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Fabrication of Poly Tetra Fluoro Ethylene to Prevent Coronary Vascular Stent-associated Infections

¹Sweda Sreekumar, ¹B. Elayarajah, ²R. Rajendran, ¹B. Venkatrajah, ¹Soumya Sreekumar and ¹Selin Jacob

¹Department of Microbiology and Bioinformatics, CMS College of Science and Commerce, Coimbatore, India

²Post Graduate and Research Department of Microbiology, PSG College of Arts and Science, Coimbatore, India

Corresponding Author: B. Elayarajah, Department of Microbiology and Bioinformatics, CMS College of Science and Commerce, Coimbatore, India

ABSTRACT

Coronary artery disease usually requires angioplasty-involving stents that help in keeping the artery open for proper blood flow. Such stents may present surfaces for colonization of biofilm forming bacteria thereby causing stent-associated infections. The purpose of this study was to provide anti-infective Poly Tetra Fluoro Ethylene (PTFE) stents for the prevention of biofilm formation. Anti-infective mixture of synergistic drugs with biodegradable carrier, DL-lactic Acid (DLA) was used to coat PTFE stents by means of dip-coating procedure. FTIR analysis of PTFE stent was used to determine the presence of bound anti-infective drugs. Antibacterial activity of anti-infective PTFE stents was determined qualitatively and quantitatively. *In vitro* challenge test was monitored to determine the persistence of drugs on anti-infective PTFE stent surface. The Fourier Transform Infrared (FTIR) analysis showed two major peaks at 3101.64 and 1141.94 nm for the O-H stretching and C=O stretching of COOH. Antibacterial activity was analysed by qualitative (Agar diffusion test) and quantitative (Bacterial adherence test) methods. The former showed largest inhibition zone for *Pseudomonas aeruginosa* (29 mm) and the latter confirmed that the number of adhered bacteria in drug-carrier coated stents ($p < 0.05$) were less than the number of adhered bacteria in carrier-coated stents ($p > 0.05$). *In vitro* challenge test clearly demonstrated that bacterial growth failed to develop even after three consecutive challenge doses. These drug-eluting stents could be of great interest for coronary stenting to prevent stent-associated infections if these results are confirmed *in vivo*.

Key words: Ofloxacin and ornidazole, antimicrobial coating, biodegradable, drug carrier, biofilm, bacterial adherence

INTRODUCTION

Among the various grave diseases that threaten human wellbeing, coronary artery disease is considered as the main cause of mortality (Amien and Lin, 2007) despite several advancements in the medical interventions (Upaganlawar *et al.*, 2011). In less than 20 years from now, this fatal disease resulting from the blockage of coronary artery is expected to hold first place in the list of leading causes of disability prepared by the World Health Organization (Sajid *et al.*, 2009). The majority of angioplasty procedures are done using highly effective and safe method involving

implants like coronary vascular stents, cardiac valves, vascular patches and catheters made of Poly Tetra Fluoro Ethylene (PTFE). The PTFE is chemically inert, exhibit little tendency to dilate, has strong electronegative luminal charge and are hydrophobic until wetted by body fluids (Cannon, 1983). The specific application of the PTFE stents would be primarily in human blood vessels to combat Coronary Artery Disease (CAD) (Israle, 2004). Several cases have been reported in which such stents were easily accessible to pathogens, like *Staphylococcus aureus* and *Staphylococcus epidermidis* (Khardori and Yassien, 1995; Barton, 1996), *Escherichia coli*, *Enterococcus fecalis* and *Streptococcus viridians* (Khossravi *et al.*, 2009). Bouchart *et al.* (1997) reported a case of a coronary stent bacterial infection due to *Pseudomonas aeruginosa*, shortly after implantation of the stent in the left circumflex artery which presented as an acute pericarditis. If infection by such bacteria is not prevented, it leads to complications like additional surgery, antibiotic therapy and sometimes even renewed disability (Widmer, 2001). These pathogens adhere to the patient's proteins that are present on the graft producing a biofilm on the PTFE implant (Schmitt *et al.*, 1986), hence presenting challenging complications (Parsek and Singh, 2003). Infectious microorganisms use biofilm formation as a means to resist antimicrobial agents and cause chronic infections (Khan *et al.*, 2011). For combating drug resistance, focus must be on proper management of antibiotic treatment (Murugan *et al.*, 2011). Matl *et al.* (2008) coated several drugs and chemical compounds on coronary stents for proving that they are biocompatible and effective in preventing bacterial adhesion. He demonstrated the development of a drug delivery system consisting of lipid-based polymers with incorporated gentamicin or teicoplanin in order to release high drug concentrations locally, in the area of implant infection. Gollwitzer *et al.* (2003) reported that a combination of Poly DL-lactic Acid (PDLA) with either gentamicin or teicoplanin or both antibiotics when coated on the implant reduced bacterial growth considerably. A combination of rifampicin and clindamycin used for coating hydrocephalus shunt catheter has been reported to prevent implant associated infections to a large extend (Bayston *et al.*, 1989). The problem with straight forward antimicrobial loading into a device by coating or immersion is the generation of resistance. Antimicrobial resistance has a significant impact on patient outcome by enhancing virulence, delaying the administration of appropriate therapy and subsequent recovery (Cosgrove and Carmeli, 2003). Even though different types of antimicrobial agents used for coating the devices were proved to be effective in antibacterial activity (Borschel *et al.*, 2006) still due to the generation of resistance by microorganism they are not considered as biocompatible. The accepted clinical practice to treat biofilm-associated infections was the use of combination therapy in which two or more antimicrobials are blended at different combinations. So that broader spectrum of activity is achieved at a lower concentration resulting in more effective therapy and decreased resistance (Saginur *et al.*, 2006). Similarly, Boeckh *et al.* (1990) suggested expansion of the antibacterial spectrum by combining quinolones with other antibacterial agents for preventing biofilm formation. Hence, in the present study two different groups of synergistic antimicrobial drugs fluoroquinolone (Ofloxacin) and nitroimidazole (Ornidazole) with appropriate carrier (DL-lactic acid) were added at the surface level of PTFE stents. Synergistic combination of Ofloxacin and Ornidazole has been demonstrated as a good choice in preventing infections caused by aerobic as well as anaerobic bacteria (Elayarajah *et al.*, 2011). The carrier was used to bind the antibacterial agents and also to enhance the antibacterial activity (Gollwitzer *et al.*, 2003). Both fluoroquinolone and nitroimidazole drugs proves their synergism by acting on the DNA of bacteria targeting the inhibition of DNA synthesis and replication.

Taking into consideration of the above facts, the present research work was designed with the specific objective of developing a process for rendering antimicrobial coating to the PTFE stent. Also, the study aims to determine the effect of the cross-linking of antimicrobial agent to the product substrate on the antimicrobial efficacy and persistence properties.

The specific objectives are as follows:

- To investigate the biofilm forming ability of test bacteria on PTFE stent
- To select and optimize the coating of drugs on PTFE stent
- To evaluate the antibacterial activity of synergistic anti-infective drug-coated PTFE stent
- To characterize the drug coated surfaces of PTFE stent using a chemical method

MATERIALS AND METHODS

In the present study, dip-coating method, antibacterial activity and *in vitro* challenge test was carried out in Microbiology laboratory, CMS College of Science and Commerce, Coimbatore, India, from March, 2010 to May 2011. FTIR test and tissue reaction test was carried out in Microbiology laboratory, PSG College of Arts and Science, Coimbatore, India, in April, 2011.

Bacterial strains used for the study: Biofilm-forming strains of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* obtained from a clinical laboratory was used for the *in vitro* studies. All the strains were cultured to late logarithmic growth phase on blood agar plates at 37°C for 24 h before testing. The cells were then resuspended in normal saline and adjusted to 1.6×10^5 CFU mL⁻¹ by visual comparison with a 0.5 McFarland standard.

Exit-site challenge test: The biofilm forming capacity of the bacterial strains used for the study is analysed by observing the migration of bacteria from the exit site down the PTFE stent track i.e., the outside of the stent (Bayston *et al.*, 2009).

Drug carrier: Food and medical grade DL-lactic acid (Hi media) was used as the cross-linker between the PTFE material and antibacterial drugs. It was considered to be an effective and cheaper drug-carrier due to their surface binding properties, biodegradable in nature and sustained release of drugs.

Preparation of anti-infective agents: Ofloxacin (Merck, India) and Ornidazole (Merck, India) suspension with drug carrier was prepared according to the method described by Matl *et al.* (2008) by suspending in 99% ethanol (Sigma chemicals). The resulting suspension was added to the drug carrier DL-Lactic acid, for building up a drug concentration of 10%.

Drug coating: Before coating with anti-infective agents, the commercial stent was cut into pieces ($n = 3$, length-1 cm, dia-1 mm). Sterile stent pieces were coated with the suspension of anti-infective agents by dip-coating method (Matl *et al.*, 2008). The coated stents were referred below as drug-carrier-coated stent and uncoated stents as carrier-coated stent.

Analysing the drug coated PTFE stent

Determining the weight of the PTFE stents: The weight of PTFE stent was measured before and after coating with drug in order to determine the quantity of drug bound to the stent after the dip-coating procedure (Matl *et al.*, 2008).

FTIR analysis of drug coated PTFE stents: The FTIR absorption spectra of the uncoated, drug coated and drug-carrier coated PTFE stents were recorded in the range of 400-4000 cm^{-1} by KBr disc method using FTIR spectrophotometer. Briefly, the samples were cut transversely, made into discs (dia-1 cm) and mixed with KBr (200 mg) to avoid moisture. Each disc was dried under radiation to remove excess moisture content. The peaks for C=O stretching and O-H stretching of COOH and the drugs were analysed (Patel *et al.*, 2009).

Tissue response of chick chorio-allantoic membranes to PTFE material: To understand the allergic reactions of drug-carrier coated PTFE material on the live tissues, the materials were placed on the surface of Chorio-Allantoic Membrane (CAM) of embryonated chick eggs. The experiment was carried out based on the method described by Valdes *et al.* (2002).

Antibacterial activity

Qualitative test-agar diffusion test: Qualitative antibacterial activity was tested by agar diffusion (Bayston *et al.*, 1989). Three samples were tested from each preparation (drug-carrier coated stents, carrier coated stents and uncoated stents). Antibacterial activity was expressed as the zone of inhibition around the test sample.

Quantitative test-bacterial adherence studies: Quantitative bacterial adherence studies were carried out by the method described by Gollwitzer *et al.* (2003).

In vitro challenge method: The *in vitro* challenge test was described based on the method proposed by Bayston and Barsham (1988).

RESULTS

Exit-site challenge test: After incubation, the biofilm around the uncoated control stent was observed. In Fig. 1 biofilm the tracking of bacteria along the abluminal surface indicated formation.

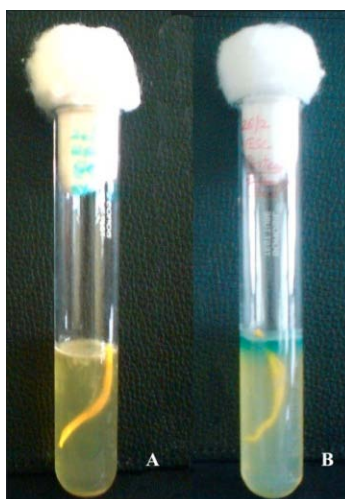


Fig. 1: Exit-site test to determine the biofilm forming efficiency of test bacteria. A: Biofilm producing *S. epidermidis* B: Biofilm producing *P. aeruginosa*

Table 1: Determining the weight of the PTFE stents

PTFE stent samples	Weight of the PTFE samples ^a	Number of stents used
Uncoated	16.1 mg	3
Drug-carrier coated	27.2 mg	3

^aMean weight of the samples

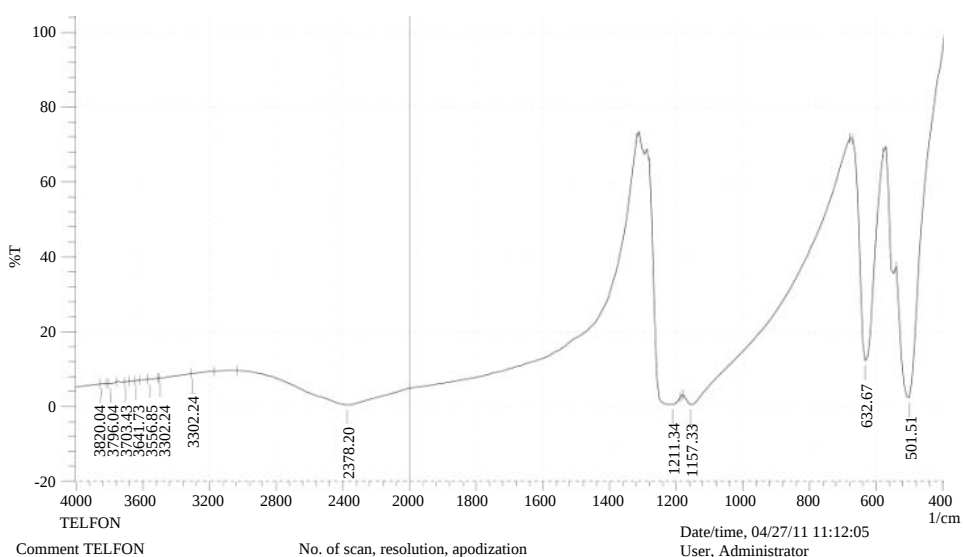


Fig. 2: FTIR analysis of uncoated PTFE stents. C=O stretch and O-H stretch was spectroscopically analysed for uncoated PTFE

Analysing the drug coated PTFE stents

Determining the weight of drug coated PTFE: The weight of PTFE stent piece measured before coating was 16.1 mg and that after coating was 27.2 mg. Hence it was expected that 11.1 mg of anti-infective agent was bound to the stent piece. In Table 1, the weight of the coated and pre-coated PTFE stent samples was reported.

FTIR analysis of drug coated PTFE stents: The FTIR spectra of uncoated PTFE, drug-carrier coated PTFE and drug coated PTFE were recorded. The results were shown in Fig. 2-4. In the FTIR spectrum of the uncoated PTFE sample (Fig. 2) the presence of the trace amount of COOH (carboxyl) functional group was evident from the absorption in the region 3200 to 2800 cm^{-1} (O-H stretch) and in the region 1500 to 1200 cm^{-1} (C = O stretch). In the FTIR spectrum of the drug-carrier coated PTFE sample (Fig. 3), two peaks at 3101.64 and 1141.94 cm^{-1} for the O-H stretching and C = O stretching of COOH were observed. The peaks at 1049.31, 1273.06, 1759.14 and 2630.99 cm^{-1} showed as major peaks for drug coated PTFE (Fig. 4). All the above peaks were also observed in drug-carrier coated PTFE (Fig. 3) that confirms the presence of drug in the polymer without any interaction.

Tissue response of CAM to drug coated PTFE stents: Drug-carrier coated PTFE implant materials were placed on to the surface of CAM using the method of Valdes *et al.* (2002). The test

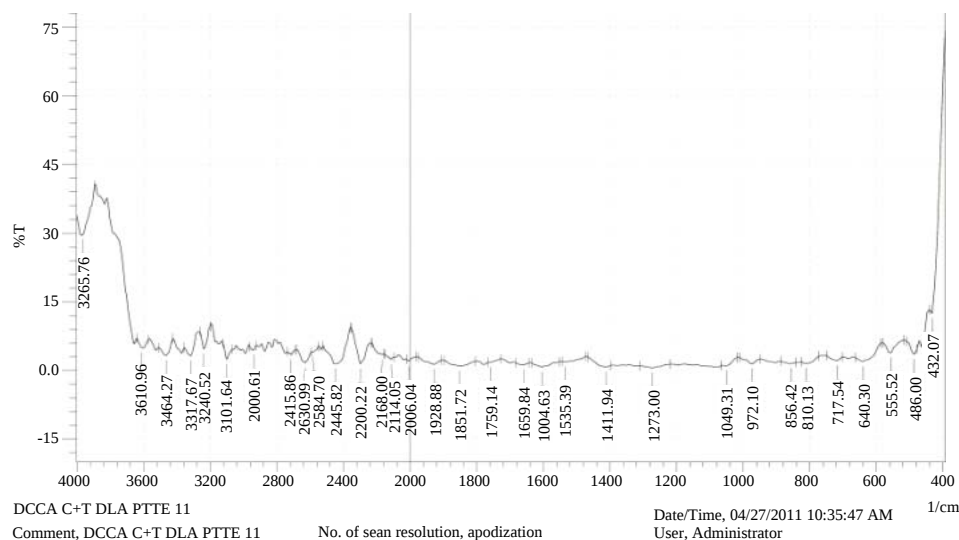


Fig. 3: FTIR analysis of drug-carrier-coated PTFE stents 3101.64 and 1141.94 nm for the O-H stretching and .C=O stretching of COOH were observed for drug-carrier-coated PTFE stents

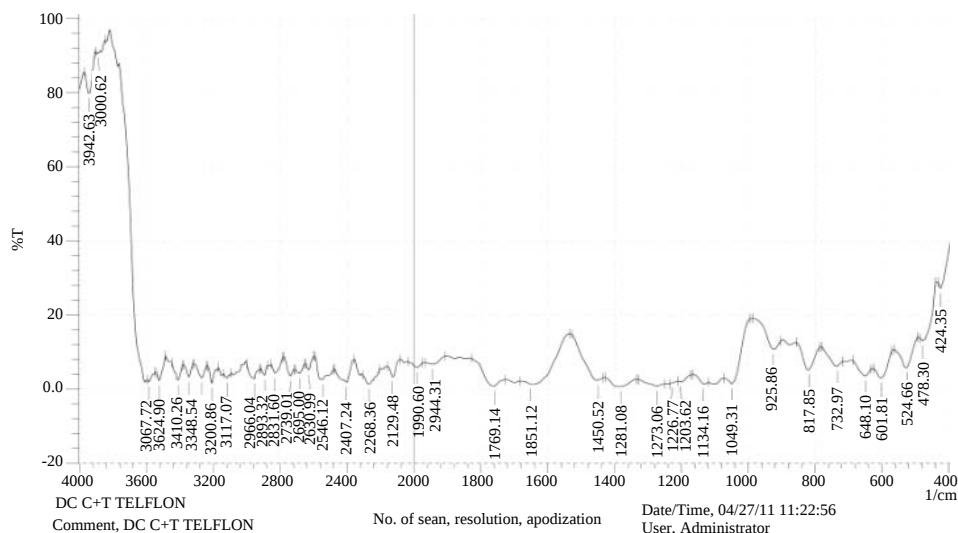


Fig. 4: FTIR analysis of drug-coated PTFE stents 1049.31, 1273.06, 1759.14 and 2630.99 nm showed as major peaks for drug-coated PTFE

was carried out to examine any tissue reactive signs on the developing CAM and the developing blood vessels. Bright-field microscopic analysis was done after the incubation period of 7 days. This showed no degenerative cells on the CAM and the developing blood vessels (Fig. 5).

Antibacterial activity

Qualitative test-agar diffusion test: The ability of the anti-infective agents to diffuse from the coated stent pieces to inhibit the growth of the bacterial strains seeded on MHA plate was calculated



Fig. 5: Tissue reactions of drug-coated stents on CAM, no visible signs of degenerated cells on tissues were recorded

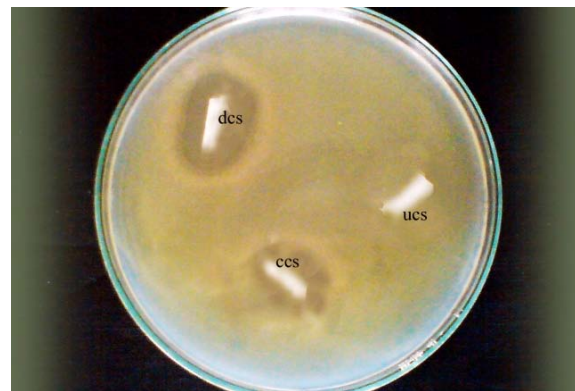


Fig. 6: Agar diffusion test showing zone of inhibition for *S. epidermidis* and *P. aeruginosa*
dcs: Drug-carrier-coated stents, ccs: Carrier-coated stents, ucs: Uncoated stents

Table 2: Agar diffusion test against *S. epidermidis* and *P. aeruginosa*

Bacterial strains	Zone of inhibition (mm)		
	dccs	ccs	Uncoated
<i>Staphylococcus epidermidis</i>	23	12	0
<i>Pseudomonas aeruginosa</i>	29	13	0

dccs: Drug-carrier-coated stents, ccs: Carrier-coated stents

based on the zone of inhibition. In Table 2, the zone of inhibition produced by Ofloxacin-ornidazole coated stents measured 23 and 29 mm for *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. The zone of inhibition for carrier-coated stents was 12 and 13 mm. The uncoated stents showed no zones of inhibition (Fig. 6).

Quantitative test-bacterial adherence studies: The study on bacterial adherence was carried out for *S. epidermidis* and *P. aeruginosa* separately. Drug-carrier coated stents and carrier-coated

Table 3: Bacterial adhesion studies of drug-carrier coated stents and carrier-coated stents

Bacterial strains	ID (CFU×10 ³)	Drug-carrier coated stents			Carrier-coated stents		
		Db ^a (CFU×10 ³)	Rb ^b (CFU×10 ³)	Chi-square test	Db ^a (CFU×10 ³)	Rb ^b (CFU×10 ³)	Chi-square test
<i>S. epidermidis</i>	122	1	12	p<0.05	40	96	p>0.05
<i>P. aeruginosa</i>	137	5	17	p<0.05	49	103	p>0.05

Test isolates-*Staphylococcus epidermidis* and *P. aeruginosa*. ID: Inoculum dose, DB: detached bacteria, RB: Remaining bacteria,

^aMean data for the adhered bacteria on the stent surface after 3 h incubation, ^bMean data for the remaining bacteria in PBS after 3 h incubation

Table 4: Ability of the bacterial strains to colonize stents under *in vitro* condition

Bacterial strains	Drug-carrier coated stents		Carrier-coated stents		Stents tested
	Challenge-1	Challenge-2	Challenge-1	Challenge-2	
<i>S. epidermidis</i>	-	-	+	+	3
<i>P. aeruginosa</i>	-	-	+	+	3

-, No colonization, +, Colonization. Challenge organisms-*S. epidermidis* and *P. aeruginosa*. Challenge dose-1: dcs and ucs flasks were inoculated with challenge dose of bacterial cultures and incubated for 7 days time, dcs inhibits the growth of challenge organism whereas ucs does not inhibit the growth of bacteria. Challenge dose-2: dcs and ucs flasks were inoculated with challenge dose of bacterial cultures and incubated for 7 days time, dcs inhibits the growth of challenge organism whereas ucs does not inhibit the growth of bacteria

stents were inoculated with the bacterial strains and adherence was studied *in vitro* based on the effect of inoculum dose. Effect of inoculum dose was calculated by dividing the CFU of detached bacteria by the CFU of inoculum dose for drug-carrier coated stents. This was compared simultaneously with carrier-coated stents. In order to see whether the drug-carrier coated stents could inhibit bacterial growth during 3 h incubation period, the remaining bacteria was calculated based on the ratio of CFU of remaining bacteria to CFU of the inoculum dose and compared simultaneously for carrier-coated stents.

The results were recorded in Table 3. For *S. epidermidis*, the table shows that the Detached Bacteria (DB) for drug-carrier coated stents (dcs) was only 1×10³ CFU whereas for carrier-coated stents (ccs), it was 40×10³ CFU. Similarly for *P. aeruginosa*, the Detached Bacteria (DB) for dcs was only 5×10³ CFU whereas for ccs, it was 49×10³ CFU. Difference in the number of bacterial counts for dcs and ccs after sonication showed the antimicrobial efficacy of Ofloxacin-Ornidazole (Fig. 2). Effect of inoculum dose (*S. epidermidis* or *P. aeruginosa*) for drug-carrier coated stents when compared with carrier-coated stents showed that the numbers of adhered bacteria in drug-coated-carrier stents (p<0.05) were less than the number of adhered bacteria in carrier-coated stents (p>0.05).

The growth inhibitory action of drug-carrier coated stents and carrier-coated stents against the remaining bacteria (*S. epidermidis* or *P. aeruginosa*) during 3 h incubation period was checked. The ratio of CFU of the remaining bacteria to CFU of the inoculum dose was then calculated. It was thus evident that the growth of remaining bacteria was inhibited to significant level for drug-carrier coated stents (p<0.05) than the carrier-coated stents (p>0.05).

***In vitro* challenge method:** Table 4 shows the results of *in vitro* challenge of stents. The drug-carrier coated stents and carrier-coated stents were tested in triplicates against two challenge test isolates, *S. epidermidis* and *P. aeruginosa*. Resistance to colonization of drug-coated-carrier

stents were observed even after 2 consecutive challenge doses, whereas, carrier-coated stents were colonized after the first challenge dose of test isolates, indicating the absence of antimicrobial coatings on their surface.

DISCUSSION

Early studies reported that the routine use of metallic stents failed to provide any additional benefit when compared with angioplasty alone (Clark, 2004). To improve safety, polymers with long-term biostability are being investigated (Simmons *et al.*, 2008) and there is interest in using biodegradable polymers which may promote less long-term inflammatory response (Grube *et al.*, 2004). The application of a biodegradable polymer, Poly DL-lactic Acid (PDLA) containing gentamycin on the surface of orthopedic implants has shown to result in drastic decrease in infection rate and a better recovery after infection (Knetsch and Koole, 2011). The polymer has excellent features with respect to implant coating, with high mechanical stability and excellent biocompatibility *in vivo* (Schmidmaier *et al.*, 2001). Gollwitzer *et al.* (2003) demonstrated that the antibacterials gentamicin and teicoplanin can be incorporated into the PDLA to give local drug-delivery systems that reduce bacterial adhesion *in vitro*. In our experiments, the biodegradable DL-lactic acid was used which provided similar results.

PTFE-covered stents have the advantage of reducing distal thrombotic embolization (Lefkovits *et al.*, 1995). Allie *et al.* (2004) reported that expanded PTFE is able to withstand the biomechanical forces that are exerted on it in the peripheral circulation without the structural damage such as fractures. Increased platelet activation has been reported in the case of PTFE-covered coronary stent grafts (Beythien *et al.*, 2004). Anti-infective coatings of PTFE stents was described by Matl *et al.* (2008) using gentamicin sulphate or teicoplanin with PDLA, tocopherol acetate or Dynasan 118 as drug carriers. This completely inhibited the proliferation of *S. aureus* while preserving biocompatible and hemocompatible characteristics. Ouedraogo *et al.* (2008) ensured a sustained release of the antibiotic gentamicin sulphate using a biodegradable and bioadhesive monoglyceride called monoolein which gave encouraging effects. Coatings containing combinations of antibiotics and antiseptics like minocycline and rifampin or chlorhexidin and silver-sulfadiazine have been applied to the internal and external surface of catheters. In several studies these antimicrobial coated catheters were compared to non-coated catheters and a reduction of catheter colonization and catheter related blood-stream infections were found (Hernandez-Richter *et al.*, 2003). Right coronary artery drug-eluting stent implantation was carried out successfully by Tsioufis *et al.* (2011). Carter *et al.* (2004) found that rapamycin-eluting stents inhibited intimal hyperplasia for 30 days; however, long-term inhibition was not sustained. In our study, synergistic activity of the anti-infective agents Ofloxacin and Ornidazole along with the drug carrier DLA is shown to inhibit microbial growth effectively with sustained release thereby preventing biofilm formation for a longer duration.

Matl *et al.* (2008) coated PTFE prostheses with the carrier containing the drug by two dip-coating procedures, ensuring a regular polymer coating. The weight of each coating was assessed by the difference in the weight of the PTFE graft before and after the coating procedure. Growth of MRSA was seen to be inhibited in the antimicrobial modified silicone catheter used in the exit-site challenge test carried out by Bayston *et al.* (2009). Likewise, in the present study, the drug-coated stent failed to show any colonization by biofilm forming bacteria.

Patel *et al.* (2009) after performing FTIR for the evaluation of ocular inserts, obtained major peaks for the drug Gatifloxacin sesquihydrate at 1663.19, 2843.01, 2975.69 and 821.53 nm which were present in drug loaded ocular inserts. This confirmed the presence of drug in the polymer without any interaction. The peak for the .C=O stretching of .COOH was obtained at 1281.59 nm. In our study, similar assumption was made. The major peaks obtained for the drug were 1049.31, 1273.06, 1759.14 and 2630.99 nm whereas the peak for .C=O stretching of .COOH was obtained at 1141.94 nm.

The effects of exposure of the drug-carrier coated PTFE stent was determined by examining the tissue response of chick chorio-allantoic membranes in chick eggs. Similar work was carried out by Valdes *et al.* (2002) to determine the tissue response of CAM against different dental implant materials. In their study, epoxy resins showed a mild response with minimal alteration of tissue elements and metal implant showed a mild response of some loss of ectoderm and increased fibrous depositions under the membrane. In contradiction to this result, we obtained no visible signs of tissue reactions on the CAM even after incubating for a period of 7 days. Qualitative test was done using diffusion method which was accepted to be a genuine antimicrobial susceptibility test (Zuridah *et al.*, 2008). In the qualitative tests done by Matl *et al.* (2008) using gentamicin coatings on PTFE grafts, no inhibition zones were obtained against *S. aureus* lawns. In contrast, this study confirmed the inhibition of colony formation by *S. epidermidis* and *P. aeruginosa* over the MHA plates under effect of the synergistic antibiotic combination (Ofloxacin and Ornidazole). Gollwitzer *et al.* (2003) carried out quantitative test involving bacterial adhesion in which the combination of PDLLA with either gentamicin or teicoplanin or both antibiotics on the implant together was indicative of reducing viable counts to almost undetectable levels ($p < 0.05$). We have used a combination of Ofloxacin and Ornidazole that has a proven synergistic effect which when coated onto PTFE stents along with DLLA showed a reduction in viable counts that was comparable to the former experiment.

The persistence of anti-infective agents on the stent has been proved by means of *in vitro* challenge test in present study. We have obtained results in support to the work of Elayarajah *et al.* (2011) in which a synergistic combination of norfloxacin-metronidazole was used along with the drug carrier tocopherol acetate. No colonization was observed as per their study even after two challenge doses of *S. epidermidis* and *E. coli*. Hence, in the present study, an effective way to prevent coronary vascular stent-associated infection is suggested by the use of a combination of Ofloxacin-Ornidazole with DLLA.

CONCLUSION

In the present study, the coating of PTFE stents with a synergistic combination of Ofloxacin and Ornidazole with the drug carrier DLLA is demonstrated in order to establish a local sustained release of anti-infective agents thereby preventing infection and also to decrease antimicrobial resistance. In our research, the drug-coated stents were shown to have prolonged antimicrobial activity and high efficacy to inhibit the colonization of coronary pathogens (*Staphylococcus epidermidis*, *Pseudomonas aeruginosa*). The drug carrier used here is highly biodegradable and safe since it does not cause any tissue reactions. *In vivo* confirmation of these findings could be an immensely great future perspective in the medical field where prevention of stent associated infection is a major concern.

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