE),

Klebsella sp., *Pseudomonas aeruginosa* and *E. coli*.

Chemicals

Gentamicin sulphate (Winlab), levofloxacin hydrochloride and ascorbic acid (El-NasrPharmaceutical Chemical Co. Abu-Zaabal) were used.

Hemodialysis catheters

Commercially available dual lumen short term polyurethane hemodialysis catheters (Ameco medical industries, Egypt) were cut into 1 cm segments.

Preparation of drug coated catheter segments

One centimeters catheters segments were impregnated with gentamicin (20, 40 and 100 mg/ml), levofloxacin (20, 40 and 100 mg/ml), ascorbic acid (20, 40 and 100 mg/ml) each alone and in combinations by immersion in an agitated solutions of the previous concentrations followed by drying overnight and rinsing to remove any unbound compound (Hanna et al., 2006). Commercially available uncoated polyurethane catheters were also tested as controls.

In vitro biofilm adherence

One centimeter-long segments of uncoated control catheters and the aforementioned coated catheters were tested in triplicate for inhibition of biofilm formation by a biofilm forming strains which were isolated from infected hemodialysis catheters. The catheters were placed into sterile 24-well tissue culture plates containing 1 ml of human donor plasma to enhance the formation and binding of blood proteins and were incubated for 24 h at 37°C. The plasma was then replaced with 5.0×10^5 cells of various organisms in Mueller-Hinton broth and the plates were incubated for an additional 24 h. After incubation, the microbial inoculum was discarded and segments were washed with shaking for 30 min in 1 ml of 0.9% sterile saline. The segments were then removed with sterile sticks, placed in 5 ml of 0.9% saline and sonicated for 15 min at 40 kHz. After sonication, each sample was vortexed for 5 s and 100 μ l of liquid from each segment was serially diluted and spread onto Trypticase soy agar plus 5% sheep blood for quantitative culture of bacterial species. The plates were then incubated at 37°C, inverted for 24 h and counted for colony growth. The lower limit of detection was 5 colonies. The experiments were repeated twice (Raad et al., 2012).

Determination of zones of inhibition (ZI) and antimicrobial durability

One centimeter-long segments of antibiotic/ascorbic acid-impregnated and control (without drugs) catheters were individually placed in human serum at 37°C for 7, 14, 21 and 30 days, with a weekly change of serum. Catheter segments removed from sera and baseline catheter segments (no serum incubation) were assessed for ZI, using a modified Kirby-Bauer diffusion assay (Mansouri et al., 2009) against biofilm forming strains. Duplicates of catheter segments were vertically embedded in Mueller- Hinton agar plates coated with biofilm forming bacterial strains. The plates were incubated overnight at 37°C and the zones of inhibition produced around catheter segments were measured and recorded as the diameters, in millimeters, across the centers of the

embedded catheter segments.

Scanning electron microscope (SEM)

Hemodialysis catheter segments (controls and that with drugs) were fixed in 2.5% (v/v) glutaraldehyde in Dulbecco PBS (Phosphate Buffered Saline) (pH 7.2) for 1.5 h, rinsed with PBS and then dehydrated through an ethanol series. Samples were critical point dried and gold palladium coated. SEM examinations were made on JSM-840 SEM (Camargo et al., 2005).

Statistical analysis

One-way ANOVA was employed to detect any significant difference between the values achieved without the drug (controls) and the values achieved in the presence of different drug concentrations. Differences were done using graphpad prism 5 software. *P* values of <0.05 indicated statistical significance.

RESULTS

In vitro biofilm adherence

The drug coated catheter segments either by an antibiotic or ascorbic acid each alone or in combinations demonstrated an anti-adherence ability against all tested biofilm forming strains; the anti-adherent ability was concentration dependent as the inhibitory effect of gentamicin at a concentration of 100 mg/ml was in the range of 78.2-98% of controls while, at concentrations of 20 and 40 mg/ml the inhibitory effects were in the range of 50-84.2 and 76.6-92.9% of the controls, respectively.

Similarly, levofloxacin at concentration 100 mg showed also an inhibitory effect ranging from 95.8-99.8% on all tested bacterial strains, that of 40 mg was 93.6-99% of controls and that of 20 mg was 88.7-98.2% of controls. Data showed that ascorbic acid at a concentration of 100 mg/ml had a significant inhibitory effect on bacterial adherence (75-97.1% of the controls) while, the effects of 20 and 40 mg/ml were in the range of 30.8-84.1 and 67.8-90% inhibitions of controls of the controls, respectively (Table 1).

The highest inhibitory effects (99.9-100% of controls) were recorded when a combination of levofloxacin/ascorbic acid at 100/100 mg/ml were used followed by 40/40 mg/ml (99-99.9% of controls) and 20/20 mg/ml (94.3-99 % of controls).

Again, similar pattern of inhibitory effect on bacterial adherence to catheter surfaces when gentamicin/ascorbic acid combinations were used. GENT/AS at 100/100 mg/ml showed the highest effect on bacterial adherence (98-99.9%) while, GENT/AS at concentrations of 20/20 and 40/40 mg/ml reduced the bacterial adherence by 60.6-91.2 and 93.3-97.7% in comparison to controls, respectively (Table 1).

These data revealed that ascorbic acid increased the therapeutic effect of gentamicin and levofloxacin resulting

in a significant inhibition in the synthesis of biofilm to catheter surfaces. Also, this study showed that levofloxacin at low concentrations either alone or in combination with ascorbic acid had a higher reductive effect than gentamicin.

Scanning electron microscope (SEM)

SEM colonization images were compatible with the adherence assay results; SEM images revealed that considerably fewer bacterial cells of MRSA and *E. coli* were attached to GENT/AS coated catheters than control catheter segments while no bacterial cells were observed on LEV/AS coated catheters. In addition, no biofilm mass was observed on both types of coated catheter after incubation with the tested organisms (Figure 1A to F).

Determination of zones of inhibition and antimicrobial durability

Figures 2 show the results of baseline tests for GENT/AS and LEV/AS coated segments and their efficacies against the tested microorganisms after being soaked in serum for 4 weeks. It was revealed that LEV/AS coated catheter segments showed the most effective, widest spectrum and longest durability of antimicrobial activity against all tested pathogens. Zones of inhibition produced by LEV/AS and GENT/AS coated segments measured 26-42 and 10-33 mm at baseline, respectively. After being immersed in serum (changed at weekly intervals), LEV/AS coated catheters still produced zones of inhibition (15-27 mm) for 4 weeks for all tested organisms while GENT/AS coated segments had lost their activity against MRSA and *E. coli* but still produced zones of inhibition (11-18 mm) only for MRSE, *K. pneumoniae* and *P. aeruginosa*. Representative images of these zones are shown in Figures 3A to F.

DISCUSSION

Despite many infection control procedures, the prevention of infections associated with medical devices mainly hemodialysis catheters remains challenging. The excessive morbidity, mortality and increasing cost associated with catheter related bloodstream infections are incredible (Raad et al., 2007). Patients often spend 10 to 40 additional days in hospitals as a result of acquiring these infections (Safdar et al., 2005).

Recently, promising strategies have been developed to improve biofilm eradication (matrix degradation or destabilization or the development of anti-persister compounds, targeting the most tolerant bacterial cells inside the biofilm) that may help to develop novel methodologies to prevent or treat these frequent

Table 1. The effect of catheters impregnation with different concentrations of (gentamicin, ascorbic acid) and (levofloxacin, ascorbic acid) each alone and in combination on the number of adhering bacterial cells.

Bacteria	Ascorbic acid					Gentamicin				Levofloxacin				Gentamicin/ascorbic acid				Levofloxacin/ascorbic acid			
	Conc. (mg/ml)	Mean no. of CFU/ml X10 ⁴ ± SEM	% Reduction	P Value	Conc. (mg/ml)	Mean no. of CFU/ml X10 ⁴ ± SEM	% Reduction	P Value	Conc. (mg/ml)	Mean no. of CFU/ml X10 ⁴ ± SEM	% Reduction	P Value	Conc. (mg/ml)	Mean no. of CFU/ml X10 ⁴ ± SEM	% Reduction	P Value	Conc. (mg/ml)	Mean no. of CFU/ml X10 ⁴ ± SEM	% Reduction	P Value	
MRSA	CTR											9.4± 3.3									
	20	6.5±2.3	30.8	<0.05	20	4.7± 2.08	50	<0.05	20	0.56± 0.88	94	<0.05	20/20	3.7± 1.5	60.6	<0.05	20/20	0.41± 0.11	95.6	<0.05	
	40	2.5± 0.17	73.4	<0.05	40	2.2± 1.15	76.6	<0.05	40	0.28± 0.11	97	<0.05	40/40	0.52± 0.5	94.5	<0.05	40/40	0.094± 0.01	99	<0.05	
	100	0.99± 0.1	89.5	<0.05	100	1.2± 0.57	78.2	<0.05	100	0.019± 0.03	99.8	<0.05	100/100	0.13± 0.023	98.6	<0.05	100/100	0± 0	100	<0.05	
MRSE	CTR											26.5± 2.5									
	20	5± 0.8	81.1	<0.05	20	10.2± 1.5	61.5	<0.05	20	3± 0.033	88.7	<0.05	20/20	2.4± 0.8	90.9	<0.05	20/20	1.5± 0.06	94.3	<0.05	
	40	3.1± 0.5	88.3	<0.05	40	4.05± 1.7	84.7	<0.05	40	1.7± 0.066	93.6	<0.05	40/40	1.06± 0.6	96	<0.05	40/40	0.06± 0.02	99.8	<0.05	
	100	1.2± 0.16	95.5	<0.05	100	1.3± 1.5	95.1	<0.05	100	1.1± 0.011	95.8	<0.05	100/100	0.29± 0.14	98.9	<0.05	100/100	0.03± 0	99.9	<0.05	
<i>E. coli</i>	CTR											180± 1.4									
	20	90± 0.86	50	<0.05	20	75± 1.2	68.3	<0.05	20	4.5± 1.2	97.5	<0.05	20/20	36± 0.6	80	<0.05	20/20	3.6± 0.8	98	<0.05	
	40	58± 0.6	67.8	<0.05	40	42± 0.3	76.7	<0.05	40	2.5± 0.3	98.6	<0.05	40/40	12± 0.15	93.3	<0.05	40/40	1.8± 0.64	99	<0.05	
	100	45± 0.4	75	<0.05	100	32± 0.17	82.2	<0.05	100	0.54± 0.16	99.7	<0.05	100/100	3.6± 0.01	98	<0.05	100/100	0.18± 0.05	99.9	<0.05	
<i>k. pneumoniae</i>	CTR											47.5± 1									
	20	11.5± 0.8	75.8	<0.05	20	7.5± 0.8	84.2	<0.05	20	0.87± 0.98	98.2	<0.05	20/20	6.2± 0.1	86.9	<0.05	20/20	0.6± 0.4	98.7	<0.05	
	40	9.15± 0.3	80.7	<0.05	40	3.35± 0.6	92.9	<0.05	40	0.45± 0.75	99	<0.05	40/40	2.6± 0.08	94.5	<0.05	40/40	0.04± 0.2	99.9	<0.05	
	100	2.7± 0.12	94.3	<0.05	100	2.15± 0.1	95.5	<0.05	100	0.09± 0.4	99.8	<0.05	100/100	0.47± 0	99	<0.05	100/100	0± 0	100	<0.05	
<i>P. aeruginosa</i>	CTR											59.3± 2.8									
	20	9.4± 1.7	84.1	<0.05	20	59.3± 2.8	83.6	<0.05	20	1.5± 1.1	97.5	<0.05	20/20	5.2± 0.23	91.2	<0.05	20/20	0.6± 0.03	99	<0.05	
	40	5.9± 1	90	<0.05	40	9.7± 1.8	89.2	<0.05	40	0.99± 0.52	98.3	<0.05	40/40	1.35± 0.1	97.7	<0.05	40/40	0.1± 0.003	99.8	<0.05	
	100	1.7± 0.5	97.1	<0.05	100	6.4± 1.6	98	<0.05	100	0.09± 0.44	99.8	<0.05	100/100	0.04± 0	99.9	<0.05	100/100	0.005± 0	99.99	<0.05	

*P-value <0.05: Significant difference. CTR, Control without drug.

infections (Gominet et al., 2017).

Novel antimicrobial catheters have been shown to be effective in preventing catheter-related bloodstream infections (Wenzel et al., 1999). New Centers for Disease Control and Prevention (CDC) guidelines have recommended the use of antimicrobial coated catheters (chlorhexidine/silver sulfadiazine or minocycline/rifampin) in

adults whose catheters are expected to remain indwelling for more than 5 days if the employment of other antiseptic procedures did not control the rate of these infections (O'Grady et al., 2011).

In this study, it was found that coated catheter segments either by gentamicin, levofloxacin or ascorbic acid were effective in preventing bacterial adherence in comparison to uncoated

catheters ($P < 0.05$). Catheter segments coated with combinations of antibiotic (gentamicin or levofloxacin) and ascorbic acid showed higher activity than that coated with antibiotic alone. Ascorbic acid was found to increase the activity of gentamicin and levofloxacin considering that antibiofilm/antimicrobial agent combination would be synergistic (Olofsson et al., 2003). Ascorbic

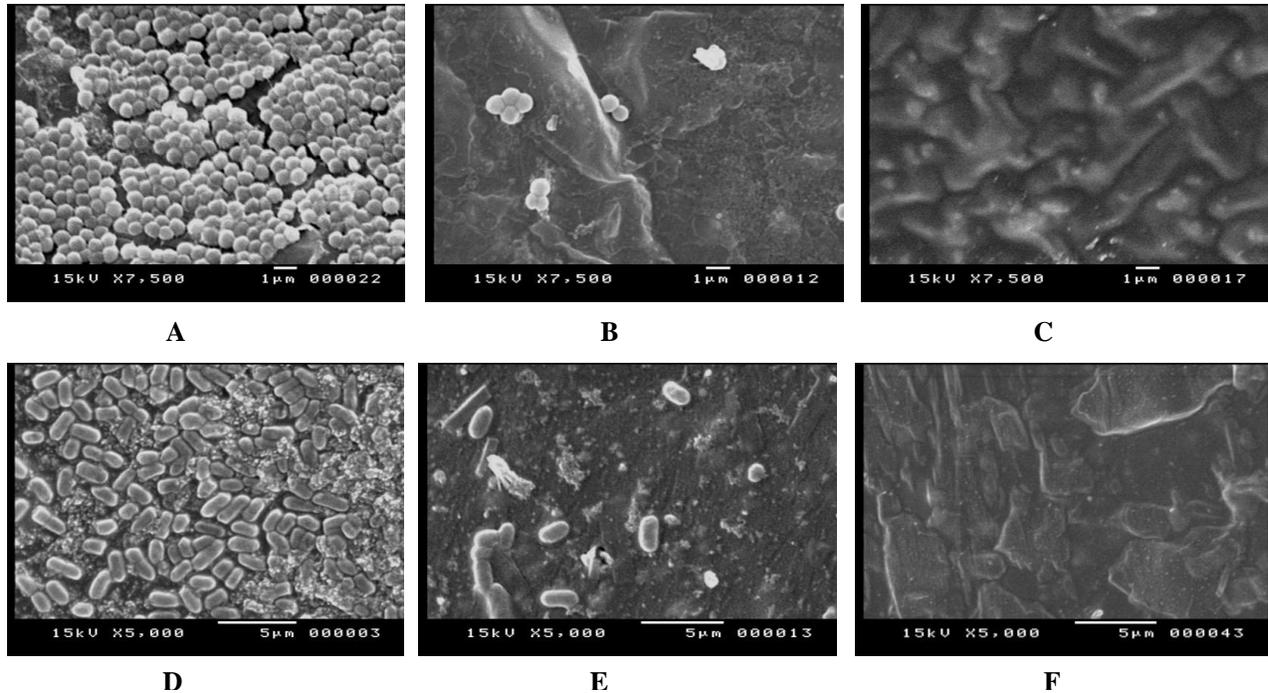


Figure 1. Scanning electron micrographs showing growth and adherence MRSA and *E. coli* on the surface of equally sized hemodialysis catheters segments incubated for 24 h. A, Initial growth and adherence of MRSA without any treatment (control). B, Growth and adherence of MRSA treated with combination of GENT/AS at 100/100 mg/ml. C, Growth and adherence of MRSA treated with combination of LEV/AS at 100/100 mg/ml. D, Initial growth and adherence of *E. coli* without any treatment (control). E, Growth and adherence of *E. coli* treated with combination of GENT/AS at 100/100 mg/ml. F, Growth and adherence of *E. coli* treated with combination of LEV /AS at 100/100 mg/ml.

acid (vitamin C) is a naturally occurring furanone (Colin, 1999). Ascorbic acid and sodium ascorbate inhibited quorum sensing in *Clostridium perfringens* (Novak and Fratamico, 2004). In addition, ascorbic acid was considered as efflux pump inhibitor in *E. coli* (Serry, 2008). The antibiofilm mechanisms of ascorbic acid may be due to quorum sensing inhibition and efflux pump inhibition which was noticed by Hisham et al. (2012) in *P. aeruginosa*. Previously, El-Gebaly et al. (2012) reported that levofloxacin and ascorbic acid combinations had better inhibitory effect on biofilm formation on the surface of urethral catheters than that recorded for individually used drugs. Similar synergistic effect on inhibition of biofilm formation was achieved when gentamicin was combined with salicylate (antibiofilm agent) (El-Banna et al., 2012).

In this study, LEV/AS coated catheters had the greatest antibiofilm activity and produced the largest inhibition zones against MRSA, a key pathogen associated with hemodialysis catheter infections and *P. aeruginosa* the most resistant and virulent form of Gram-negative bacteria, which contribute to certain cases of catheter related blood stream infection compared to GEN/AS coated segments that lost their activity after immersion in serum for 4 weeks so LEV/AS was the most durable. Since pathogenic colonization of a catheter can occur

days after its insertion, it would be ideal for antimicrobial coated catheters to have a sustained activity for as long as it is safe and necessary. This property was achieved in the LEV/AS coated catheter segments. A similar pattern was reported by Mansouri et al. (2013); where N. acetyl cysteine/levofloxacin coated catheters produced the most active and durable antimicrobial effect against both Gram-positive and Gram-negative isolates and significantly reduced colonization by all tested pathogens compared to control catheters. However, ascorbic acid is more advantageous than N-acetylcysteine due to both terms; safety and cost.

Another in vitro study was made by Hanna et al. (2006) who investigated the activity and durability of novel antimicrobial catheters in preventing the colonization of microbial organisms to the surfaces of the Catheters. Novel antimicrobial catheters were impregnated with antibiotics (minocycline and rifampin), Oligon agent (silver, platinum, and carbon black), antiseptics (chlorhexidine and silver sulfadiazine) or a novel antiseptic agent (gendine) which contains gentian violet and chlorhexidine. Gendine-coated catheter segments protected against the bacterial adherence of MRSA and *P. aeruginosa*, significantly more than all other types of antimicrobial coated catheter segments and also demonstrated the most prolonged antimicrobial activity in

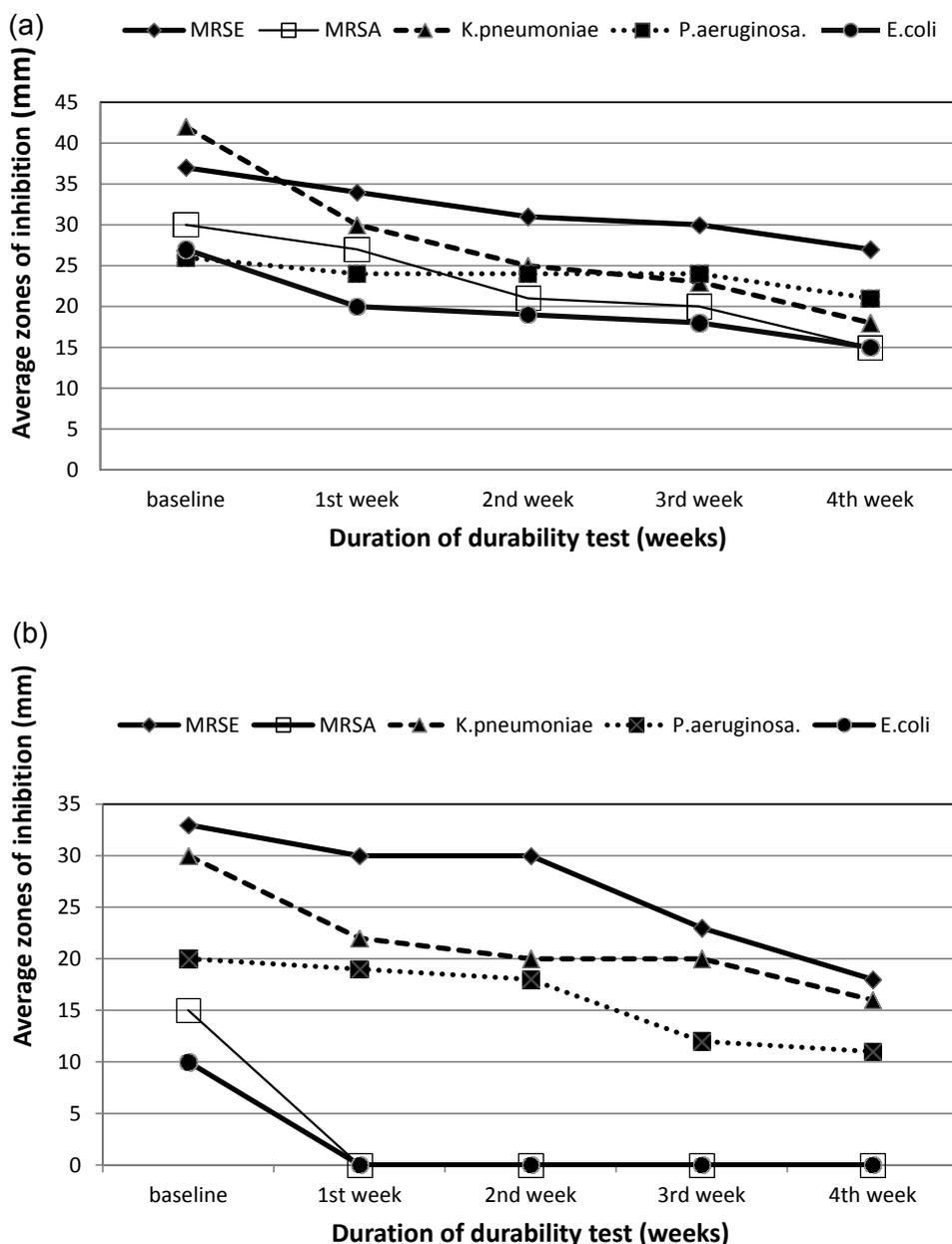


Figure 2. Antibacterial activity of LEV/AS (a) and GENT/AS (b) impregnated central venous catheters against common clinical pathogens based on zone of inhibition.

serum. These findings were confirmed in a recent study that reported that gentine coated catheters completely inhibited the adherence of all pathogens from 24 h to 1 week, while minocycline/rifampicin, chlorhexidine coated and control catheters failed to inhibit the adherence (Jamal et al., 2015).

On the other hand, GENT/AS coated segments had lost activity against MRSA and *E. coli* but still produced zones of inhibition (11-18 mm) only for MRSE, *K. pneumoniae* and *P. aeruginosa*. Their limited antimicrobial durability is similar to that obtained by Logghe et al.

(1997) who reported the lack of efficacy of chlorhexidine-silver sulfadiazine antiseptic catheters in preventing catheter related blood stream infections in leukemia and lymphoma patients who had central venous catheters with an average stay time of 20 days.

In conclusion, these *in vitro* results suggest that LEV/AS catheters may protect against hemodialysis catheter colonization by gram-positive and gram-negative bacteria and their prolonged antimicrobial durability against these organisms recommend that this antimicrobial catheters may prove to be successful *in vivo*.

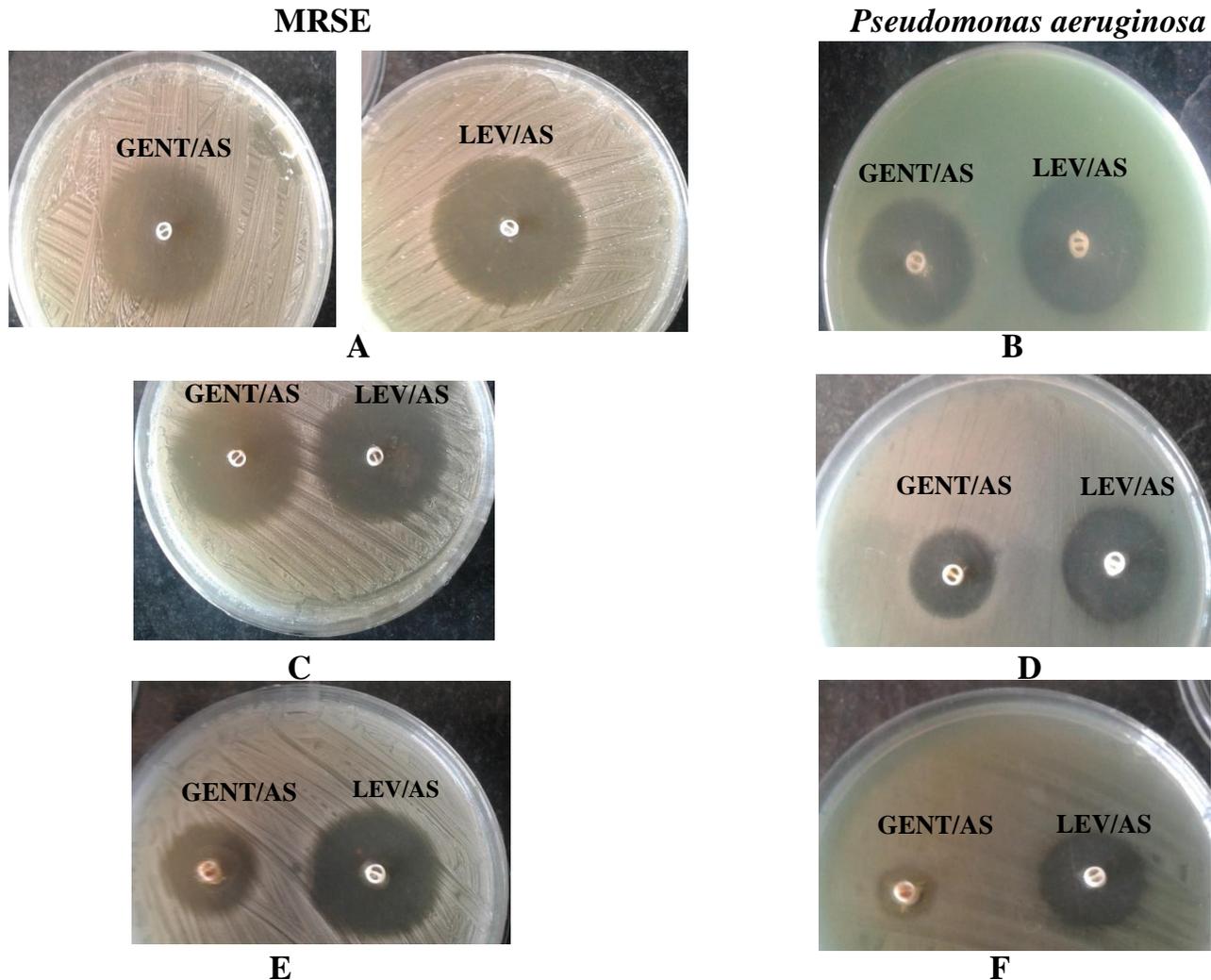


Figure 3. Antimicrobial activity and durability of LEV/AS and GENT/AS coated catheters against MRSE and *P. aeruginosa*. **(A)** LEV/AS and GENT/AS coated catheters showed 37.33 mm growth inhibition zones of MRSE, respectively (baseline). **(B)** LEV/AS and GENT/AS showed 26.20 mm growth inhibition zones of *P. aeruginosa*, respectively (baseline). **(C)** LEV/AS and GENT/AS coated catheter (immersed in serum for 2 weeks) showed 31.30 mm growth inhibition zone of MRSE, respectively. **(D)** LEV/AS and GENT/AS coated catheter (immersed in serum for 2 weeks) showed 24.18 mm growth inhibition zone of *P. aeruginosa* respectively. **(E)** LEV/AS and GENT/AS coated catheter (immersed in serum for 4 weeks) showed 27.18 mm growth inhibition zone of MRSE, respectively. **(F)** LEV/AS and GENT/AS coated catheter (immersed in serum for 4 weeks) showed 21.11 mm growth inhibition zone of *P. Aeruginosa*, respectively.

Zone of inhibition testing is a basic estimation of antimicrobial activity against the free-floating form of microorganisms, while adherence testing estimated the antimicrobial activities against organisms in biofilm. So, LEV/AS-coated catheter segments showed efficacy when tested by both methods and this represents a promising methodology for prevention of hemodialysis catheter associated infection and colonization.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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