**Escherichia coli** Variants in Periprosthetic Joint Infection: Diagnostic Challenges with Sessile Bacteria and Sonication

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The microbiologic diagnosis of prosthetic joint-associated infection is hampered by the phenotypic change of bacteria into a sessile and resistant form, also called biofilm. With sonication, adherent bacteria can be dislodged from the prosthesis. Species identification may be difficult because of their variations in phenotypic appearance and biochemical reaction. We have studied the phenotypic, genotypic, and biochemical properties of *Escherichia coli* variants isolated from a periprosthetic joint infection. The strains were collected from synovial fluid, periprosthetic tissue, and fluid from the explanted and sonicated prosthesis. Isolates from synovial fluid revealed a normal phenotype, whereas a few variants from periprosthetic tissue and all isolates from sonication fluid showed different morphological features (including small-colony variants). All isolates from sonication fluid were beta-galactosidase negative and nonmotile; most were indole negative. Because of further variations in biochemical properties, species identification was false or not possible in 50% of the isolates included in this study. In contrast to normal phenotypes, variants were resistant to aminoglycosides. Typing of the isolates using pulsed-field gel electrophoresis yielded nonidentical banding patterns, but all strains were assigned to the same clonal origin when compared with 207 unrelated *E. coli* isolates. The bacteria were repeatedly passaged on culture media and reanalyzed. Thereafter, most variants reverted to normal phenotype and regained their motility and certain biochemical properties. In addition, some variants displayed aminoglycoside susceptibility after reversion. Sonication of an explanted prosthesis allows insight into the lifestyle of bacteria in biofilms. Since sonication fluid also reveals dislodged sessile forms, species identification of such variants may be misleading.

The microbiologic diagnosis of prosthetic joint-associated infections (PJIs) is often based on cultures of periprosthetic tissue. However, this method is insufficient since microorganisms are attached as a biofilm on the prosthesis (5). Under this condition, bacteria change their phenotype to a sessile form and adhere strongly to the device. They may remain surface adherent even if the whole prosthesis is cultured in broth, leading to false-negative culture results. In contrast, sonication can dislodge pathogens from implants (16). Recently, Trampuz et al. (15) showed that culture of samples obtained by sonication from removed hip and knee prostheses was more sensitive than conventional culture from periprosthetic tissue. However, since this method reveals isolates in sessile forms also, showing variations in both phenotypic appearance and biochemical reactions (17), species identification of such bacteria may be false or misleading. This should be considered in routine laboratory testing. In the present report, we demonstrate the phenotypic, biochemical, and genotypic properties of *Escherichia coli* variants (including small-colony variants [SCVs]) that were involved in a PJI. Bacterial variants were isolated from periprosthetic tissue and by sonication of the explanted prosthesis. We also illustrate potential diagnostic difficulties in species identification.

**Case report.** A 67-year-old man complained of gradually increasing joint pain, fever, and impaired general condition. These symptoms started approximately 2 years after a total hip arthroplasty. They persisted for several months and were initially attributed to a rheumatic disorder. Therefore, the patient received treatment with prednisone (50 mg/day) for 14 days. Shortly thereafter, a sinus tract developed at the implant site. In the meantime, loosening of the implant was evident by X-ray. The patient was referred to our center with the diagnosis of a PJI. His personal history included recurrent urinary tract infections with *E. coli* and *Klebsiella pneumoniae*.

*E. coli* grew in culture from the synovial fluid obtained by a preoperative puncture. During surgery, the sinus tract was excised, all foreign material was removed, and a spacer was implanted. Nine biopsy samples from periprosthetic tissue were obtained before administration of antimicrobial treatment (2 g ceftriaxone intravenously, once daily). Gram-negative rods grew in eight of the nine biopsy specimens. In contrast to the rapidly growing *E. coli* isolated from the synovial fluid, the bacterial culture from periprosthetic tissue grew slowly (i.e., detection time of 4 to 12 days) and displayed various morphological characteristics in all eight biopsy specimens. In two of them, the culture displayed SCVs (Fig. 1A). In one of

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the eight culture-positive specimens, two commercial identification systems, namely, API20E and Vitek2 (bioMérieux, Marcy l’Etoile, France), misinterpreted the pathogen as Citrobacter freundii. Cultures of sonication fluid from the explanted hip prosthesis also revealed Gram-negative rods, but with five different colonial variants. In these specimens, both API20E and Vitek2 failed to identify the bacteria in two samples (“identification not conclusive”) and misinterpreted the bacteria as Salmonella enterica serovar Typhi in another sample. Antibacterial susceptibility testing revealed that all isolates were susceptible to amoxicillin, cefazolin, cefuroxime, ceftizoxime, imipenem, ciprofloxacin, and co-trimoxazole. The isolates of two biopsy samples from the periprosthetic tissue and all isolates from the sonication fluid were resistant to aminoglycosides (Fig. 1B). In contrast, isolates from preoperative puncture (sonyval fluid) and six biopsy samples from periprosthetic tissue were susceptible to gentamicin (GM10).

Materials and Methods

Bacterial isolates. Eight clinical isolates and one control strain were included in this study. They consisted of E. coli strains obtained from the synovial fluid by preoperative puncture (n = 1, SF), from periprosthetic tissue displaying an atypical morphology (SCVs, n = 2, PPT 1 and 2), and from fluid cultures of the sonicated explanted prosthesis, displaying various morphological features (n = 5, SON 1 to 5). E. coli ATCC 25922 was used as a control. Sonication was applied as described elsewhere (15).

Culture media. Both the synovial fluid and the biopsy specimens were obtained in an operation room under sterile conditions. Each specimen was collected in a sterile and separate box to avoid cross contamination and transported to the microbiology laboratory within 2 h. Specimens were inoculated in various culture media (brain heart bouillon, Columbia sheep blood, chocolate agar, and brucella agar; bioMérieux, France) and incubated aerobically and anaerobically (brucella agar, incubator filled with 10% H2, 10% CO2, and 80% N2; residual O2 is removed through the use of a palladium catalyst; incubation temperature, 35±2°C). Then, each biopsy specimen was also inoculated in a separate tube with liquid medium (brain heart bouillon; bioMérieux, France) and incubated for 14 days. Subcultures were performed on agar plates as soon as growth in the liquid medium was visible. Routine subculturing was performed from the remaining tubes on days 2 and 10 of incubation.

Selecting culture media for analyzing phenotypic properties and antimicrobial susceptibilities. The isolates from the two biopsy specimens included in this study (PPT 1 and 2) were subcultured with an inoculum of a single small colony on each of three plates of Columbia sheep blood agar, Mueller-Hinton agar, Mueller-Hinton agar supplemented with 5% sheep blood, chocolate agar, MacConkey agar, brucella blood agar, and Trypticase soy agar plates for 24 and 48 h. Incubation conditions were as described above. Based on the number and morphology of colonies, as well as the reversion time, the Columbia sheep blood agar plate was identified as the optimal culture medium.

Auxotrophisms. Impregnated disks were used on Mueller-Hinton agar plates to test auxotrophism for hemin (Sigma-Aldrich, Buchs, Switzerland), thymidine, and menadione, as described elsewhere (20).

Comparative assays. Bacteria were passaged three times using a single colony, cultured on Columbia blood agar and incubated aerobically for 24 and 48 h. Comparative assays (i.e., before and after passaging) included biochemical analysis, motility testing, and antimicrobial susceptibility testing for aminoglycosides. All results were interpreted by both an author (P.S.) and a laboratory technician. All comparative assays were performed in duplicate and repeated three times.

Biochemical analysis, motility, and antimicrobial susceptibility testing. All isolates were subjected to 70 biochemical reactions. These tests were performed by the API20E and Vitek2 identification systems (bioMérieux). In addition, oxidase production (Becton Dickinson, Franklin Lakes, NJ) and indole positivity (James reagent; bioMérieux) were tested. Motility was examined by using M medium (bioMérieux). Antibacterial susceptibility was determined by Vitek2 according to the manufacturer’s guidelines and standard disk diffusion tests according to CLSI guidelines. MICs for gentamicin and amikacin were ascertained by Etest according to the manufacturer’s guidelines (AB Biodisk, Solna, Sweden). The same inoculum was used for Etest and disk diffusion tests. Disk zone diameters were interpreted according to the CLSI guidelines. Results of Etest were rounded up to the next higher 2-fold dilution of microdilution assays and interpreted according to CLSI guidelines.

Species identification. In addition to the diagnostic systems API20E and Vitek2, 16S rRNA (500-bp) partial gene sequencing (ABI Prism 3130; Applied Biosystems Inc., Foster City, CA) was applied (7).

Given the strong sequence similarities of E. coli and Shigella spp., an agglutination assay (Remel Europe Ltd., Dartford, Kent, United Kingdom) for Shigella spp. was performed to exclude the latter (i.e., no agglutination).

PFGE and cluster analysis. Before passaging, the isolates were molecularly typed by pulsed-field gel electrophoresis (PFGE) as described elsewhere (14). In brief, after digestion of the genomic DNA by XbaI, the restriction fragments
were separated by PFGE using a temperature-controlled CHEF DR III system (Bio-Rad, Richmond, CA). Following staining with ethidium bromide, the fragments were visualized by a UV transilluminator and documented by use of a gel system (GeneFlash; Syngene, Cambridge, United Kingdom). For PFGE pattern and cluster analysis, the software GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) was applied using Pearson’s correlation coefficient and the unweighted pair-group method with arithmetic mean. To establish the clonal relatedness and diversity among our clinical isolates, we compared their PFGE pulotypes with those of 207 unrelated strains. This is, however, in line with the expected clinical presentation of joint pain, effusion, erythema, and warmth at the implant site. In contrast, our patient reported several months of symptoms that are commonly associated with a low-grade infection. This is, however, in line with the expected clinical presentation of SCV infections (9). Similarly, Roggenkamp et al. (11) reported a case of PJI caused by SCV E. coli in which the patient also presented with a chronic course. A feature of these bacterial variants that has been attributed to the pathogenesis of relapsing or persistent staphylococcal infections is their ability to persist within host cells that are not naturally phagocytic, such as epithelial cells and endothelial cells (12). SCVs are

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<th>Assay</th>
<th>ATCC 25922</th>
<th>SF</th>
<th>PPT 1</th>
<th>PPT 2</th>
<th>SON 1</th>
<th>SON 2</th>
<th>SON 3</th>
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* SF, synovial fluid; PPT, periprosthetic tissue; SON, sonication cultures.

### RESULTS

Three of the five E. coli variants isolated from sonication fluid cultures (SON 2, 3, and 5) reverted to normal phenotypes after passaging. The remaining two clinical isolates from the sonication fluid cultures (SON 1 and 4) and from the periprosthetic tissue (PPT 1 and 2) did not revert or only partially reverted. Urease activity was not detected in any of the isolates. In contrast, the isolates from the periprosthetic tissue and those from sonication fluid cultures were nonmotile (Table 1). However, after passaging, all but one morphotype from the sonication fluid cultures (SON 4) demonstrated motility. In the tested isolates, 44 of 70 biochemical reactions were classified as identical or similar (i.e., minor variability within the triplicates) to those of E. coli ATCC 25922. In addition, results for oxidase, citrate, and H_S2O reactions proved to be negative in all assays. Of the remaining 26 biochemical reactions with significant discrepancies, 9 are listed in Table 1. They represent important biochemical characteristics for the identification of E. coli. The preoperatively obtained isolate from the patient’s synovial fluid revealed expected biochemical reactions for E. coli. In contrast, the isolates from the periprosthetic tissue and from the sonication cultures were beta-galactosidase negative. Further, six of the eight isolates were indole negative and three did not produce lysozyme. Six isolates tested negative for alkaline phosphatase activity, and two tested negative for tyrosine arylamidase activity. Alkalization of lactate was not detected in four isolates, and that of succinate was missing in five isolates. Finally, the results of Ellman’s esterase assay were negative in two isolates. After the isolates were passaged three times on Columbia sheep blood agar, the majority of strains regained their expected biochemical activity (Table 1). Consequently, and in contrast to the prepassaged state, both diagnostic systems (API20 and Vitek2) identified seven of eight isolates as E. coli. One variant from sonication fluid (SON 4) failed to reveal the expected biochemical reactions. However, the E. coli species identification was confirmed by 16S rRNA gene sequencing and a negative agglutination assay result for Shigella spp.

Two isolates from biopsy samples (PPT 1 and 2) and all isolates from the sonication fluid (SON 1 to 5) were resistant to aminoglycosides (Table 2). To test whether this resistance was related to the phenotype, we passaged isolates and restested aminoglycoside susceptibility. Completely reverted phenotypes (SON 2, 3, and 5) regained susceptibility to both gentamicin and amikacin (Table 2).

PFGE of the isolates was performed prior to passaging to evaluate whether all isolates belonged to the same clone. Among the eight isolates (Fig. 2A), all strains clustered into one group, suggesting a clonal origin when isolates were compared with 207 unrelated E. coli isolates (Fig. 2B).

### DISCUSSION

Gram-negative bacteria cause about 5% of PJI episodes (21). They often develop after hematogenous seeding from an exogenous source (i.e., recurrent urinary tract infection, as was likely in our case). They typically manifest with an acute onset of joint pain, effusion, erythema, and warmth at the implant site. In contrast, our patient reported several months of symptoms that are commonly associated with a low-grade infection. This is, however, in line with the expected clinical presentation of SCV infections (9). Similarly, Roggenkamp et al. (11) reported a case of PJI caused by SCV E. coli in which the patient also presented with a chronic course. A feature of these bacterial variants that has been attributed to the pathogenesis of relapsing or persistent staphylococcal infections is their ability to persist within host cells that are not naturally phagocytic, such as epithelial cells and endothelial cells (12). SCVs are
naturally occurring subpopulations of bacteria. They differ from the normal phenotype not only in their small colony size and slow growth but also in their decreased pigmentation and hemolysis. These organisms require specific compounds to grow normally because they are unable to synthesize essential nutrients (auxotrophism). *Staphylococcus aureus* SCVs are commonly auxotrophic for hemin, thymidine, or menadione (10). Auxotrophism in *E. coli* is incompletely understood, but deficiencies in the pathway of δ-aminolevulinic acid and hemin synthesis have been discussed (11). Our analysis did not identify a specific compound to which the bacteria were auxotrophic. However, given the slow growth (i.e., 4 to 12 days) and phenotypic patterns (Fig. 1A), the presence of small colonies was evident. Yet, our phenotypes may represent not SCVs in the strict biochemical sense but rather microcolonies dislodged from biofilm: bacteria are known to form microcolonies when developing biofilm architecture (17). Nevertheless, our treatment concept for PJI in the case of SCVs is irrespective of the biochemical basis of these colonies: these phenotypic variants commonly express increased adhesion proteins (18), and most antimicrobial agents do not act on surface-adhering pathogens. Therefore, we avoid the use of a spacer in cases with difficulty-to-treat microorganisms (13, 21). Thereby, we intend to prevent bacterial adherence to the newly inserted foreign device.

Species identification of the Gram-negative rods proved to be difficult in this case. Sessile growth, encasement in a hydrated extracellular matrix, altered phenotype, and resistance to certain antibiotics are typical microbiologic characteristics of bacteria adhering to artificial surfaces (3). The extent to which these sessile bacteria regain their normal phenotype and biochemical properties when being liberated by sonication is unknown. Consequently, species identification can become difficult, in particular for Gram-negative bacilli. *Staphylococci* are often rapidly identified by use of the Gram stain, which reveals positive cocci in clusters, and by a few biochemical tests, such as catalase positivity; although SCVs of *staphylococci* can also be difficult to detect. However, species identification in Gram-negative rods is generally more elaborate. *E. coli* is motile, has beta-galactosidase and lysine decarboxylase activity, and is indole positive. Most of the clinical isolates analyzed in this study regained these properties only after reversion to normal phenotypes. Similar results, although to a lesser extent, were observed for sorbitol fermentation, lactate and succinate alkalization, tyrosine arylamidase and alkaline phosphatase activity, and the Ellman esterase assay. Taken together, these findings suggest that the sessile form of these bacteria leads to inactivation of many biochemical properties and hence makes the species identification in Gram-negative rods difficult. This observation should be considered when sessile pathogens are isolated (in particular by sonication) and routine diagnostic methods are used for the diagnosis of PJI. To our knowledge, and given the rare occurrence of such a problem, there is no established method to overcome these diagnostic difficulties. However, as illustrated in this study, repetitive subculturing in enriched media commonly leads to reversion of bacterial variants to normal phenotypes and hence facilitates genus and species identification.

The role of phenotype-dependent resistance to aminoglycosides has been previously described for SCVs of *S. aureus* (10). These variants reveal an increase in membrane potential, which is known to hinder aminoglycoside uptake (8). However, irrespective of auxotrophy, phenotypic changes as a result of differentiation from planktonic cells into a biofilm commonly accompany an increased resistance to certain antimicrobial agents (1). In our variants, aminoglycoside resistance vanished when variants completely reverted (Table 2). In nonreverted or partially reverted phenotypes, resistance persisted. We did not screen our isolates for genes known to be responsible for aminoglycoside resistance in Gram-negative rods (6). However, the evidence that the primary isolate (synovial fluid) was never exposed to this class of antimicrobial agents (1). In our variants, aminoglycoside resistance vanished when variants completely reverted (Table 2). In nonreverted or partially reverted phenotypes, resistance persisted. We did not screen our isolates for genes known to be responsible for aminoglycoside resistance in Gram-negative rods (6). However, the evidence that the primary isolate (synovial fluid) was never exposed to this class of antimicrobial agents, together with the documented clonal relatedness of all clinical isolates, suggests that the resistance is related to the phenotype (i.e., cell wall) and not to resistance genes.

Interestingly, PFGE of the isolates yielded nonidentical banding patterns among the isolates included in this study. On the other hand, microorganisms within biofilms are known to develop into organized, complex communities with structural and functional heterogeneity, resembling multicellular organisms (2). Gene expressions within such a formation are population density dependent and regulated by cell-to-cell signaling molecules (4). In addition, close contact between...
FIG. 2. (A) PFGE of the clinical isolates (before passaging) included in the study yielded nonidentical banding patterns among the eight isolates. (B) PFGE pattern and cluster analysis, comparing the clinical isolates included in the study (marked with green squares) with 207 *E. coli* strains that were not related to the case. All strains related to the case clustered into the same group, suggesting a clonal origin. For a better overview, only 42 of the 215 isolates are illustrated.
bacteria within the biofilm allows a horizontal gene transfer. Hence, it is not surprising that not all restriction patterns among our isolates were identical. However, all strains were assigned to the same clonal origin when compared with 207 unrelated E. coli isolates. This again may indicate that in the early phase, the infection was caused by one clone, presumably hematogenously seeded from the urinary tract. The phenomenon and mechanisms of bacterial gene exchange within a biofilm have shown distinct results depending on strain, medium, and biofilm growing conditions (2, 17). Here, we have demonstrated ex vivo a genetic heterogeneity of clonally related strains that were isolates from patient material.

In conclusion, sonication is a very promising method for the diagnosis of PJI. In addition, it allows insight into the bacterial lifestyle within the biofilm on patient material. However, by dislodging sessile bacteria from implants and using routine diagnostic systems, it may make species identification misleading. In clinical microbiology, this should be taken into consideration, in particular when Gram-negative rods are involved.

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