

Development of Bacterial Biofilms on Silastic Catheter Materials in Peritoneal Dialysis Fluid

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● A modified Robbins' device was used to monitor the growth of bacteria associated with clinical peritonitis in peritoneal dialysis fluid. To simulate bacterial colonization and biofilm formation on peritoneal catheters, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Escherichia coli* were allowed to adhere to silastic disks and were then grown in fresh or used dialysis fluid. Adherent bacteria formed microcolonies and biofilms on silastics within 4 to 24 hours. Our data showed that colonization of the silastic disks was related to the quantity of bacteria and there were significant differences between the growth of adherent bacteria in fresh and used dialysis fluid. Adherent *S epidermidis* and *P aeruginosa* grew better in dialysis fluids than adherent *E coli*. These results suggest that *S epidermidis* and *P aeruginosa* are more likely to colonize silastic catheters and to cause catheter-related peritonitis in peritoneal dialysis patients than *E coli*.

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INDEX WORDS: Peritoneal dialysis; bacterial biofilm; silastic catheter.

PERITONITIS is a major problem and a significant reason why patients discontinue peritoneal dialysis (PD) treatment programs. *Staphylococcus epidermidis* is the most common skin bacteria associated with peritonitis due to touch contamination of the dialysis fluids during the daily changing of bags.¹ Recently, the association of *Staphylococcus aureus* and *Pseudomonas aeruginosa* with more severe catheter exit site infection and catheter loss has received attention.^{2,3} Peritonitis can be caused by hematogenous spread of *Streptococcus viridans*, by organisms of the intestine such as *Escherichia coli*, and by fungal infections in a minority of patients undergoing PD treatment.¹ Peritoneal catheters commonly become colonized by bacterial growth in the form of adherent microcolonies in biofilms. Electron microscopic studies have shown that biofilm colonization of peritoneal catheters occurs in patients with or without clinical peritonitis.^{4,5} In a rabbit model of PD, biofilm colonization of peritoneal catheters by autochthonous skin bacteria occurred within a few days of catheter insertion. However, clinical peritonitis by skin bacteria occurred in these animals only after starting the procedure of PD.⁶ Based on these clinical and experimental observations, it was postulated that bacterial biofilms on peritoneal catheters provide a microbial reservoir that at an opportune moment of decreased host defenses during dialysis, may disseminate to cause clinical peritonitis.⁷

The focus of this study was to investigate the potential of three bacteria that are associated with clinical peritonitis to colonize silastic peritoneal

catheter material and form biofilms in the presence of dialysis fluids. Using a modified Robbins' device (MRD),⁸ we examined the growth of *S epidermidis*, *P aeruginosa*, and *E coli* on silastic disks in the presence of used and fresh dialysis fluid.

MATERIALS AND METHODS

The Modified Robbins' Device

The MRD⁸ is a multiport acrylic block that is 41.5 × 2 cm in dimension. Dialysis fluid flows through a central lumen of the MRD that has a cross-sectional area of 2 × 10 mm. Each of the 25 evenly spaced sampling ports contains a sampling plug that can be removed and replaced aseptically. One silastic disk (0.5 cm² in diameter) is attached to each sampling plug. The plug is oriented in such a way that the disk lies flush with the lumen so that it does not disturb the natural flow of dialysis fluid through the MRD (Fig 1). The entire experimental apparatus is sterilized with gaseous ethylene oxide before each experiment.⁸

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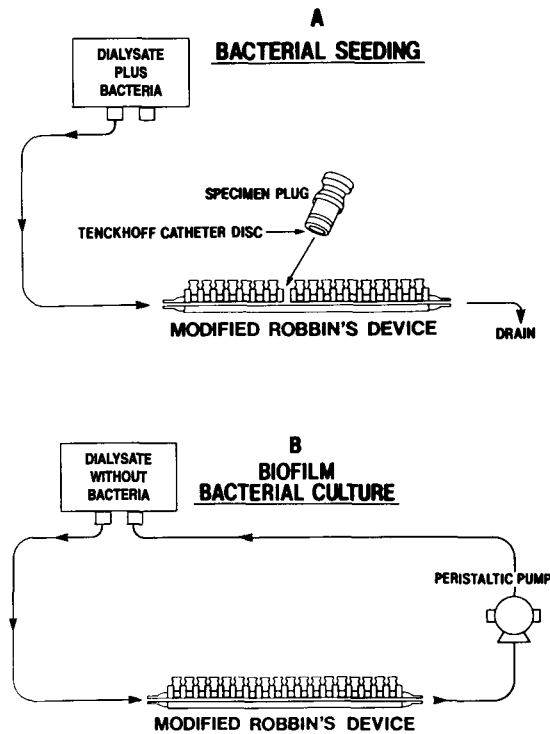


Fig 1. Diagrammatic representation of the biofilm culture system with an MRD (see text for details). The inset describes details of the sampling plug: silastic disks are attached to the tip of the plug which lie flush with the central lumen of the MRD. (A) Freshly cultured bacteria are added to the first bag of PD fluid. The fluid with bacteria is then passed through the MRD without recirculation. During this procedure, bacteria in the fluid in the planktonic phase attach to the silastic disks. This process is referred to as "bacterial seeding." (B) Dialysis fluids containing bacteria are disconnected. Bacterial culture is then continued by using a second bag of fresh or used dialysis fluid without any bacteria added. The second bag of dialysis fluid is recirculated in the MRD through a peristaltic pump as a source of nutrient for the bacteria adhered to the silastic disks. This procedure allows the growth of adherent bacterial microcolonies in biofilm on the silastic disks.⁸

Collection of Peritoneal Dialysis Effluent (Used Dialysis Fluid) From Patients

Peritoneal dialysis effluents were collected from patients that did not have clinically or microbiologically associated peritonitis. None of the patients selected for this study were receiving antibiotics. Two liters of PD fluid collected from each patient were processed by using sterile techniques. To ensure that the PD effluents did not contain bacteria, 100 mL of the dialysis fluid was aseptically removed from the dialysis bag and processed using the technique of Vas and Law.⁹ Briefly, this involved centrifuging the sample at 7,000 rpm for 30 minutes and inoculating the pellet onto blood agar, MacConkey, brain-heart infusion, and phenylethyl alcohol agar plates and into supplemented peptone broth. All growth

media were incubated for 2 to 7 days at 35°C. Isolates were identified according to standard microbiologic methods.¹⁰

Bacterial Inocula

S epidermidis, *P aeruginosa*, and *E coli*, originally isolated from the PD fluid culture of three different PD patients with peritonitis, were maintained as frozen stock cultures in skim milk at -20°C. Aliquots of the stock cultures were inoculated onto brain-heart infusion agar plates supplemented with 5% defibrinated sheep blood and grown overnight at 35°C. Bacterial colonies were then suspended in phosphate buffered saline to a density of approximately 10⁸ cells/mL using McFarland standards. The bacterial concentration was then adjusted to 10², 10⁴, 10⁶, and 10⁷ cells/mL by serially diluting the 10⁸ cells/mL sample with phosphate-buffered saline. Standard plate count techniques were used to determine the bacterial concentration of each dilution.

Experimental Design

For each experiment, 2 L of fresh or used dialysis fluid (0.5% glucose dialysis solution; Dianeal-Baxter Canada Inc. Mississauga, Ontario, Canada) was aseptically divided into two 1-L bags. The first bag was used to "seed" the silastic disks and the second bag was used to "culture" the attached bacteria. To select for the attachment of bacteria to the silastic disks, 1 mL of each bacterial dilution was added to 1 L of dialysis fluid. After gently mixing the fluid at 20°C for 2 minutes, the inoculated fluid was connected to the MRD and allowed to drain for 15 minutes (Fig 1A). During this procedure, bacteria in the planktonic phase attached to the silastic disks. We refer to this procedure as "bacterial seeding." The dialysis fluid containing the bacteria was then disconnected from the MRD.

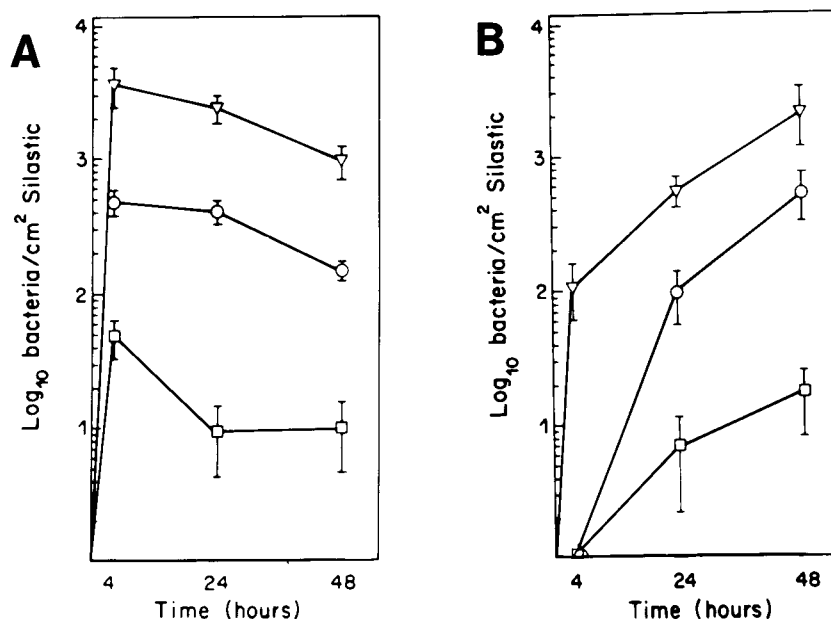
Culture of the bacteria was then continued by connecting to the MRD the second bag of fresh or used dialysis fluid without any bacteria added. The dialysis fluid was recirculated for 48 hours at 20°C by using a controlled peristaltic pump (Fig 1B). This procedure allowed the attached bacteria to form biofilms on the silastic disks.⁸

Sampling was carried out at 4-, 24-, and 48-hour intervals. At each time interval, three plugs from the MRD were rinsed with phosphate-buffered saline to wash off unattached bacteria. Biofilm bacteria attached to the silastic disks from the MRD were then scraped, sonicated, cultured in brain-heart infusion medium, and processed for colony counts as described.⁸ Bacteria were taxonomically identified by using standard methods.¹⁰

RESULTS

Inocula concentrations of 10² and 10⁴ cells/mL did not result in colonization of disks in either fresh or used dialysis fluids for *S epidermidis*, *P aeruginosa*, and *E coli*. However, inocula concentrations of 10⁶, 10⁷, and 10⁸ cells/mL provided sufficient colony counts to establish relationships between the number of bacteria attached to the silastic disks and incubation time. Figures 2 to 4 show the growth profiles of adherent bacteria

Fig 2. Colonization of silastic disks by *S. epidermidis*. Bacteria were injected into fresh (A) or used (B) PD fluids (0.5% Dianeal solution) at an inoculating dose of 10^2 , 10^4 , 10^6 , 10^7 , or 10^8 colony-forming units/mL. As there were no growths at inoculating doses of 10^2 and 10^4 colony-forming units/mL, only the results of 10^6 (boxes), 10^7 (circles), and 10^8 (triangles) colony-forming units/mL are shown. The results are expressed as a colony-forming unit per square centimeter silastic and each point represents the mean of three consecutive experiments and standard error.



grown in fresh (A) and used (B) dialysis fluid. Each datum point represents the mean and standard deviation of three consecutive experiments.

After 4 hours of incubation, the colony counts of adherent *S. epidermidis* cells grown in fresh dialysis fluid (Fig 2A) were 1.5 to 2.5 logs higher than the colony counts of adherent cells grown in used dialysis fluid (Fig 2B). The number of adherent cells in fresh dialysis fluid decreased slightly when incubation times were increased to 24 and 48 hours. This was not observed when *S. epidermidis* cells were grown in used dialysis fluid. In fact, the number of adherent bacteria increased 1 to 1.5 logs when the incubation time was increased to 48 hours (Fig 2B). Electron microscopic examination of silastic disks incubated in fresh and used dialysis fluid showed that the disk surface was covered with individual adherent coccoid cells after 4 hours of incubation. When the incubation time of the silastic disks was increased to 24 hours, the adherent cells appeared to be in the form of microcolonies covered with glycocalyx (Fig 3).

Both fresh and used dialysis fluid supported the growth of *P. aeruginosa* when inocula concentrations of 10^6 and 10^8 cells/mL were used (Fig 4). Comparisons between fresh and used dialysis fluid showed that the latter supported the growth of approximately 1 to 1.5 logs more adherent bacteria at 4 hours than the former. Elec-

tron microscopic examination supported these findings (Fig 3).

Although fresh and used dialysis fluid supported the growth of adherent *E. coli*, there was a dependence on the inoculum concentration used to seed the silastic disks. After 24 hours of incubation, an inoculum concentration of 10^6 cells/mL grew in fresh dialysis fluid; however, the same concentration did not grow in used dialysis fluid (Fig 5). An inoculum concentration of 10^8 *E. coli* cells/mL grew in both fresh and used dialysis fluid; however, the colony counts of the former were 3 logs higher than the colony counts of the latter. Moreover, growth of adherent *E. coli* cells did not significantly change after 24 or 48 hours in used dialysis fluid, suggesting that some factor(s) in the fluid might be inhibiting cell growth for this bacterium (Fig 5B).

Of the three bacterial species investigated, growth of adherent bacteria appeared to be optimal for *P. aeruginosa* and *S. epidermidis* in PD effluent. *E. coli* did not appear to grow well in either used or fresh dialysis fluid.

DISCUSSION

The growth of bacterial biofilm in dialysis fluid was investigated because autochthonous skin bacteria that attach to peritoneal catheters provide sources of contamination that possibly lead to catheter-related peritonitis in PD patients. To

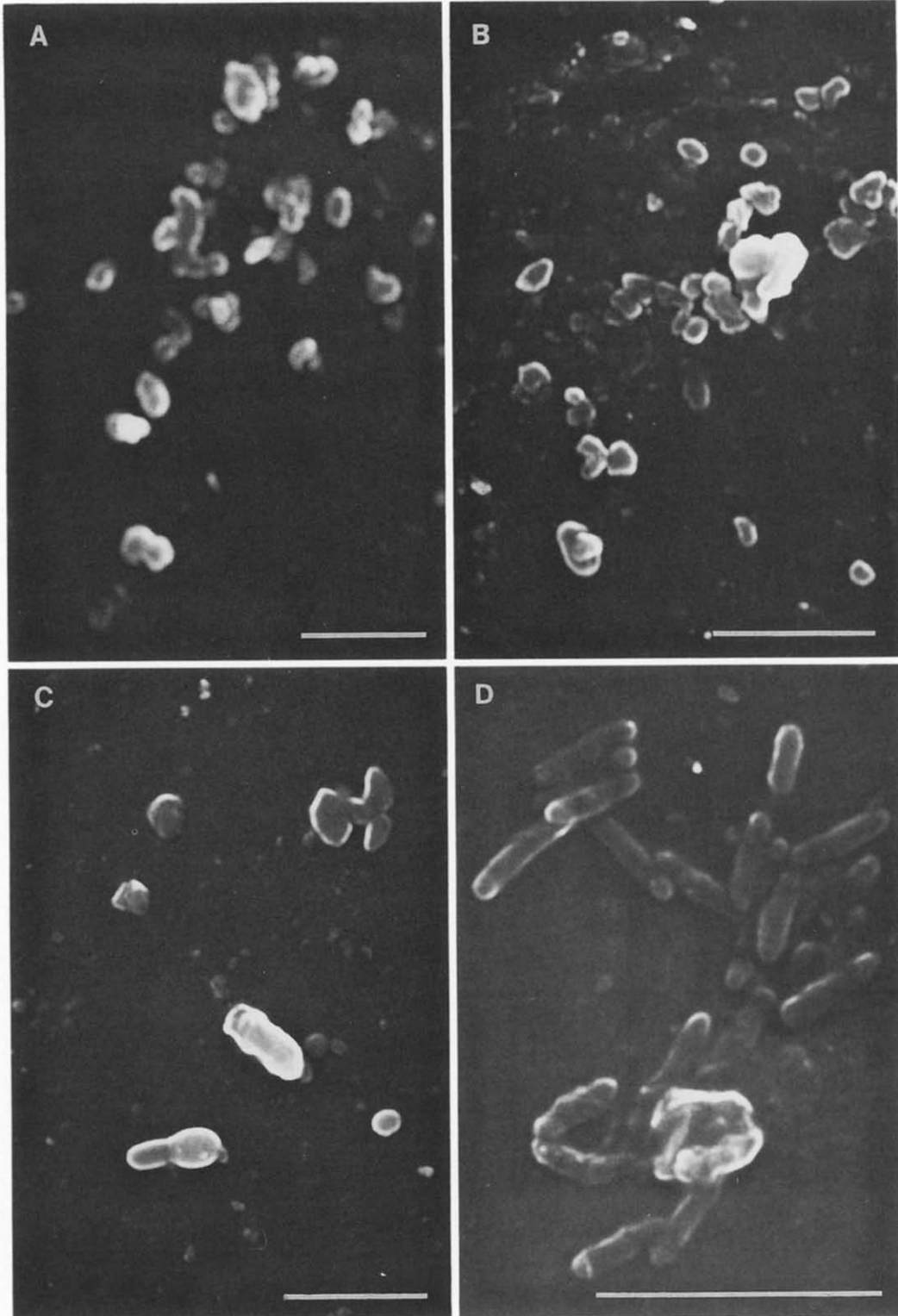


Fig 3. Scanning electron micrographs of the surfaces of silastic disks were exposed to colonization by *S. epidermidis* (A and B) or to *P. aeruginosa* (C and D). High bacteria inocula (1×10^8 cells/mL) were prepared in PD fluids (0.5% Dianeal solution) in minutes prior to irrigation with 0.5% sterile Dianeal solution. The bacteria adhere initially (4 hour) as individual cells (A and C) and then proceed (24 hours) to proliferate to form adherent microcolonies (B and D), within which they are surrounded by the dehydration-condensed residue of their developing glycolyses or biofilm. The bars indicate 5.0 μm .

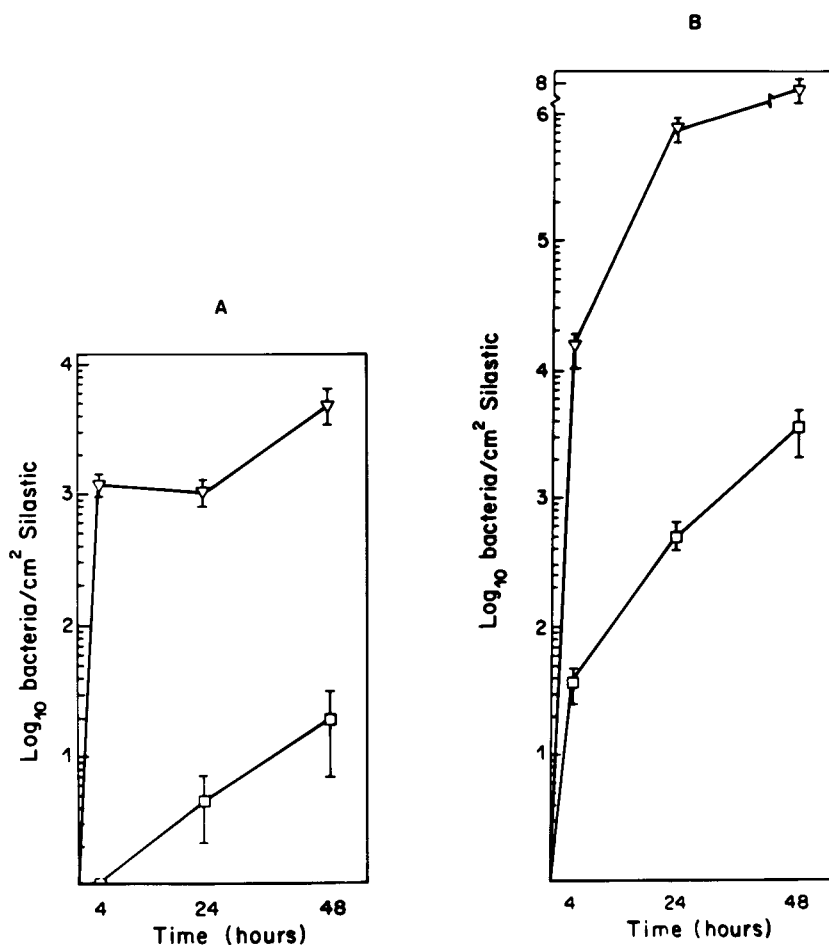


Fig 4. Colonization of silastic disks of *P aeruginosa* in fresh (A) and used (B) PD fluids (0.5% Dianeal). Inoculating dosages and symbols are the same as for Fig 2. The dose of 10^7 colony-forming units/mL is omitted.

test this hypothesis, an experimental model of biofilm bacterial culture was designed by using three bacterial species that are relevant in clinical peritonitis. In this model, the clinical setting of PD was simulated with the use of used and unused dialysate flowing through silastic disks that were made out of the same material as peritoneal catheters. Three bacteria at several inocula concentrations were used to seed the silastic disks. Bacteria that attached to silastic disks were cultured in fresh or used dialysis fluid for 48 hours. By monitoring the colony counts of adherent bacteria with incubation time, we modelled the growth of biofilm bacteria on silastic catheters in PD patients.

Of the three organisms used in this model, only *S epidermidis* is a native skin bacterium that is commonly associated with touch contamination or exit site infection.¹ *P aeruginosa* and *E coli* were used in the experimental model because they

enter the peritoneal fluid through the enteric or exit tunnel routes.^{1,11}

Since it was difficult to estimate the number of bacteria required to cause an infection in the clinical setting, our experimental model used several different inocula concentrations. The data revealed that an inoculum concentration of at least 10^6 bacteria/mL was required to colonize the silastic surfaces of our model. This is equivalent to a final concentration of 10^3 cells/mL, a realistic number of bacteria associated with touch contamination.

The experimental model was sampled after 4 hours of incubation because this was an adequate amount of time for bacteria to colonize a peritoneal catheter of a dialysis patient. We also sampled at 24 and 48 hours to determine whether poor survivors such as *E coli* would form detectable microcolonies.

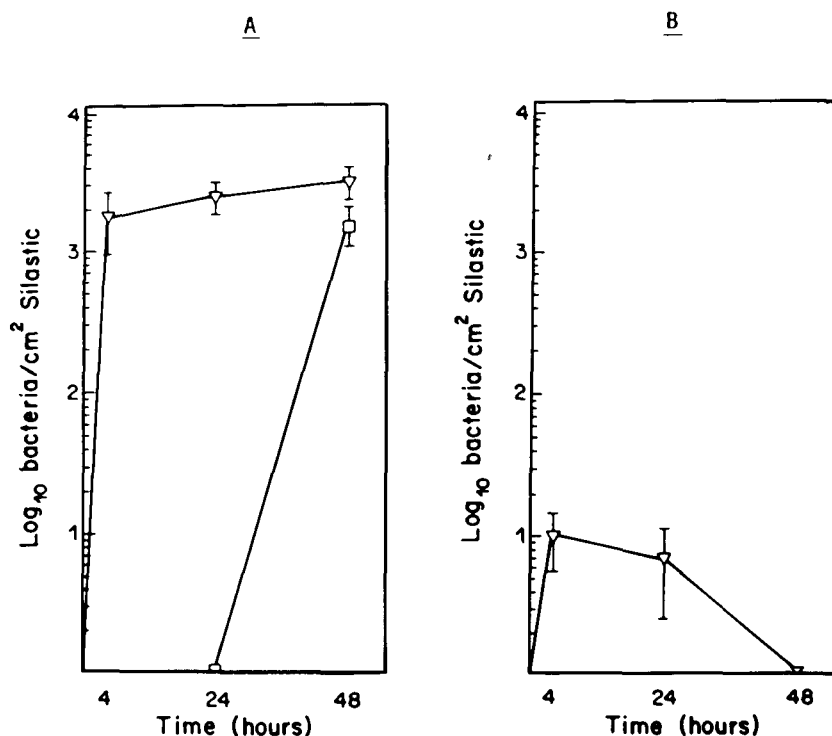


Fig 5. Colonization of silastic disks of *E. coli* in fresh (A) and used (B) PD fluids (0.5% Dianeal). Inoculating dosages and symbols are the same as for Fig 2. The dose of 10^7 colony-forming units/mL is omitted.

One difference between our model and that of the clinical setting is the temperature used to incubate the adherent bacteria. In the clinical setting, part of the peritoneal catheter subject to biofilm growth is extracorporeal and therefore at room temperature (20°C), while the remainder lies within the peritoneal cavity at body temperature (37°C). Differences in incubation temperature undoubtedly influenced the rate of biofilm growth for all bacteria examined in the study. To accommodate these differences, we incubated the samples at 20°C for 24 and 48 hours. Although bacteria in our experimental model had slower doubling times than those in the body cavity, there should be no difference in biofilm development of pure cultures as a function of growth rate.

The attachment and subsequent growth of bacteria are governed by several factors, such as nutrient availability, pH, and the presence of antibodies and complements.¹² However, these factors were not analyzed in this study as a part of the study design. One reason *S. epidermidis* grows better in fresh than in used dialysis fluid is because the former contains more glucose than the latter.¹³ Utilization of glucose by *S. epidermidis* results in the production of acetic and lactic acids.¹⁴

The production of these acids decreases pH of the dialysis fluid, resulting in cessation of bacterial growth. Colony counts of adherent *S. epidermidis* declined with increasing incubation time, presumably because most of the glucose in fresh dialysis fluid was used and/or the pH of the dialysis fluid was too low to support cell growth. Since used dialysis fluid contains more protein and less glucose than fresh dialysis fluid,¹³ growth of *S. epidermidis* readily assimilates glucose as a carbon source while other carbon sources such as proteins are not readily assimilated. Moreover, used dialysis fluid is not as affected by decreases in pH as fresh dialysis fluid because the former contains more bicarbonate and protein than the latter.¹³ Both bicarbonate and protein participate in buffering dialysis fluid from decreasing pH values caused by bacterial growth.

We have no explanation as to why more adherent *P. aeruginosa* cells grew on silastic disks than adherent *S. epidermidis* cells. Differences in metabolism may account for this variation, or perhaps *P. aeruginosa* was more resistant to host factors in used dialysis fluid than was *S. epidermidis*. More detailed studies are required to ascertain the reason for these differences.

Colonization of the silastic disks was significantly reduced when *E coli* was compared with the other two bacteria. This finding is in agreement with Bailie et al,¹⁵ who showed that *E coli* grows poorly in dialysis fluid. Reduced survival and adherence of *E coli* might be due to multiple factors such as the presence of specific host factors (eg, antibodies, complements, and other unknown factors). Moreover, it is possible that these factors prevent the adherence and/or growth of *E coli* used in dialysis fluid.

Although there are many causes of recurrent peritonitis in patients undergoing PD treatment, catheter-related infections are the major cause of catheter loss and technique failure in PD patients.^{2,3,16} The formation of a biofilm on PD catheters is postulated to be one of the factors that cause recurrent peritonitis. This is because biofilms protect bacteria from the effects of antibiotics¹⁷⁻¹⁹ and facilitate the entry of bacteria into the peritoneum through the catheter tract.⁶ Clinically, recurrent peritonitis associated with catheter-related infections can be eradicated by simultaneously removing the affected catheter and replacing it with a new one.^{20,21} Results of our study suggest that in the clinical setting, *S epidermidis* and *P aeruginosa* are more likely to cause catheter-related peritonitis as these bacteria adhere more to and form biofilms on silastic catheter materials than does *E coli*.

In conclusion, we have characterized the growth of three potential pathogens that attach to silastic disks by using an in vitro model. We found that colonization of the silastic disks was related to the quantity of bacteria introduced into the dialysis fluid. Each bacteria responded differently to used and unused dialysis solution in the expression of biofilm colonization of silastic catheter material. We found that pure cultures of *P aeruginosa* and *S epidermidis* readily adhered to silastic disks and formed biofilms. Similar results were not obtained for *E coli*. These results suggest that *S epidermidis* and *P aeruginosa* are more likely to colonize silastic catheters and to cause catheter-related peritonitis in PD patients. In addition, lower rates of *E coli* peritonitis in PD patients may be related to the observation that biofilm colonization of silastic catheter material is inhibited by dialysate. Further studies are required to investigate the specific

factors responsible for preventing or enhancing the attachment and growth of these bacteria.

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