Biofilm-related infections of cerebrospinal fluid shunts
C. A. Fux1,2, M. Quigley3, A. M. Worel4, C. Post5, S. Zimmerli2, G. Ehrlich5 and R. H. Veeh1

1Center for Biofilm Engineering, Montana State University, Bozeman, USA, 2Institute for Infectious Diseases, University of Bern, Bern, Switzerland, 3Department of Neurosurgery, Allegheny General Hospital, Pittsburgh, USA, 4Department of Pediatric Surgery, Spitalzentrum Biel, Biel, Switzerland, and 5Center for Genomic Sciences, Allegheny-Singer Research Institute, Pittsburgh, USA

ABSTRACT
Cerebrospinal fluid (CSF) shunts carry a high risk of complications. Infections represent a major cause of shunt failure. Diagnosis and therapy of such infections are complicated by the formation of bacterial biofilms attached to shunt surfaces. This study correlated the pathophysiology and clinical course of biofilm infections with microscopical findings on the respective shunts. Surface irregularities, an important risk-factor for shunt colonisation with bacteria, were found to increase over time because of silicone degradation. Scanning electron-microscopy (SEM) documented residual biological material (dead biofilm), which can further promote extant bacterial adhesion, on newly manufactured shunts. Clinical course and SEM both documented bacterial dissemination against CSF flow and the monodirectional valve. In all cases, biofilms grew on both the inner and outer surfaces of the shunts. Microscopy and conventional culture detected all bacterial shunt infections. Analyses of 16S rDNA sequences using conserved primers identified bacteria in only one of three cases, probably because of previous formalin fixation of the samples.

Keywords: Biofilm, cerebrospinal fluid shunts, colonisation, electron-microscopy, pathogenesis, shunt infections, pathogenesis

INTRODUCTION
Tens of thousands of cerebrospinal fluid (CSF) shunts are implanted annually to relieve cranial pressure caused by hydrocephalus [1]. CSF is generally shunted, via subcutaneous tubing, from the cerebral ventricle into the peritoneal cavity. The high rate of complications is illustrated by the fact that the ratio of shunt revisions to primary shunt placements reaches 3 : 1 at many institutions [2]. In 5–30% of cases, infections are a major cause of shunt failure [2,3]. Manifestations range from local disease (e.g., ventriculitis, peritonitis) to shunt nephritis or septicemia, and are associated with a higher risk of seizures, decreased intellectual performance, and a two-fold increase in long-term mortality [2].

Corresponding author and reprint requests: R. H. Veeh, Center for Biofilm Engineering, Montana State University, 366 EPS Building, PO Box 173980, Bozeman, MT 59717, USA E-mail: rick_v@erc.montana.edu

Diagnosis and treatment of device-related infections are notoriously difficult because the causative bacteria form biofilms, i.e., communities of surface-adherent organisms embedded in an extracellular matrix [4]. Diagnostic cultures of aspirates and swabs are often falsely negative, presumably because the microorganisms adhere to the surface of the device and very few cells are found in the planktonic state [5]. Biofilm eradication is limited by the antimicrobial tolerance of embedded bacteria as a result of complex adaptations to high cell-density and nutritional starvation within biofilms [6]. Furthermore, the extracellular matrix inhibits the bactericidal activity of inflammatory host cells [4].

Several studies have suggested the usefulness of PCR-based assays for the evaluation of biofilm-related disease [5,7–9] The present study had two main goals: (1) to use scanning electron-microscopy (SEM) to document the morphology and extent of biofilm formation on infected shunts; and (2) to use PCR amplification of
bacterial 16S rDNA retrieved from infected shunts to identify the bacteria present.

MATERIALS AND METHODS

In-hospital assessment

The proximal and distal tips of explanted catheters from three patients were resected aseptically, sonicated in broth, and cultured under aerobic and anaerobic conditions [10]. Organisms were identified by standard biochemical methods [11]. One (patient 1), two (patient 2; proximal and distal) or three (patient 3; proximal, distal and intermediate) segments of the shunt fixed in formaldehyde 4% v/v in phosphate-buffered saline (PBS) were available for microscopical and 16S rDNA analyses. Clinical data were collected retrospectively by independent physicians unaware of the SEM and PCR results.

Scanning electron-microscopy

Half of the fixed shunt material was bisected longitudinally, dehydrated and coated with 15–20 nm of Au/Pd using a Hummer VII Sputtering System (Anatech Ltd, Alexandria, VA, USA). Pictures were taken with a JSM-6100 scanning electron-microscope (Joel, Peabody, MS, USA) via a BLAST search. Nucleic acid extraction and purification were performed using a FastDNA SPIN Kit for Soil and a FastPrep FP120 beadbeater (BIO101Systems; QBiogene, Inc., Carlsbad, CA, USA) at the 5.5 distance of 13 mm. For patients 1 and 3, unused sterile control shunts were obtained from the manufacturers and processed in the same manner.

Analysis of 16S rDNA sequences

For each patient, total DNA was extracted from four to six longitudinally bisected shunt pieces of 0.5–1 cm in length. Nucleic acid extraction and purification were performed using a FastDNA SPIN Kit for Soil and a FastPrep FP120 beadbeater (BIO101Systems; QBiogene, Inc., Carlsbad, CA, USA), and 1 l of DNA template, 25 l AccuPrime Supermix II (Invitrogen, Carlsbad, CA, USA), and 1 l each of 20 mM forward and reverse primers were added to 11 l of nuclease free water (Sigma, St. Louis, MO, USA). After 25 cycles, the length of the PCR products was confirmed by gel electrophoresis. Purified bands were cloned using the TOPO TA Cloning Kit (Invitrogen) and the BigDye Terminator v3.1 kit (PE Applied Biosystems, Foster City, CA, USA), then sequenced on an ABI Prism 310 Genetic Analyser (PE Applied Biosystems) with a 47-cm capillary column (PE Applied Biosystems). The sequences obtained were analysed for homology to existing 16S rDNA sequences contained in the GenBank database (NCBI, Bethesda, MD, USA) via a BLAST search.

RESULTS

Clinical data

Patient 1 was a male aged 31 years with spina bifida and symptomatic hydrocephalus. During a 10-year period of recurrent infections, the patient had undergone multiple CSF-shunt exchanges and antibiotic courses. Bacterial cultures of CSF and portions of removed shunt intermittently revealed methicillin-resistant _Staphylococcus aureus_ and coagulase-negative staphylococci. Recurrent symptomatic ventriculitis was cleared only after the discovery and removal of a retained shunt fragment from a previous procedure. While inflammatory signs and coagulase-negative staphylococci had been identified in the untreated CSF (Table 1), the shunt fragments extracted and cultured following antibiotic treatment for 1 week remained sterile.

Table 1. Diagnostic features for three cases of ventriculo-peritoneal shunt infection

<table>
<thead>
<tr>
<th>Shunt location</th>
<th>CSF</th>
<th>CSF and/or shunt</th>
<th>Culture</th>
<th>Shunt PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells x 10⁶/L (PMN)</td>
<td>Protein (g/L)</td>
<td>Glucose (mM/L)</td>
<td>Gram's stain</td>
</tr>
<tr>
<td>Normal value</td>
<td>1-4 (&lt; 1%)</td>
<td>&lt; 0.45</td>
<td>≥ 1.8</td>
<td>Negative</td>
</tr>
<tr>
<td>Patient 1</td>
<td>167 (50%)</td>
<td>1.3</td>
<td>2.1</td>
<td>Leukocytes +</td>
</tr>
<tr>
<td>Patient 2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Gram-positive cocci +</td>
</tr>
<tr>
<td>Patient 3</td>
<td>1330 (83%)</td>
<td>1.2</td>
<td>2.5</td>
<td>Leukocytes ++</td>
</tr>
<tr>
<td>1st episode</td>
<td>35 (35%)</td>
<td>1.1</td>
<td>1.8</td>
<td>Gram-positive cocci +</td>
</tr>
<tr>
<td>2nd episode</td>
<td>35 (35%)</td>
<td>1.1</td>
<td>1.8</td>
<td>Gram-negative bacilli +</td>
</tr>
</tbody>
</table>

CSF, cerebrospinal fluid; PMN, polymorphonuclear neutrophils; CoNS, coagulase-negative staphylococci; MRSA, methicillin-resistant _Staphylococcus aureus_; NA, not available.

*See main text (Clinical data) for details.
Patient 2 was a male aged 48 years with symptomatic lumbar myelocompression caused by a lipomyelomeningocele. Persistent post-operative leakage of CSF from the surgical site necessitated the placement of a lumbo-peritoneal shunt. A sinus connecting skin and shunt material had developed after 6 months. Swab cultures revealed *Staphylococcus epidermidis*. Despite thorough debridement and antibiotic therapy, putrid secretion persisted and the shunt had to be removed. After antibiotic treatment of *Corynebacterium* spp., isolated from cultured shunt material, the wound healed without further complications.

Patient 3 was a female aged 17 years with a cystoperitoneal shunt draining a congenital subarachnoidal cyst, who underwent surgery for acute, non-perforating appendicitis. The peritoneal tip of the shunt was found adjacent to the inflamed appendix. Appendectomy with 5 days of antibiotic treatment was followed by a series of infectious complications ascending along the shunt: (1) an abdominal abscess surrounding the distal tip of the shunt occurred after 10 weeks and cleared following empirical antibiotic therapy for 3 weeks; (2) a retroperitoneal phlegmone occurred 1 week after termination of antibiotic treatment and cleared following empirical antibiotic therapy for another 3 weeks; (3) bacterial meningitis occurred after a further 6 weeks, with *Streptococcus salivarius* being isolated in CSF from both a spinal tap and the shunt reservoir (Table 1, 1st episode). Despite treatment for 10 days with cefepime and vancomycin, the meningitis recurred. The CSF cultures from spinal taps and the shunt reservoir indicated a polymicrobial infection (Table 1, 2nd episode). Meningitis was controlled only after shunt removal and another antibiotic course. Cultures of both the peritoneal and the intracerebral tip confirmed the polymicrobial infection. A new shunt was implanted 10 days after removal of the infected material, with no further complications.

**Scanning electron-microscopy (SEM)**

Explanted silicone shunts and unused controls (Fig. 1A) showed major surface irregularities. Cross-sections of a silicone shunt explanted after 5 years (Fig. 1B) revealed areas with intramural cavities and amorphous granular material suggestive of material decay. Both control shunts showed isolated areas with surface-adherent endoluminal biological material (Fig. 1C). Coccoid

*Fig. 1.* Material properties of ventriculo-peritoneal silicone shunts as visualised by scanning electron-microscopy. Surface irregularities such as those found on newly manufactured shunts (A) are an established risk-factor for bacterial colonisation. Intramural cavities with crystalline debris in a shunt that had been explanted after 5 years (B) suggest silicone decomposition with time. Sterile residues of biofilms inoculated during the manufacturing process – such as that found in an unused shunt (C) – may further promote bacterial attachment.
structures, c. 1 mm in diameter, and the amorphous matrix indicated the presence of a bacterial biofilm on these unused shunts. SEM documented bacterial shunt infection with biofilm formation in all three patients. Biofilms were found on inner (Fig. 2A–C) and outer (Fig. 2D–F) surfaces. For patient 3, the biofilm extended from the distal end to the proximal shunt segments. Biofilm-embedded bacteria from patient 1 were coccoid in shape, while bacteria from patient 2 showed elongated forms. For patient 3, SEM confirmed the polymicrobial infection found in conventional culture by detecting a mixture of coccoid and bacillus-like morphotypes (Fig. 2C).

Fig. 2. Scanning electron-microscopy of infected cerebrospinal fluid shunts. Biofilm formed both on the inner (A–C) and outer (D–F) surfaces of the shunts. Shunts from patients 1 (A, D) and 2 (B, E) show monomorphous bacteria, whereas the shunt from patient 3 (C, F) shows cocci as well as bacilli. The extracellular polymeric substance (E) has shrunk during the dehydration process necessary for electron-microscopy.
Analysis of 16S rDNA sequences

No confirmatory 16S rDNA was amplified from the shunts of patients 1 and 3, whereas the shunt from patient 2 provided confirmatory PCR products from the proximal (19 clones) and distal (nine clones) segments. Of the 28 clones sequenced, 26 identified most closely to Corynebacterium spp., the same genus identified by traditional culturing methods.

DISCUSSION

This study provides insight into the pathophysiology of CSF shunt infections. As in patients 1 and 2, most such infections are caused by representatives of the skin flora. S. epidermidis accounts for c. 50% of these infections, followed by S. aureus, accounting for c. 25% [13]. Shunt colonisation occurs commonly during surgery and leads to symptomatic infection within 1 month [13]. As exemplified by patient 2, bacterial growth in biofilms may delay symptoms for months or years [4]. Alternatively, delayed infections have been attributed to bacteria ascending from abdominal foci or to haematogenous seeding [14]. In patient 3, acute appendicitis preceded shunt infection. Haussler et al. [15] described secondary CSF infections in nine of 21 children with appendicitis, including all four patients with perforating appendicitis, but only two of seven patients with non-ulcerative appendicitis. However, no infections of the central nervous system occurred in another series of six children, although the appendix was perforated in three patients [3]. Physical contact of the shunt tip with the inflamed appendix or an ensuing abdominal abscess may explain the polymicrobial infection typical of intestinal perforation found in patient 3 [16]. Perforation into the gut lumen is an uncommon complication of peritoneal shunts, associated with a 45% risk of ascending cerebral infections [16].

Explanted shunts were examined by conventional culture, SEM and sequencing of PCR-amplified bacterial 16S rDNA. SEM and conventional culture detected all bacterial shunt infections, whereas analysis of the 16S rDNA sequences identified bacteria in only one of three cases. In all cases, the bacterial morphologies observed by SEM were consistent with conventional culture results. SEM revealed surface-adherent bacterial communities on all the shunts. Surface attachment is a crucial step in biofilm formation, as it triggers phenotypic changes that make bacteria more tolerant to antibiotics and host defences [17,18]. Surface irregularities, as documented on unused (Fig. 1A) and explanted silicone shunts (Fig. 1B), are known to promote bacterial attachment [13,19]. The intramural cavities with biotic debris found in a catheter explanted after 5 years (Fig. 1B) document progressive material decay, increasing the risk of colonisation with time. Previous SEM studies have documented long-term degradation of silicone [20,21], and it has also been suggested that substrates favouring bacterial growth may thereby be released [22]. Bacterial colonisation is further promoted by surface-adherent thrombi [23] or preformed biofilm (J.W. Costerton, personal communication). Even if completely sterile, this biomaterial represents a considerable risk for subsequent shunt infections. Remarkably, dead biofilm was detected on unused sterile shunts provided from the manufacturers (Fig. 1C). The current manufacturing process for shunts is finalised by shunt sterilisation using alcohol and gaseous ethylene oxide. Based on the findings of the present study, an additional step to mechanically remove any surface-adherent biological material should be considered.

The sequence of complications with patient 3 (i.e., abdominal abscess, retroperitoneal abscess and meningitis) suggests an ascending spread of bacteria along the shunt. Indeed, SEM documented bacterial biofilm along the entire length of the shunt. In other words, bacteria disseminated against the flow of CSF and overcame the monodirectional valve. Pressure within a shunt decreases in the supine position [24], and flow may be inverted during increased abdominal pressure (valsalva manoeuvre), thereby allowing the upstream spread of planktonic bacteria and detached biofilm aggregates. Biofilm formation occurred not only on the inner (Fig. 2A–C), but also on the outer tubing surface (Fig. 2D–F). This spread of non-motile bacteria along the shunt–tissue interface in the absence of a carrier fluid suggests continuous growth or bacterial migration through degrading silicone (Fig. 1C). For example, Pseudomonas aeruginosa has been shown to penetrate 0.22–0.45 μm pore-size filters [25].

Amplification of 16S rDNA has been used successfully to detect non-cultivable bacteria from
blood [26], CSF [12], middle-ear effusions [9], endocarditis vegetations [8] and joint prostheses [5,7]. However, considerable limitations of this technique have also been noted [27–29]. Several reports have described species-specific difficulties with nucleic acid extraction, particularly with staphylococci. Jaffe et al. [30] reported that a minimum of $10^9$ CFU/mL were necessary for PCR detection of target sequences from *S. aureus* with two independent methods of DNA extraction, whereas only $10^5$ CFU/mL were required for detection of *E. coli*. In the present study, shunt-adherent biofilms were disrupted by bead-beating, and DNA was extracted with a commercial kit that has been shown to be as effective as nucleic acid extraction by phenol-chloroform [31] or guanidinium thiocyanate [27].

In the present study, 16S rDNA-based molecular methods confirmed only one in three cases of bacterial shunt infection. This low sensitivity may be related to formalin-fixation as there are conflicting results concerning the efficacy of conventional PCR for the study of formalin-treated samples [32–35]. Formalin inhibits cell disruption and nucleic acid extraction by cross-linking proteins within cell membranes [32,36]. Thus, formalin may be particularly protective for matrix-embedded biofilm cells. This could explain why proteinase K, although known to be capable of solubilising formalin-fixed tissue and releasing nucleic acids [34,36], failed to improve 16S rDNA detection from samples from patient 3. For further studies, sonication before bead-beating may promote detachment of biofilm cells [5]. An alternative explanation for limited sensitivity could be the chemical modification and degradation of nucleic acids by formalin, which prevented the amplification of DNA fragments $>200$ bp in previous studies [32,34]. In some cases, nucleic acid degradation has been overcome by heat treatment to remove formalin-induced methylol groups from nucleic acids [32,34,36], and by performing nested PCR [34,35].

Overall, this study illustrates the importance of biofilm formation in the pathophysiology of bacterial shunt infections. Molecular methods hold great promise for the detection of culture-negative bacterial infections, especially in the context of sub-acute chronic infections [9] or following antibiotic treatment [12]. However, amplification of 16S rDNA failed to detect infection reliably with formalin-fixed shunt material. Modifications in DNA extraction and amplification protocols may improve sensitivity, but unfixed material should be used whenever possible.

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