BACTERIA ON CATHETERS IN PATIENTS UNDERGOING PERITONEAL DIALYSIS

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♦ **Background:** Peritonitis is the leading cause of morbidity for peritoneal dialysis (PD) patients, and microbial biofilms have previously been identified on catheters from infected patients. However, few studies of catheters from patients without clinical signs of infection have been undertaken. The aim of the present study was to investigate the extent to which bacteria are present on catheters from PD patients with no symptoms of infection.

♦ **Methods:** Microbiologic culturing under aerobic and anaerobic conditions and confocal laser scanning microscopy were used to determine the distribution of bacteria on PD catheters from 15 patients without clinical signs of infection and on catheters from 2 infected patients. The 16S rRNA gene sequencing technique was used to identify cultured bacteria.

♦ **Results:** Bacteria were detected on 12 of the 15 catheters from patients without signs of infection and on the 2 catheters from infected patients. Single-species and mixed-microbial communities containing up to 5 species were present on both the inside and the outside along the whole length of the colonized catheters. The bacterial species most commonly found were the skin commensals *Staphylococcus epidermidis* and *Propionibacterium acnes*, followed by *S. warneri* and *S. lugdunensis*. The strains of these micro-organisms, particularly those of *S. epidermidis*, varied in phenotype with respect to their tolerance of the major classes of antibiotics.

♦ **Conclusions:** Bacteria were common on catheters from patients without symptoms of infection. Up to 4 different bacterial species were found in close association and may represent a risk factor for the future development of peritonitis in patients hosting such micro-organisms.

The advantages of peritoneal dialysis (PD) over hemodialysis are the higher relative freedom and the lower cost for patients, but the major drawback is the risk of infections such as peritonitis. Peritonitis gives rise to symptoms including abdominal pain and fever (1), and it is a leading cause of morbidity and technique failure in PD (2,3). The average incidence of peritonitis in the PD population varies considerably depending on factors such as country, center, PD technique, and patient category, ranging from more than 2 episodes per patient per year (4) to fewer episodes than 1 every 4th year (1). The organisms that most commonly cause peritonitis are coagulase-negative staphylococci (CoNS), including *Staphylococcus epidermidis* and *S. aureus* (2,5–8). However, infections involving gram-negative bacteria—for example, *Klebsiella*, *Escherichia coli*, *Pseudomonas*—or mixed populations are generally more severe, leading to more hospitalizations, catheter removals, PD dropouts, and deaths (8–11). Under microscopy, PD catheters removed from patients experiencing infections have been shown to be covered with microbial biofilms (12,13), consisting of single-species (14) or mixed-microbial populations (13,14). Despite the large number of potential niches in biofilms, medical device-associated biofilms often show a low degree of microbial diversity (15,16).

Naturally, much work has been conducted in PD patients with infections, but only a few studies have investigated microbial biofilms on catheters from patients without clinical signs of infection, with results ranging from an absence of bacteria to lightly colonized areas and mature biofilms (12–14). The latter studies focused primarily on revealing the presence and surface coverage of biofilms rather than on identifying the bacterial species present, although cultures of catheter scrapings revealed *S. epidermidis*, *S. aureus*, and species of the *Enterobacteriaceae* family (12–14). Normally, analysis of dialysis effluent is used to isolate and identify bacteria in PD patients with infections (6,8,17), but how well effluent cultures correlate with what is actually on the

Perit Dial Int 2013; 33(1):51-59 www.PDIConnect.com
epub ahead of print: 01 Aug 2012 doi:10.3747/pdi.2011.00320

KEY WORDS: Biofilm; 16S rRNA gene sequencing; anaerobic culture; confocal microscopy.

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Received 21 December 2011; accepted 31 January 2012

catheter is not known, and studies of actual catheters are therefore important.

We set out to identify the range of aerobic and anaerobic bacterial species on PD catheters from patients without infections and to compare those results with results obtained from catheters in PD patients with infections. We also studied the phenotypic variation of the bacterial strains isolated. Data presented here show bacterial colonization, commonly by skin commensals, on most catheters. Bacteria were found in single-organism or mixed-microbial populations that showed a varied phenotypic pattern of antibiotic tolerance.

METHODS

PATIENTS

All patients undergoing removal of a PD catheter at Skåne University Hospital, Malmö, Sweden, were asked to participate in the study, and those who agreed gave written consent. The study was approved by the regional ethical committee in Lund, Sweden (Dnr 236/2008).

The 17 patients (10 men, 7 women; mean age: 56 ± 14 years) who participated had catheters that had been in place for 1–49 months (median: 17 months) before removal. In 2 patients, corticosteroid treatment had been given before catheter removal. The reasons for dialysis were chronic glomerulonephritis (6 patients), diabetic nephropathy (4 patients), polycystic kidney disease (3 patients), chronic interstitial nephritis (3 patients), and vasculitis (1 patient). In 2 patients, the catheter was removed because of the presence of clinical symptoms of infection (peritonitis and exit-site infection); in the others, the catheter was removed in conjunction with renal transplantation. Including the 2 patients who had clinical signs of infection, 7 patients had been treated with antibiotics in the year before catheter removal, and 5 of those treatments were for catheter-related infection.

After removal, the catheter was divided into a subcutaneous (S) part and an intraperitoneal (I) part, placed into sterile dialysis fluid, and kept at 4°C. The catheter was then divided into 2-cm pieces, half of which were used for microbial culture and half for confocal laser scanning microscopy (CLSM) studies, as described next.

BACTERIAL CULTURE

Catheter pieces used for culturing were split longitudinally into two pieces that were placed into Bacto Todd Hewitt broth (TH: Becton–Dickinson, Mountain View, CA, USA) or BBL Fluid Thioglycollate Medium (FTM: Becton–Dickinson) solidified using 0.2% agar. The pieces were

cultured aerobically in 5% CO₂ at 37°C or anaerobically in 5% H₂ and 95% N₂ at 37°C for at least 3 months. The medium for anaerobic cultures was pre-reduced.

Because the agar was semisolid, the catheter pieces could be placed in the middle of the test tubes surrounded by medium, which facilitated growth detection on both the external and luminal side of each catheter piece. Where growth was seen, colonies were streaked on blood agar and re-cultured. All colony-forming units (CFUs) showing different morphologies were subjected to standard microbiology evaluation techniques—Gram staining, catalase test, oxidase test, Pastorex Staph Plus agglutination test (Bio-Rad Laboratory, Hercules, CA, USA)—and were grown on 110 agar.

Representatives of each CFU type were subjected to 16S rRNA gene sequencing and identified to the species level using universal primers as previously described (18). To investigate phenotypic variation, the isolates from the microbial cultures were tested for susceptibility to antibiotics of different classes. The antibiotics tested were amoxicillin (10 µg, penicillinase-stable penicillin), penicillin V (10 µg, penicillinase-sensitive penicillin), ciprofloxacin (10 µg, quinolone), clindamycin (15 µg, lincosamide), and tetracycline (30 µg, tetracycline). Anaerobes were also tested for sensitivity to metronidazole (10 µg, imidazole derivative). Bacteria were spread on blood agar plates, antibiotic discs were placed on top, and the plates were then incubated under aerobic or anaerobic conditions as described earlier. Bacteria were classified as “resistant” or “sensitive” according to the breakpoints given for zone diameters recommended by the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/eucast_disk_diffusion_test/breakpoints).

DIRECT MICROSCOPY

The LIVE/DEAD *BacLight* Bacterial Viability method (Kit L7007, molecular probes: Life Technologies Corporation, Burlington, ON, Canada) was used to study the viability of the bacteria on catheters. To 1 mL NaCl (0.9%), we added 1.5 µL of each component, and 10 µL of the resulting solution was placed directly onto the longitudinally cleaved catheter pieces. The pieces were then left in the dark at room temperature for at least 15 minutes before being studied with CLSM. Catheter pieces were placed in a flattening device consisting of two stainless steel plates screwed together around the sample and pieces of glass. An Eclipse TE2000 inverted CLSM (Nikon Corporation, Tokyo, Japan) was used with a 60× oil-immersion objective and a numerical aperture of 1.4. Green fluorescence was provided by an Ar laser

(488 nm laser excitation), and red fluorescence was emitted by a G-HeNe laser (543 nm laser excitation).

RESULTS

BACTERIA RECOVERED BY CULTURING

The PD catheter pieces were cultured under aerobic and anaerobic conditions in semisolid TH or FTM agar and studied daily over 3 months for growth. Most isolates (82%) were recovered within 2 – 14 days; only 3 isolates (4%) appeared later than 27 days. Bacteria growing in association with the catheter were plated on blood agar, and each colony type was identified using standard microbiology methods and 16S rRNA gene sequencing. As reported in Table 1, bacterial growth was detected on 11 of 17 catheters and resulted in 71 isolates. Of those

TABLE 1
Bacterial Species Found on Peritoneal Catheters^a in
15 Patients Without Symptoms of Infection and in
2 Patients with Infection

Pt ID	Growth ^b		Bacterial species isolated
	Aerobic	Anaerobic	
No symptoms of infection			
A	+	+	<i>Staphylococcus epidermidis</i>
B	–	+	<i>Micrococcus</i> species
	+	+	<i>S. lugdunensis</i> , <i>S. warneri</i>
C	+	–	<i>S. epidermidis</i>
D	+	–	<i>Corynebacterium</i> species
E	+	+	<i>S. epidermidis</i> , <i>Propionibacterium acnes</i>
	+	–	<i>S. warneri</i>
F	+	+	<i>S. epidermidis</i>
G	–	+	<i>S. warneri</i>
H	+	+	<i>Proteus mirabilis</i>
	–	+	<i>P. acnes</i>
I	+	+	<i>P. acnes</i>
	+	–	<i>S. epidermidis</i> , <i>Streptococcus</i> <i>sanguinis</i> , <i>Rothia mucilaginosa</i>
	–	+	<i>S. lugdunensis</i>
J, K, L, M, N, O	–	–	
Infection present			
P	–	+	<i>P. acnes</i>
Q	+	+	<i>S. aureus</i>

Pt = patient.

^a Diagnostics based on 16S rRNA gene analysis of isolates recovered on blood agar.

^b + = growth; – = no detectable growth.

71 strains, 37 were obtained after anaerobic culture (mostly in TH). *Micrococcus* species were recovered only under anaerobic conditions, as were some strains of *S. warneri*, *Propionibacterium acnes*, and *S. lugdunensis*. The other 34 isolates were obtained through aerobic culture (mostly in FTM) and *Corynebacterium* species, *Streptococcus sanguinis*, and *Rothia mucilaginosa* were detected only under aerobic conditions, as were some strains of *S. epidermidis* and *S. warneri*. These results indicate the importance of using both aerobic and anaerobic culture conditions to detect all bacteria.

Of the 17 catheters, 6 were culture-negative. From the remaining 11 catheters, the species identified were *S. epidermidis*, *S. aureus*, *S. lugdunensis*, *S. warneri*, *P. acnes*, *Proteus mirabilis*, *Strep. sanguinis*, *R. mucilaginosa*, *Corynebacterium* species, and *Micrococcus* species. Most of the isolates found were CoNS species (*S. epidermidis*, *S. lugdunensis*, *S. warneri*), which were recovered from 7 of the culture-positive patients; *P. acnes* were obtained from 4 of those patients. As can be seen in Table 1, 7 of the patient catheters (A, C, D, F, G, P, and Q) were colonized by a single species, *S. epidermidis*, *Corynebacterium* species, *S. warneri*, *S. aureus*, or *P. acnes*. At the other extreme was patient I, whose catheter was colonized by a 5-species consortium (*P. acnes*, *S. epidermidis*, *Strep. sanguinis*, *R. mucilaginosa*, and *S. lugdunensis*). In addition, 3-species consortia of *Micrococcus* species, *S. lugdunensis*, and *S. warneri*, and of *P. acnes*, *S. epidermidis*, and *S. warneri* were also found (patients B and E respectively). *Staphylococcus epidermidis*, *P. acnes*, and *S. warneri* were found to colonize catheters both as single species and as part of a polymicrobial colonization. *Rothia mucilaginosa*, *Proteus mirabilis*, *S. lugdunensis*, and *Strep. sanguinis* were found only in polymicrobial colonizations; *Corynebacterium* species and *S. aureus* were found only in single-species cultures. With regard to bacterial species, no great differences were seen in catheters from patients with and without clinical symptoms of infection, and our results showed single-species colonization by CoNS or polymicrobial colonization to be the most frequent.

DISTRIBUTION OF BACTERIA AS SHOWN BY CULTURING

To investigate the distribution of bacteria along the catheter and the species that could coexist, each catheter was divided into 1 S part and 3 I parts (I1 – I3) with I1 being closest and I3 being most distal to the peritoneum. Most bacterial growth was isolated from parts S, I1, and I2. Only on 3 catheters was bacterial growth detected on part I3. Most bacterial strains were recovered from the external side of the catheter. As Figure 1 shows,

Figure 1 — Presence of bacterial species on various parts of the peritoneal dialysis catheters, detected by microbiologic culture. S = subcutaneous part of the catheter; I1 = intraperitoneal part of the catheter closest to the peritoneum; I2 = intraperitoneal part of the catheter farther away from the peritoneum; I3 = intraperitoneal part of the catheter most distal to the peritoneum; *S. epi* = *Staphylococcus epidermidis*; *M. spp* = *Micrococcus* species; *S. lug* = *Staphylococcus lugdunensis*; *S. war* = *Staphylococcus warneri*; *C. spp* = *Corynebacterium* species; *P. acn* = *Propionibacterium acnes*; *P. mir* = *Proteus mirabilis*; *R. muc* = *Rothia mucilaginosa*; *S. san* = *Streptococcus sanguinis*; *S. aur* = *Staphylococcus aureus*.

exclusively external colonization was seen in 6 of the 11 culture-positive catheters; no catheter was colonized solely on the luminal side.

On the catheters from symptom-free patients, bacterial growth showed that most were generally colonized in 3 or 4 parts, had both luminal and external growth, and had polymicrobial colonization (catheters B, E, and I). The catheters with the smallest amount of growth usually harbored fewer species—often just one. The catheters from infected patients differed in the amounts of bacteria harbored: one harbored external *P. acnes* on the I2 part; the other was colonized by *S. aureus* on both the luminal and the external sides, which was found on 3 parts (S, I1, I2).

The consortia found on the same catheter parts included *S. lugdunensis* and *S. warneri* (patient B) and *P. acnes* in combination with one other species [*S. epidermidis* (patient E) or *Proteus mirabilis* (patient H)], with 2 other species [*S. epidermidis* and *S. warneri* (patient E) or *S. epidermidis* and *Strep. sanguinis* (patient I)], and with 3 other species [*S. epidermidis*, *S. lugdunensis*, and *R. mucilaginosa* (patient I)]. Our results clearly indicate bacterial coexistence on the catheter, with *P. acnes* and *S. epidermidis* in particular being able to coexist with a range of different species.

DISTRIBUTION OF BACTERIA AS SHOWN BY DIRECT MICROSCOPY

As a complement to culturing, catheter pieces were studied directly using CLSM after LIVE/DEAD *BacLight* staining. Approximately one quarter of the total catheter surface was studied using microscopy, which showed the growth of bacteria to be rather limited. Microscopy found bacteria on 9 catheters (B, C, F, H, I, J, N, O, and Q). Of those 9, 3 were negative on culturing (J, N, O), suggesting that the bacteria were unable to grow in our media. The catheter parts most associated with the presence of bacteria were the S ($n=6$) and I1 ($n=5$) parts, as had been the case for bacterial isolates obtained in cultures. On the I2 parts, bacteria were detected on 3 catheters; only 1 catheter showed the presence of bacteria on the I3 part. Bacteria were found both on the external and luminal sides of the catheter pieces, and in contrast to culturing, most findings (56%) came from the luminal side.

Bacteria were heterogeneously spread along the catheter, and LIVE/DEAD *BacLight* staining showed most of them to be green and therefore viable. Very few red-stained bacteria (corresponding to membrane-compromised or dead cells) were observed.

Microscopy revealed the presence of bacteria, but showed no heavy biofilms. Bacteria were generally

clustered in small groups, examples of which can be seen in Figure 2(A–C). The total biomass was low even though the bacteria were flushed in glucose-rich dialysis fluid and bathed in host proteins, which should provide necessary nutrients. A few bacteria were surrounded by a matrix-like substance [Figure 2(A)]. Evidently, the bacteria were strongly adhered to the surface, or they would have been flushed away by daily exchanges of dialysis fluid. Most bacteria detected were rod-like [Figure 2(A,B)], but cocci [Figure 2(C)] were also found, possibly corresponding to *P. acnes* and staphylococci found in the bacterial cultures. In addition to bacteria, red [Figure 2(E)] and white [Figure 2(F)] blood cells were observed. The catheter surface was generally seen to be coated with a deposit, possibly corresponding to proteins derived from the peritoneal fluid [Figure 2(D)].

PHENOTYPIC VARIATION OF ISOLATED BACTERIA

Phenotypic variation between strains of the same species recovered from the same or different catheters was studied using antibiotic susceptibility patterns. The *S. epidermidis* strains showed a varied pattern of antibiotic sensitivity, ranging from sensitive to all of the tested antibiotics to resistant to 5 of the tested antibiotics, indicating a varied phenotypic pattern (data not shown). This pattern could be seen even for strains isolated from the same patient. Phenotypic differences with respect to antibiotic susceptibility could also be seen in the strains isolated from *P. acnes* and *Proteus mirabilis* (data not shown).

DISCUSSION

In addition to PD catheters, indwelling medical devices such as central venous catheters, urinary catheters, contact lenses, mechanical heart valves, and orthopedic devices can attract biofilms (19). Although hundreds of species form natural biofilms in the body (20), the films on devices are often composed of one or a few bacterial species (15,16). In the present study, 15 PD catheters were removed before renal transplantation—that is, from patients with no symptoms of infection—and 2 were removed because the patients had clinical signs of infection. After examination using both CLSM and culturing, 3 catheters, all from symptom-free patients, showed no signs of bacteria. However, most of the catheters—14 of 17 (82%)—were found, by one or both investigation methods, to contain bacteria. Of the 14 catheters, 6 were positive by both methods, 5 showed only culture growth, and 3 were positive only by CLSM. Those findings accord with other findings of the presence of viable microbial

Figure 2 — Confocal laser scanning microscopy images of peritoneal dialysis catheters. On the catheters, (A,B) rod-like bacteria, some of which were imbedded in (A) a matrix-like substance, and (C) cocci could be found. Most often, (D) a deposit, possibly corresponding to proteins, could be seen on the surface. (E) Red and (F) white blood cells could also be detected. Bacteria were stained using the LIVE/DEAD *BacLight* bacterial viability and counting kit (Life Technologies Corporation, Burlington, ON, Canada), and most were green, indicating live bacteria; few bacteria were red, indicating compromised membranes or death.

biofilms on 81% of PD catheters removed before renal transplantation in a study by Gorman *et al.* (14). Other studies have also shown the presence of biofilms on catheters removed from patients without clinical signs of infection upon renal transplantation (13) or membrane failure and abdominal hernia repair (12).

Using CLSM, catheters were shown to be sparsely and heterogeneously colonized by bacteria, and no substantial differences were seen between catheters obtained from patients with and without signs of infection. The results revealed one group of patient catheters that were culture-positive without bacteria being found using CLSM. Although it cannot be completely ruled out that those catheters became contaminated during handling, contributing to the positive culture result, the most likely explanation for the observation is that, because of the relatively small area analyzed, it was more difficult to detect the sparsely distributed bacterial colonies with CLSM than with culturing. In addition, the conditioning film formed on the catheter could have concealed the presence of bacteria. LIVE/DEAD *BacLight* uses SYTO 9 to stain all bacteria green; by contrast, propidium iodide (red) enters only membrane-compromised or

dead bacteria. In damaged or dead cells, propidium iodide reduces the SYTO 9 fluorescence, and thus viable cells appear green, while those that are damaged or dead appear red. Despite the fact that most bacteria on the PD catheters appeared green (that is, viable), one catheter group was CLSM-positive, but no bacterial growth was detected in culture even though both aerobic and anaerobic culture conditions and 2 complex media (FTM and TH, which would be expected to support the growth of a wide range of bacteria) were used. That finding might be explained by the presence of bacteria that were non-cultivable under the conditions used in this study—a phenomenon that has previously been described (21). Thus, it can be concluded that microscopy and culture techniques complemented each other in the present work.

It is worth noting that, in many studies of peritonitis, the atmospheric conditions for microbial growth are not mentioned (2,4,6–8,12), and it is therefore difficult to compare our results with those reported by others. In our study, half the isolated strains were recovered using aerobic culture conditions, and half, using anaerobic culture conditions. Some strains, including *Micrococcus*

species, *S. warneri*, *P. acnes*, and *S. lugdunensis*, were recovered only under anaerobic conditions, and some strains of *S. epidermidis*, *Corynebacterium* species, *S. warneri*, *Strep. sanguinis*, and *R. mucilaginosa* were recovered only under aerobic conditions. Our results indicate the importance of using aerobic and anaerobic culture conditions both—as recommended by the International Society for Peritoneal Dialysis (17)—to gain a more complete picture of microbial colonization.

To identify the bacterial species, we used 16S rRNA gene sequencing. Based on that method, the most common colonizing bacterium was *S. epidermidis*, found on 5 of 11 culture-positive catheters. On a further 2 catheters, we found CoNS, identified as *S. warneri* and *S. lugdunensis*. That result is consistent with results in studies using culture techniques, which reported *S. epidermidis* or CoNS as the most common cause of peritonitis (6–8). Thus, *S. epidermidis* appears to have a dual nature, being the most common cause of infection, but also able to exist in balance with the host immune system without giving rise to clinical signs of infection (22). Although the reasons for that duality are not known, it seems likely that if the balance were to change, the presence of those bacteria might be a risk factor for the development of peritonitis. Furthermore, in terms of tolerating the major classes of antibiotics used in clinical practice, *S. epidermidis* showed a high degree of phenotypic variation. Antibiotic resistance is known to be widely spread in *S. epidermidis* strains. In a study of 75 peritonitis patients, 53% of the *S. epidermidis* strains isolated were methicillin-resistant, and similar levels of resistance have been reported in Europe and the United States (23). Phenotypic variation such as the antibiotic resistance seen here is thought to be promoted in biofilms because of processes such as mutation and horizontal gene transfer (24).

Our results showed that no *S. aureus* was present on catheters from symptom-free patients. Generally, *S. aureus* leads to more severe peritonitis than is seen with other gram-positive bacteria (11)—although on occasion, *S. aureus* has been found on catheters from symptom-free patients (13,14). In our study, *S. aureus* was present in patient Q, who was known to have an exit-site infection with that bacterium. The bacterium was present on the external and luminal sides of the catheter and was found on parts distal to the exit site, suggesting that the catheter bacteria originated from the exit site and spread to the luminal surface of the catheter by exchange of dialysis fluid.

In the present study, we found *P. acnes* in 4 patients, and in 2 cases, the organism was widely spread over the catheter surface (S, I1–I3; and I1–I3). The presence of

P. acnes is not surprising, being that it is a commensal bacteria on the skin (25) and that touch contamination is a common route for bacterial transfer to catheters. However, studies have shown that *Propionibacterium* rarely causes infections in PD patients (26,27) and that, on occasion, it is present in effluent from symptom-free patients (27). Those results may reflect the fact that these bacteria have often not been cultured under anaerobic conditions. A similar phenomenon has been reported for hip prostheses (28,29). We often found *P. acnes* in proximity to staphylococci, and *in vitro*, *S. epidermidis* has been shown to rapidly consume oxygen (30), which could create an anaerobic environment on catheters suitable for *P. acnes*. In other constellations, similar cooperation could exist.

Our results show that single-species and mixed-microbial populations can be present on catheters removed from symptom-free patients (Figure 1). Polymicrobial peritonitis is commonly reported to be more severe than single-species peritonitis (9). We found several species in close proximity to each other on the catheter parts—for example, a 4-species mixed population comprising *P. acnes*, *S. lugdunensis*, and *R. mucilaginosa*, and a 3-species mixed consortium of *S. epidermidis*, *P. acnes*, and *S. warneri*. Thus, the patients were generally colonized by mixed gram-positive flora that have been shown to result in a significantly lower risk of catheter removal, PD dropout, and death than is seen either with mixed gram-negative or with mixed gram-positive and -negative peritonitis (9).

Our results indicate that polymicrobial colonization of PD catheters is common in symptom-free patients and that these microbial consortia might interact in ways that improve bacterial survival on medical devices, but that don't necessarily increase virulence. Further studies of bacterial cooperation in systems mimicking *in vivo* conditions need to be undertaken.

CONCLUSIONS

Bacteria were found heterogeneously spread on PD catheters removed from patients without clinical signs of infection. The most common colonizer was *S. epidermidis*, followed by *P. acnes*; both could be found as single species and as part of mixed-microbial communities, indicating that they can coexist with other species. The bacterial strains showed a wide degree of phenotypic diversity in terms of antibiotic tolerance. The techniques used in this study (CLSM and culturing under aerobic and anaerobic conditions) complemented each other, yielding a more complete picture of bacterial content than could be achieved using just one technique.

ACKNOWLEDGMENTS

We thank Ulrika Troedsson, Birgitta Liedberg, and Agnethe Henriksson for excellent technical support. We are also grateful to the staff of the surgery ward and nephrology ward 23 at Skåne University Hospital, Malmö, Sweden. We express our gratitude to Associate Professor Ann Cathrine Petersson, Skåne University Hospital, Lund, Sweden, and Professor David Beighton, KCL Dental Institute, London, UK, for sequencing bacterial strains. Helena Jeppsson and Anders Wieslander, Gambro AB, are thanked for initiating the idea and facilitating clinical contacts. The study was financed by the Knowledge Foundation (Biofilms-Research Centre for Biointerfaces), Sweden.

DISCLOSURES

No financial conflicts of interest exist.

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