Epidemiol. Infect. (1994), **112**, 551–559

Incidence and nature of peritoneal catheter biofilm determined by electron and confocal laser scanning microscopy

S. P. GORMAN, C. G. ADAIR AND W. M. MAWHINNEY

School of Pharmacy, Medical Biology Centre, The Queen's University of Belfast, Belfast BT9 7BL, UK

(*Accepted 30 November 1993*)

SUMMARY

Thirty-two Tenckhoff catheters retrieved from continuous ambulatory peritoneal dialysis patients with a history of peritonitis were examined for microbial biofilm. Confocal laser scanning microscopy was successfully employed to visualize bacteria in biofilm occluded from view by scanning electron microscopy. Occluded but viable microbial biofilm was associated with 17 (81%) catheters from patients free from infection following renal transplant. Mixed isolate biofilm with two or more isolates of coagulase-negative staphylococci or *Staphylococcus aureus* was found on 41% of these catheters. Clearly visible viable biofilm consisting exclusively of *Pseudomonas aeruginosa* occurred on all four catheters removed due to recurrent peritonitis. Five (71%) catheters retrieved from patients transferred to haemodialysis had viable biofilm. Antibiotic sensitivities of the biofilm isolates were similar in profile to those reported for non-biofilm isolates from infected dialysate. Persistence of catheter biofilm despite direct contact with therapeutic levels of antibiotics in peritoneal dialysate requires that attention be directed towards improving antibiotic efficacy against peritonitis-causing bacteria in biofilm form.

INTRODUCTION

Continuous ambulatory peritoneal dialysis (CAPD) has become the accepted form of dialysis treatment for patients with end-stage renal disease. Infections often occur and peritonitis remains the predominant complication in CAPD [1]. Coagulase-negative staphylococci are most frequently encountered although *Staphylococcus aureus* may also present problems. Gram-negative rods account for up to 30% infections [2]. Because of the wide range of organisms implicated in peritonitis, antibiotic therapy can be difficult and may be complicated by the problem of relapsing peritonitis.

Microbial biofilm is a complex structure that forms on the surface of biomaterials by the growth of adherent microorganisms exuding an exopolysaccharide matrix (glycocalyx) protecting them from host immune-defence mechanisms, antibiotics and biocides [3]. Biofilms are implicated in the persistence of many severe or recurrent infections and reports of its occurrence on prosthetic devices include, for example, heart valves [4], bladder catheters [5], cardiac catheters [6] and endotracheal tubes [7]. Formation of a biofilm on peritoneal catheters has been suggested as a possible cause of relapsing peritonitis [8]. The presence of biofilm

on such devices may not always be recognizable by conventional microscopic techniques as the entrained microorganisms are often obscured by the biofilm matrix or overlying erythrocytes and inflammatory cells. Additionally, difficulties arise in distorted views due to preparation techniques for electron microscopy. The frequent occlusion of the biofilm and the tenacity of the biofilm for the surface can also present difficulties in isolation and identification of the infecting bacteria by standard techniques. These problems may be alleviated by the use of the relatively new technique of confocal laser scanning microscopy (CLSM) [9]. CLSM operates by the optical sectioning of specimens in the x, z rather than in the x, y plane. The images generated of sections taken down to the sub-micrometre level are digitally enhanced to provide a three-dimensional, non-invasive image of sub-surface organelles. Artifacts introduced by preparing specimens for scanning electron microscopy are avoided.

Previous studies have revealed, by electron microscopy, the presence of microbial biofilm on peritoneal catheters [10, 11]. The aim of this study was to investigate further the incidence and types of microbial biofilm on catheters retrieved from different groups of dialysis patient by application of confocal microscopy and to assess the antibiotic sensitivities of catheter biofilm isolates.

MATERIALS AND METHODS

CAPD catheters

Thirty-two Tenckhoff peritoneal dialysis catheters (silicone, double cuffed) were obtained following surgical removal due to recurrent peritonitis, following renal transplantation (RT) or for other reasons such as catheter blockage or patient unsuitability for CAPD. Excluding patients with recurrent peritonitis, there was no clinical infection at the time of catheter removal although each patient had experienced peritonitis during the period of catheterization. Each catheter was placed immediately into a sealed, sterile, labelled specimen bag and stored at 4 °C until collection which was not later than 2 h after removal. Sections (6 × 1 cm) cut from the cuff and intraperitoneal regions were split longitudinally into two portions. One portion was placed in 5% (w/v) glutaraldehyde in cacodylate buffer (0·1 mol/l, pH 7·4) and stored at 4 °C for subsequent microscopic examination and the other portion retained for identification of adhered bacteria.

Isolation of adhered microorganisms

Biofilm attached to catheter surfaces was dislodged into sterile artificial spent dialysate (ASD) [12]. The dislodgement was effected by a combination of gentle scraping with a sterile scalpel blade, vortex mixing (30 sec) and ultrasonication (30 sec in a 150 W ultrasonic bath operating at a nominal frequency of 50 Hz). These procedures have been shown to be harmless to the infecting microorganisms [13].

Identification of biofilm isolates

Catheter biofilm suspension in ASD was streaked onto Columbia blood agar, chocolate blood agar and MacConkey agar (Oxoid Ltd., UK) for single colony isolation. Plates were incubated for 48 h at 37 °C aerobically and with 5% carbon dioxide enrichment. All Gram-positive cocci were catalase tested. Those testing

positive were regarded as staphylococci and were slide coagulase tested by the Staph. aurex® slide agglutination method (Wellcome, UK) and tube coagulase tested. Organisms testing positive to both the slide and tube coagulase tests were identified as *Staphylococcus aureus*. Other catalase-positive, coagulase-negative isolates were identified as coagulase-negative staphylococci (CNS). Catalase-negative Gram-positive cocci were tested for Lancefield carbohydrate group antigens A, B, C, D, F and G by the Streptex® method (Wellcome, UK). They were all also sub-cultured onto bile aesculin agar to test for Group D streptococci/enterococci.

Gram-negative rods were oxidase tested, oxidase-positive organisms being identified by the API 20 NE system and oxidase-negative microorganisms by the API 20 E system. Each isolate was inoculated onto MacConkey agar (Life Technologies Ltd., UK) to determine the fermentation of lactose, onto Blood agar (Life Technologies Ltd., UK) to determine the presence or absence of haemolysis and onto Mannitol Salt agar (Oxoid, UK) to demonstrate the fermentation of mannitol.

Each isolate was inoculated into the cryopreservative fluid of a Protect Bacterial Preserve System (Technical Service Consultants Ltd., UK) and stored at −20 °C.

Sensitivity testing

Antibiotic susceptibilities were determined by comparative disk-diffusion assays using penicillin (P) 5 μg, methicillin (ME) 10 μg, gentamicin (G) 10 μg, amikacin (AK) 30 μg, streptomycin (ST) 25 μg, neomycin 30 μg, vancomycin (VA) 30 μg, tetracycline (T) 50 μg, minocycline (M) 30 μg, erythromycin (E) 5 μg, clindamycin (CL) 2 μg, fusidic acid (FU) 10 μg, rifampicin (RI) 2 μg, trimethoprim (TR) 2·5 μg, bacitracin (BA) 0·04 μg, chloramphenicol (C) 50 μg, novobiocin (NO) 5 μg, nitrofurantoin (F) 100 μg and teicoplanin (TE) 30 μg. All tests were carried out on Blood DST agar (Oxoid, UK) and incubated at 37 °C for 18 h, except for methicillin which was carried out on Columbia Blood agar at 30 °C for up to 48 h.

Biofilm microscopy

The preparation of glutaraldehyde-fixed catheter portions for electron microscopy was as described by Gorman and colleagues [9]. Prepared samples were scanned at 10 KV with a Jeol 35 CF scanning electron microscope and viewed at 100 kV in a Phillips 400 transmission electron microscope. For confocal laser scanning microscopy (CLSM) of biofilm, catheter portions were attached to glass microscope slides with epoxy resin [9]. Examination of the specimens was conducted at a wavelength of 488 nm with a Biorad Lasersharp MRC 500 microscope.

RESULTS

Scanning electron microscopy of a typical catheter retrieved from a patient with recurrent peritonitis shows a surface with heavy deposits and a large mass of material blocking a catheter pore (Fig. 1*a*). Higher magnification of this material reveals bacilli, subsequently identified as *Pseudomonas aeruginosa*, enmeshed in a thick, protective glycocalyx (Fig. 1*b*) and the nature of this biofilm is further

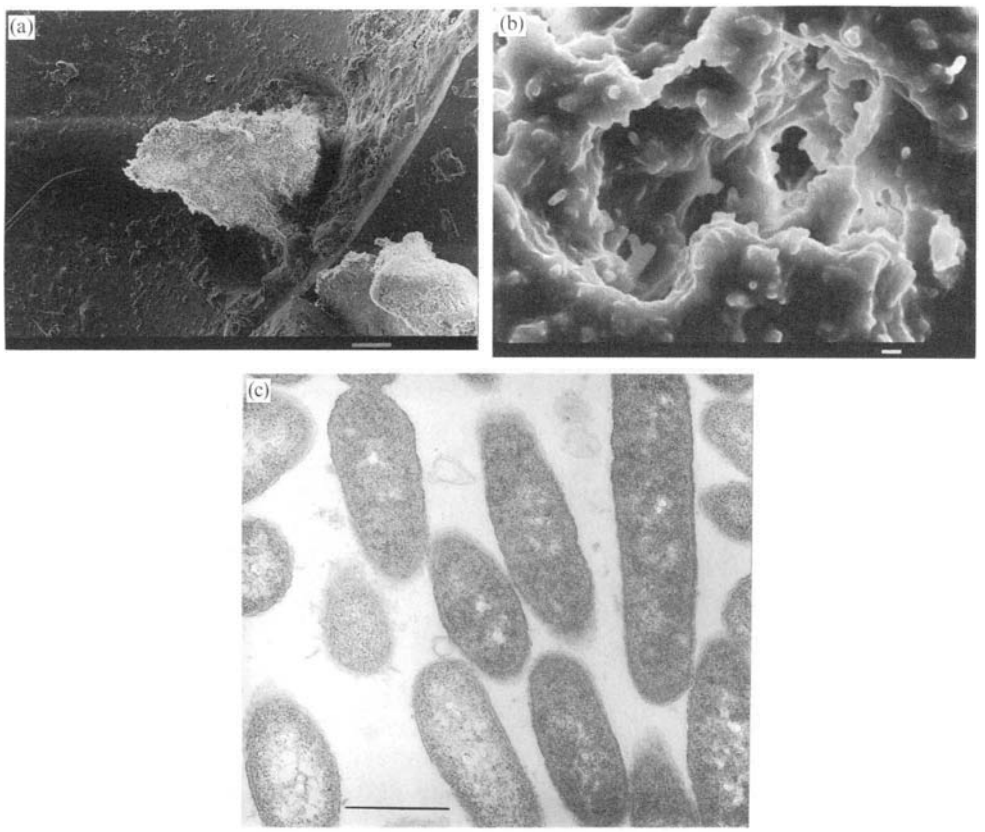

Fig. 1. (*a*) Scanning electron micrograph (bar = 100 μm) of a typical catheter retrieved from a dialysis patient with recurrent peritonitis showing surface deposition of material and blockage of a pore. (*b*) higher magnification view (bar = 1 μm) of the pore deposit showing gross microbial biofilm of bacilli encased in glycocalyx, subsequently identified as *Pseudomonas aeruginosa*. (*c*) transmission electron micrograph of this biofilm indicating the proximity of cells to one another (bar = 1 μm).

illustrated by TEM (Fig. 1*c*). Such clear views of biofilm are often not possible by SEM, as shown in Figure 2*a*, when, in a typical catheter from RT patients, other cell types such as erythrocytes, inflammatory cells and fibrous material can obscure the microbial biofilm. Using CLSM to penetrate this occluded biofilm to a depth of 22 μm, discrete entities of 1–2 μm are visible in close proximity to areas of high intensity fluorescence representing clusters of cells (Fig. 2*b*). *Staphylococcus epidermidis* was subsequently identified in this biofilm. Surface areas presenting difficulties for microbiological examination also may be accessed readily by CLSM. Figure 2*c* shows a low magnification view of biofilm as a fluorescent band circling the rim of a catheter pore and detached from part of the pore surface. Distinct microcolonies may be observed at higher magnification of this area and the high intensity fluorescence in some areas is indicative of gross bacterial presence (Fig. 2*d*). This was subsequently identified as a mixed isolate biofilm of coagulase-negative staphylococci.

Of the 32 catheters examined in this study, approximately 66% were obtained from RT patients. 12% were removed due to peritonitis, with 22% removed for reasons such as catheter blockage or transferral to haemodialysis (Table 1). Viable

https://doi.org/10.1017/S0950268800051256 Published online by Cambridge University Press

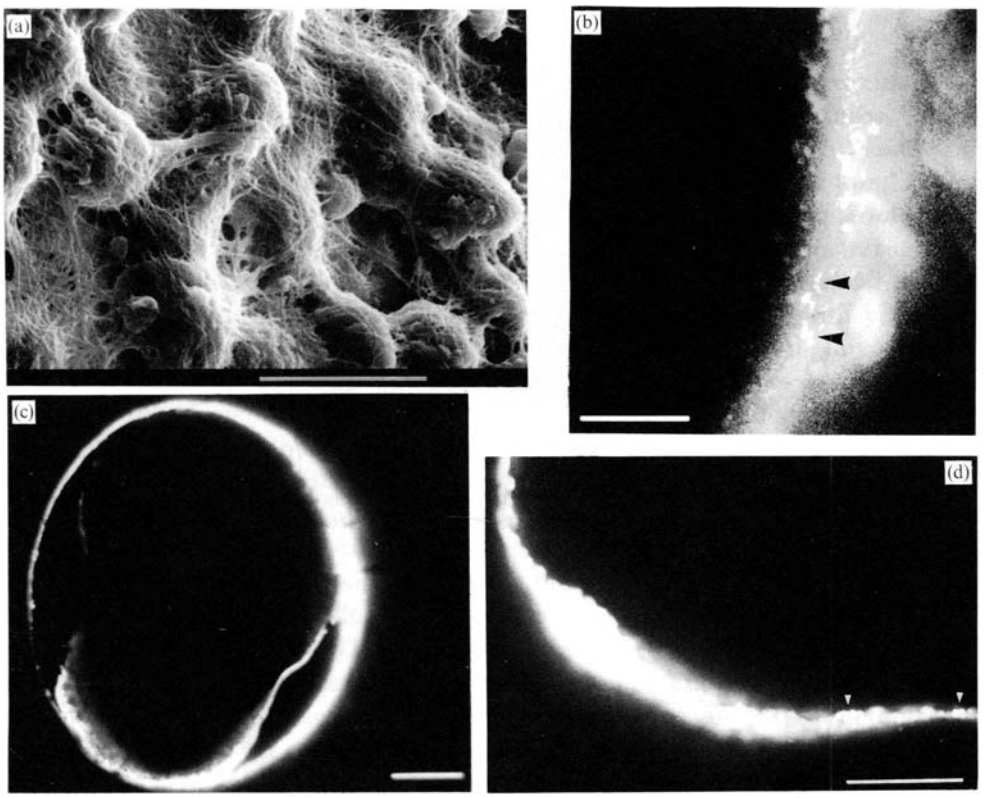

Fig. 2. (*a*) Scanning electron micrograph of typical occluded biofilm on the surface of catheters retrieved from patients following renal transplant. Erythrocytes may be seen enmeshed in extensive fibrous material (bar = 10 μm). (*b*) confocal laser scanning micrograph of the occluded biofilm encompassing dispersed circular entities of approximately 1–2 μm in diameter (arrows). *Staphylococcus epidermidis* was subsequently identified in this biofilm (bar = 10 μm). (*c*) CLSM of a deposit, becoming detached, within a peritoneal catheter pore showing high intensity fluorescence indicative of gross bacterial presence (bar = 100 μm). (*d*) higher magnification view by CLSM of the pore deposit showing (arrows) a number of distinct microcolonies of a staphylococcal biofilm (bar = 100 μm).

Table 1. *Incidence of microbial biofilm on Tenckhoff peritoneal catheters removed for various reasons*

Reason for catheter removal	Number (% of 32) of catheters collected	% of catheters with viable biofilm	% of total (39) isolates from biofilm
Peritonitis	4 (12·5)	100	10·3
Renal transplant	21 (65·6)	81	64·1
Miscellaneous*	7 (21·9)	71	25·6

* Catheter blockage, transfer to haemodialysis.

microbial biofilm was identified on all catheters removed due to peritonitis. Viable microbial biofilm was also present on 81% of the RT catheters and on 71% of catheters removed for other reasons. Of the 39 biofilm isolates obtained in total, 25 (64%) were from RT catheters with 41% of infected catheters having 2 or more biofilm isolates (Fig. 3). Single isolate biofilms only, of *Pseudomonas aeruginosa*, were identified on catheters removed due to peritonitis.

https://doi.org/10.1017/S0950268800051256 Published online by Cambridge University Press

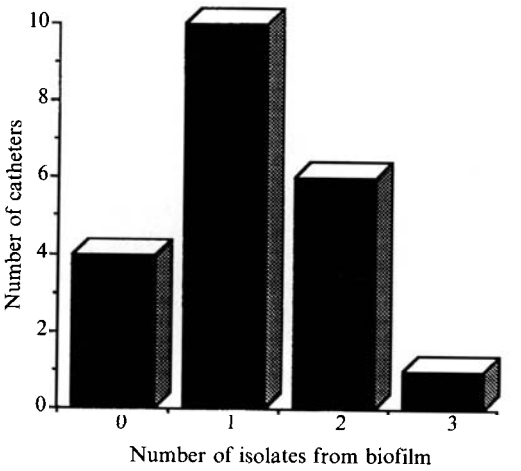

Fig. 3. Incidence and number of biofilm isolates associated with catheters from renal transplant patients during study period.

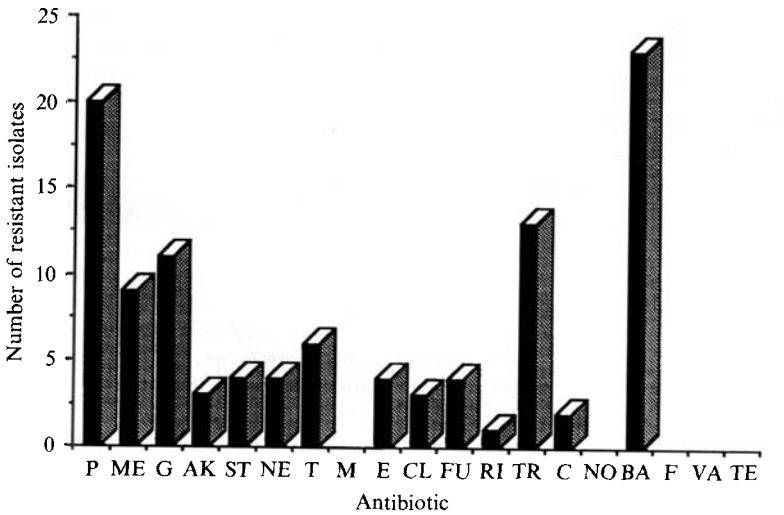

Fig. 4. Antibiotic resistance profile of coagulase-negative staphylococci isolated from CAPD catheter biofilm. Antibiotic abbreviations are as described in Methods.

Coagulase-negative staphylococci accounted for more than 62% of all isolates and over 65% of RT catheter isolates. Of these, *S. epidermidis* was the most frequent biofilm isolate, accounting for 33% of all isolates and 35% of RT isolates with a lesser incidence observed of *S. haemolyticus* (13%) and *S. hominis* (8%). *S. aureus* was isolated in 24% of all cases (31% RT catheter isolates). Other less frequently isolated organisms were *S. saprophyticus*, *S. simulans*, *S. warnerii*, *Micrococcus* spp., *Enterococcus faecalis* and *Enterobacter cloacea*.

Sensitivity testing of CNS showed no resistance to either vancomycin or teicoplanin. These antibiotics were also effective against *S. aureus* isolates. Nearly half of all CNS isolated were shown to be resistant to penicillin and approximately 40% were methicillin-resistant (Fig. 4). *P. aeruginosa* isolates were all sensitive to gentamicin.

https://doi.org/10.1017/S0950268800051256 Published online by Cambridge University Press

DISCUSSION

Biofilm was observed on 90% of the 32 CAPD catheters examined by microscopy and was confluent on both outer and inner catheter surfaces. This is consistent with the 100% prevalence observed in other studies with 7 catheters [14] and 25 catheters, wherein 12 were removed due to renal transplant and 13 due to infection [11]. The propensity of biofilm to accumulate in and around the catheter pores is of interest as catheter surface roughness, or microrugosity, increases with dwell time in the peritoneum [15]. Although increased bacterial adherence is associated with increased surface microrugosity, differences in dialysate flow characteristics in the region of the catheter pores and planar surface areas may have a greater influence on biofilm formation in these areas.

Considerable benefit is to be gained from CLSM of occluded biofilms as microbiological isolation of the entrained bacteria is frequently difficult by conventional techniques with the consequent inevitability of misleading negative culture. Obtaining SEM evidence of bacterial presence in biofilms can also be difficult [16] as the micrographs of biofilm on catheters from RT patients show. Problems also arise in determination of the biofilm structure as estimates of the degree of biofilm shrinkage due to dehydration and embedding techniques vary from 50% [17] to 99% [18]. The penetrative view by CLSM, with minimal sample preparation, allows visualization of biofilm presence and structure below the superficial, occluding material associated with biofilms on RT catheters. The organization of microorganisms within a biofilm may be structured to ensure maximum benefit and/or protection from the environment. Consequently, the absence of distinct microcolonies in mature pseudomonal biofilms formed *in vitro* has been noted in a CLSM study [19], whereas in our investigation distinct microcolonies were observed within the matrix of mature pseudomonal and staphylococcal biofilms. The maturity of our biofilms formed under adverse conditions *in vivo* (> 12 months catheter dwell-time) with their constant exposure to dialysis solutions and, inevitably, antibiotic therapy may influence biofilm structure and account for this difference. As in the latter study, the microcolonies in our *in vivo* biofilms were more concentrated close to the material (catheter) surface.

CAPD catheters with no visible biofilm gave a negative culture, while 89% of those catheters with visible biofilm gave a positive culture. This figure is in keeping with previous CAPD catheter studies: 90% [10], 70% [14], and 100% viability of biofilm examined [11]. In contrast, none of 17 CAPD catheter biofilms examined gave positive cultures indicating that a non-infectious biofilm may also occur [20]. In this respect, our observation of viable biofilm in 81% of catheters removed due to renal transplant is interesting as clinical evidence of peritonitis post-transplant was absent. Host defence factors are the main determinants of whether microorganisms disseminated from biofilm can cause peritonitis and in the case of RT patients these defences will have been considerably weakened by immunosuppressive therapy aimed at reducing graft rejection. That none of the RT patients developed peritonitis, although all retained their catheters for 6–24 months, may be ascribed to the discontinuation of active dialysis and the consequent removal of the physical pressure on release of biofilm bacteria into the

peritoneal cavity. Furthermore, release of biofilm bacteria would be retarded by the build-up of occluding material typically observed on these catheter biofilms. In contrast, the clearly visible biofilm on catheters removed due to recurrent peritonitis during active dialysis could readily release infectious bacteria into the peritoneum.

The antibiotic resistance profile obtained indicated that all coagulase-negative staphylococci isolated from biofilm were sensitive to vancomycin and teicoplanin. A survey of eight studies of the sensitivities of CNS isolated from infected dialysis fluid in patients with peritonitis [21] shows close agreement with the sensitivities of our biofilm isolates. All the catheters examined in our study were, at some stage *in vivo*, exposed to antibiotics for treatment of peritonitis. Many of the catheter biofilms will have had similar antibiotic exposure but bacteria remained viable within these biofilms. The sensitivities of isolates when removed from the biofilm are markedly lower than the applied therapeutic levels for peritonitis (e.g. 25 μg/ml for vancomycin and teicoplanin) indicating the resistance conferred on infecting bacteria by the biofilm mode of growth. To ensure CAPD patient recovery from peritonitis and prevention of recurrent peritonitis, antibiotic therapy must be selected to maximize activity against catheter biofilm.

ACKNOWLEDGEMENTS

Financial assistance for this study was provided by the Northern Ireland Kidney Research Fund. The excellent technical assistance of Neville Heaney and John McCluggy of the Department of Microbiology, Belfast City Hospital, and the QUB Electron Microscopy Unit is greatly appreciated.

REFERENCES

1. Mawhinney WM, Adair CG, Gorman SP. The development and treatment of peritonitis in patients undergoing continuous ambulatory peritoneal dialysis. Int J Pharm Pract 1991; **1**: 10–8.
2. Spencer RC. Infections in continuous ambulatory peritoneal dialysis. J Med Microbiol 1988; **27**: 1–9.
3. Mawhinney WM, Adair CG, Gorman SP. The nature and role of microbial biofilm in infections associated with prosthetic devices. Int J Pharm Pract 1992; **1**: 136–45.
4. Kluge RM. Infections of prosthetic heart valves and arterial grafts. Heart Lung 1983; **11**: 146–51.
5. Tenney JH, Moody MR, Newman KA, Schimpff SC. Adherent microorganisms on lumenal surfaces of long-term intravenous catheters. Arch Int Med 1986; **146**: 1949–54.
6. Nickle JC, Wright JB, Ruseska I, Marrie TJ, Whitfield C, Costerton JW. Antibiotic resistance of *Pseudomonas aeruginosa* colonising a urinary catheter *in vitro*. Eur J Clin Microbiol Infect Dis 1985; **4**: 213–8.
7. Gorman SP, Adair CG, O'Neill F, Goldsmith EC, Webb CH. Influence of selective decontamination of the digestive tract on microbial biofilm formation on endotracheal tubes from artificially ventilated patients. Eur J Clin Microbiol Infect Dis 1993; **12**: 9–17.
8. Evans RC, Holmes CJ. Effect of vancomycin hydrochloride on Staphylococcus epidermidis biofilm associated with silicone elastomer. Antimicrob Ag Chemother 1987; **31**: 889–94.
9. Gorman SP, Mawhinney WM, Adair CG. Confocal laser scanning microscopy of adherent microorganisms, biofilms and surfaces. In: Microbial biofilms: formation and control. Denyer SP, Gorman SP, Sussman M, eds. The Society for Applied Bacteriology Technical Series No. 30. Oxford: Blackwell Scientific Publications, 1993: 95–107.

10. Marrie TJ, Noble MA, Costerton JW. Examination of the morphology of bacteria adhering to peritoneal dialysis catheters by scanning and transmission electron microscopy. J Clin Microbiol 1983; **18**: 1388–98.
11. Dasgupta MK, Ulan RA, Bettcher KB, Burns V, Lam K, Dosseter JB, Costerton JW. Relationship of adherent bacterial biofilms to peritonitis in chronic ambulatory peritoneal dialysis. Perit Dial Bull 1987; **7**: 168–73.
12. Holmes CJ, Evans RE, Vonesh E. Application of an empirically derived growth curve model to characterise *Staphylococcus epidermidis* biofilm development on silicone elastomer. Biomaterials 1989; **10**: 625–9.
13. Gorman SP. Microbial adherence and biofilm production. In: Mechanisms of action of chemical biocides: their study and exploitation. Denyer SP, Hugo WB, eds. The Society for Applied Bacteriology Technical Series No. 27, Oxford: Blackwell Scientific Publications, 1991: 271–95.
14. Reed WP, Light PD, Newman KA. Biofilm on Tenckhoff catheters: a possible source for peritonitis. In: Frontiers in peritoneal dialysis. Maher JF, et al. eds. New York: Field, Rich and Associates, 1986: 176–80.
15. Gorman SP, Mawhinney WM, Adair CG, Issoukis M. Confocal laser scanning microscopy of peritoneal catheter surfaces. J Med Microbiol 1993; **38**: 411–7.
16. Howgrave-Graham AR, Wallis FM. Preparation techniques for the electron microscopy of granular sludge from an anaerobic digester. Lett Appl Microbiol 1991; **13**: 87–9.
17. Woldringh CL, De Jong MA, Van Den Berg W, Koppes L. Morphological analysis of the division cycle of two Escherichia coli substrains during slow growth. J Bacteriol 1977; **131**: 270–9.
18. Sutherland IW. Surface carbohydrates of the procaryotic cell. London: Academic Press, 1977.
19. Lawrence JR, Korber DR, Hoyle BD, Costerton JW, Caldwell DE. Optical sectioning of microbial biofilms. J Bacteriol 1991; **173**: 6558–67.
20. Verger C, Chesneau AM, Thibault M. Biofilm on Tenckhoff catheters: a negligible source of contamination. Perit Dial Bull 1987; **7**: 168–73.
21. Verbrugh HA. Organisms causing peritonitis. In: CAPD: Host defence, nutrition and ultrafiltration. Coles GA, Davies M, Williams JD, eds. Contrib Nephrol. Basel, Karger, 1990; **85**: 39–48.