MAJOR ARTICLE

# Detection of *Staphylococcus aureus* Biofilm on Tampons and Menses Components

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**Culturing has detected vaginal *Staphylococcus aureus* in 10%–20% of women. Because growth mode can affect virulence expression, this study examined *S. aureus*–biofilm occurrence in 44 paired—tampon and vaginal-wash—specimens from 18 prescreened women, using fluorescent in situ hybridization (FISH). All 44 specimens were also analyzed for *S. aureus* by standard culturing on mannitol salt agar, which produced positive results for 15 of the 44 specimens. FISH detected *S. aureus* cells in all 44 specimens, and *S. aureus* biofilm was observed in 37 of the 44 specimens. Independent confirmation of the presence of *S. aureus* in specimens from all 18 women was also obtained by amplification, via polymerase chain reaction, of an *S. aureus*–specific nuclease gene. The results of this study demonstrate that *S. aureus* biofilm can form on tampons and menses components in vivo. Additionally, the prevalence of vaginal *S. aureus* carriage may be more prevalent than what is currently demonstrated by standard culturing techniques.**

*Staphylococcus aureus* has previously been determined to transiently colonize the nares, axillae, vagina, vulva, anus, pharynx, or damaged skin surfaces of 30%–50% of healthy adults [1, 2]. This bacterial species gained prominence during the late 1970s and early 1980s, as the pathogen responsible for menstrual toxic-shock syndrome (mTSS). Although standard culturing methods have demonstrated a 10%–20% vaginal carriage rate for this bacterial species in the general population [3, 4], only 10%–20% of these colonizing *S. aureus* isolates produce toxic-shock–syndrome toxin (designated "TSST-1" and previously known as "enterotoxin F" and as "pyrogenic exotoxin C"), the toxin usually responsible for mTSS [5–7]. It is believed that mTSS develops from a site of colonization rather than from infection [8] and that the nares and genitals (specifically the labia majora) are the common locations of carriage [2, 9]. Little is known, however, about surface colonization by *S. aureus* on either tampons or menses components. The rate of commensalism of *S. aureus* in the vagina has been reported to be higher during menses, possibly because of the altered levels of iron, pH, oxygen, carbon dioxide, redox potential, and/or osmolarity. These parameters affect not only the total number of bacteria but also the number of colonizing species [4, 10, 11] and gene expression [12].

The mode of growth of an organism—that is, whether it exists as planktonic individual cells or as surface-attached biofilm clusters—can have significant effects on many bacterial behaviors, including the expression of virulence factors and antimicrobial susceptibilities [13–15]. Thus, knowledge about the mode of growth of an organism can provide considerable insight into how that organism senses and responds to its environment. Likewise, an appreciation of the unique and mutable environments presented by humans as bacteri-

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al hosts will lead to an understanding of the complex mechanisms of host/pathogen interactions. Acquisition of accurate carriage and epidemiological data could enable better prediction of conditions leading to bacterial disease and also could help guide strategies to prevent or alleviate such diseases.

Much of the current understanding of the resident vaginal microflora has been determined by basic culturing and biochemical techniques [16]. These techniques are often selective, because of their dependence on generic growth conditions. In addition, these culturing methods are time-consuming, may significantly alter the phenotypic expression of the organism, and cannot detect nonculturable or stressed organisms [16]. Culture-independent techniques such as nucleic acid–based methods have the ability to circumvent many of these limitations, yet they are still underused in many clinical investigations.

Fluorescent in situ hybridization (FISH) is a culture-independent, RNA-DNA hybridization technique that has been used to visually identify and to study spatial distributions of bacteria at various phylogenetic levels in diverse ecological settings [17–22]. The realization that both conserved and hypervariable regions in rRNA could be used as phylogenetic markers has led to the development of this technique [23]. Both 16S rRNA– and 23S rRNA–targeted, fluorescently labeled oligonucleotide probes have been designed that allow microscopic visualization of bacteria at various phylogenetic levels and that provide information about the cells' mode of growth [24, 25]. For example, planktonic cells should generally appear as discrete units not associated with a solid substratum, whereas biofilm bacteria should appear in surface-attached cell clusters. FISH is quickly becoming an extensively used cytochemical staining technique because of its specificity, sensitivity, versatility, and cost-effectiveness [26].

The present study used FISH for the detection of *S. aureus* in clinical tampon and clinical vaginal-wash samples from healthy, menstruating women, in an attempt to observe phenotypic modes of growth of *S. aureus* in the vagina during menstruation. Culturing, polymerase chain reaction (PCR), and DNA-sequencing methods were also used to confirm the presence of *S. aureus,* enabling a comparison of the sensitivity of the various methods applied to the same specimens.

## SUBJECTS, MATERIALS, AND METHODS

***Clinical-study design.*** All participants in the present study were healthy women 18–45 years old who resided in Arizona, had a menstrual cycle of 21–35 days, and were menstruating (day 2, 3, or 4 of the menstrual cycle) at the time of sampling. In addition, the subjects did not meet any of the exclusion criteria (see the Appendix). Thus, 138 women were reviewed (visit A) for eligibility to participate in this partial, single-blind study. Of the 117 women subsequently screened for the presence of *S. aureus* (by culture from a vaginal swab administered during visit B; table 1), 18 (10 culture negative and 8 culture positive) were then selected to provide tampon and vaginal-wash specimens for analysis by FISH. These 18 women provided such specimens on either 1 occasion (visit C) or 2 occasions (visits C and D), resulting in the collection of 22 pairs of tampon and vaginal-wash specimens (table 1). All 18 subjects who provided specimens were monitored for the presence of vaginal *S. aureus,* by culturing on $\geqslant$2 occasions, to ensure that both culture-negative and culture-positive women were included in the study.

Informed consent was obtained from all volunteers who participated in the present study, according to the guidelines for clinical research that have been established by the US Department of Health and Human Services. In addition, the human-experimentation guidelines of the authors' institutions were followed during the clinical research.

***Sampling protocol.*** Paired specimens from each woman consisted of a tampon (Tampax Regular; Procter & Gamble) and a vaginal wash, both collected 3 h after tampon insertion on day 2, 3, or 4 of the menstrual cycle. Tampon and vaginal-wash specimens were immediately subsampled by use of sterile swabs (culturette tubes; Becton Dickinson), for standard culturing, to determine the presence of *S. aureus.* Immediately after being swabbed for culture analyses, the tampon and vaginal-wash specimens were placed in either 50%–70% ethanol or 2% formaldehyde, in sterile 50-mL centrifuge tubes, and were stored at −70°C until transported for initial FISH analyses.

***Culturing protocol.*** Within 24 h after collection, swab subsamples of the tampon and vaginal-wash specimens were streaked on mannitol salt agar (Difco) and were incubated aerobically overnight at 37°C. Identities of presumptive *S. aureus* isolates were confirmed at Hill Top Research, Inc., by the Staphaurex slide coagulation test (Murex Diagnostics) and Gram stain, and were reconfirmed by the State of Montana Veterinary Diagnostic Laboratory, by the Analytical Profile Index (API)–staphylococcus and API-streptococcus tests (bioMerieux Vitek).

***Sample preparation.*** The outermost fibers from whole, formaldehyde-fixed tampons were aseptically removed from the sides of the tampon and from the end proximal to the cervix and were placed in filter-sterilized, deionized water. These fibers were used because preliminary experiments had revealed a dramatic decrease in both human and bacterial cells on interior parts of exposed tampons (data not shown). The fibers were teased apart with sterile tweezers and were excised with a sterilized razor blade, were rinsed once with filter-sterilized, deionized water, and then were placed in 25 $\mu$L of lysostaphin (100 $\mu$g/mL) (Sigma) for 10 min at 37°C, to permeabilize fiber-associated bacteria. Preliminary experiments were conducted to ensure that, under these treatment conditions, optimal cell permeabilization was achieved without cell lysis. Fibers were

**Table 1. Samplings and corresponding culturing results for isolation of *Staphylococcus aureus.***

| | Visit B | | Visit C[a] | | | Visit D[a] | | |
|---|---|---|---|---|---|---|---|---|
| Woman (specimen[s]) | Date | Swab result | Date | Tampon result | Vaginal-wash result | Date | Tampon result | Vaginal-wash result |
| 1 (217, 240) | 2/29/00 | + | 3/27/00 | − | + | 5/16/00 | + | + |
| 2 (105, 246) | 3/6/00 | + | 4/4/00 | − | + | 5/23/00 | + | + |
| 3 (207) | 3/16/00 | + | 5/18/00 | + | + | NA | NA | NA |
| 4 (128) | 4/17/00 | + | 5/15/00 | − | + | NA | NA | NA |
| 5 (102, 253) | 3/1500 | + | 4/13/00 | + | + | 5/12/00 | − | − |
| 6 (135, 254) | 3/9/00 | + | 4/4/00 | − | + | 5/24/00 | − | + |
| 7 (214) | 4/10/00 | + | 5/4/00 | − | − | NA | NA | NA |
| 8 (176) | 3/23/00 | − | 4/19/00 | + | + | NA | NA | NA |
| 9 (115) | 3/22/00 | − | 5/18/00 | − | − | NA | NA | NA |
| 10 (231) | 3/16/00 | − | 5/19/00 | − | − | NA | NA | NA |
| 11 (232) | 3/21/00 | − | 4/19/00 | − | − | NA | NA | NA |
| 12 (157) | 3/1/00 | − | 5/23/00 | − | − | NA | NA | NA |
| 13 (151) | 2/29/00 | + | 4/18/00 | − | − | NA | NA | NA |
| 14 (119) | 3/2/00 | − | 3/30/00 | − | − | NA | NA | NA |
| 15 (161) | 3/8/00 | − | 4/4/00 | − | − | NA | NA | NA |
| 16 (140) | 3/15/00 | − | 4/18/00 | − | − | NA | NA | NA |
| 17 (159) | 3/21/00 | − | 4/19/00 | − | − | NA | NA | NA |
| 18 (168) | 3/20/00 | − | 4/19/00 | − | − | NA | NA | NA |

**NOTE.** +, Isolation of *S. aureus;* −, no isolation of *S. aureus;* NA, not applicable (subject did not return for visit D/specimen not obtained).

[a] All specimens also were analyzed by fluorescent in situ hybridization (table 3); in addition, all vaginal-wash specimens also were analyzed by polymerase chain reaction (table 3 and figure 1).

then washed twice in 500 $\mu$L of filter-sterilized, deionized water for 5 min and subsequently were hybridized in suspension in microcentrifuge tubes.

Vaginal-wash specimens stored in 50%–75% ethanol (30–50 mL) were concentrated by centrifugation (4600 *g* for 10 min at 4°C) and were washed 2 times with 1.5 mL PBS, 2 times with 1.5 mL filter-sterilized, deionized water, and 3 times with 1.5 mL of 50% ethanol (with a 20-min incubation step between each centrifugation). The pellets were then resuspended in 1–4 mL of 80% ethanol. Cell suspensions were dried onto multiwell printed microscope slides (Erie Scientific), were permeabilized by being covered with lysostaphin solution, as described above, and then were gently rinsed with filter-sterilized, deionized water. Bacterial cultures used as control samples for FISH analyses were harvested from growth media, were repeatedly washed in filter-sterilized, deionized water, were stored in 80% ethanol, and then were treated as has been described above for the vaginal-wash specimens.

***FISH probes.*** To complement a previously published 16S rRNA–targeted, *S. aureus*–specific probe (Saur72; *Escherichia coli* bp 72–93 [5′-AGAGAAGCAAGCTTCTCGTCCG-3′]) [27], an additional 23S rRNA–targeted, *S. aureus*–specific probe (Saur327; *E. coli* bp 327–349 [5′-GATTCGTCTAATGTCGTCCTTTG-3′]) was designed and validated, as part of the present study. The 23S rRNA–targeted probe, Saur327, specific for *S. aureus* was designed by use of the GenBank database of the National Center for Biotechnology Information [28]. The Basic Local Alignment Search Tool (BLAST) was used to identify a 23-bp DNA sequence, putatively unique to *S. aureus,* within a 23S rRNA variable region that had previously been shown to afford species-level identification of another Gram-positive bacterium, *Enterococcus faecalis* [25]. In addition to the theoretical *S. aureus* specificity that the GenBank database suggested for probe Saur327, empirical specificity of Saur327 was validated by use of 5 strains of *S. aureus,* 14 strains of other staphylococci, 1 strain of *Micrococcus luteus,* and 2 species of lactobacilli (table 2). A bacterial domain–level, 16S rRNA–targeted probe (EUB338; *E. coli* bp 338–355 [5′-GCTGCCTCCCGTAGGAGT-3′]) [25] and a nonsense probe (anti-Saur835; no target; 5′-AGTGCTAAGTGTTAGGGGGT-3′) were used as positive and negative control probes, respectively, throughout the validation and specificity testing of Saur327. Similarly, the *S. aureus* specificity and signal strength of Saur72 were confirmed empirically by testing it against 5 strains of *S. aureus, Staphylococcus epidermidis, Streptococcus gordonii* (CH1), *Streptococcus mutans, Pseudomonas aeruginosa, M. luteus,* and *Enterococcus faecalis.*

**Table 2. Results of 23S rRNA–targeted fluorescent in situ hybridization using probe Saur327 against target and nontarget organisms.**

| Species (strain) | Species-confirmation method | EUB338 | Anti-Saur835 | Saur327 |
|---|---|---|---|---|
| *S. aureus* (Harrisburg) | API/BBL | + | − | + |
| *S. aureus* (MN8) | API/BBL | + | − | + |
| *S. aureus* (unknown) | API/BBL | + | − | + |
| *S. aureus* (ATCC 25923) | API/BBL | + | − | + |
| *S. aureus* (unknown) | API/BBL | + | − | + |
| *S. caseolyticus* (ATCC 13548) | No database | + | − | − |
| *S. epidermidis* (ATCC 12228) | API/BBL | + | − | − |
| *S. epidermidis* (unknown) | API/BBL | + | − | − |
| *S. epidermidis* (80-8 #2) | API/BBL | + | − | − |
| *S. epidermidis* (unknown) | API/BBL | + | − | − |
| *S. haemolyticus* (unknown) | API/BBL | + | − | − |
| *S. hyicus* (subspecies *hyicus*) | API | + | − | − |
| *S. intermedius* (clinical isolate) | API | + | − | − |
| *S. kloosii* (unknown) | BBL | + | − | − |
| *S. lentis* (unknown) | No identification | + | − | − |
| *S. saprophyticus* (#52) | No identification | + | − | − |
| *S. saprophyticus* (ATCC 16305) | BBL | + | − | − |
| *S. sciuri* (clinical isolate) | API | + | − | − |
| *S. xylosus* (ATCC 29971) | API/BBL | + | − | − |
| *Lactobacillus crispatus* (unknown) | Not tested | + | − | − |
| *L. jensenii* (unknown) | Not tested | + | − | − |
| *Micrococcus luteus* (unknown) | Not tested | + | − | − |

**NOTE.** +, Significant fluorescent signal; −, negligible fluorescent signal.

All DNA probes were custom synthesized, fluorescently conjugated, and purified commercially, by Integrated DNA Technologies. All probes were 5′-labeled with either fluorescein isothiocyanate (FITC), Cy3, or Cy5 (Amersham Biosciences). Specifically, these fluorophores were conjugated by a reaction with the 5′-terminal hydroxyl group and formation of a covalent ether linkage with the 5′-terminal nucleotide.

***FISH protocol.*** Hybridizations were performed in triplicate for each clinical specimen, according to the procedure used by Manz et al. [29] and described in detail by Neef et al. [30], by use of 40% formamide. In brief, hybridizations were conducted for ⩾2 h at 46°C, in 10–48 μL of hybridization buffer consisting of 0.9 mol NaCl, 0.02 mol Tris-HCl (pH 7.6), 40% formamide (vol/vol), 0.01% SDS (wt/vol), and 5 ng each probe/μL. All samples were hybridized with both probe Saur72 and probe Saur327 simultaneously, with each probe being labeled with either Cy3 or Cy5 and with the 2 probes combined such that each was always distinguishable from the other. Tampon-fiber samples were hybridized with a third probe present, EUB338-FITC, to serve as a counterstain for all bacteria. Immediate posthybridization washing was conducted for 15–30 min at 46°C, by submersion in 0.5–50 mL of preheated washing buffer consisting of 0.05 mol NaCl, 0.02 mol Tris-HCl (pH 7.6), and 0.01% SDS (wt/vol). After being washed, samples were rinsed or were soaked gently for a few minutes in filter-sterilized, deionized water and then were allowed to air-dry in darkness on a glass microscope slide. In all cases, tampon fibers were transferred between hybridization/wash solutions by sterile forceps. Nonfiber samples were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma), applied at 1 μg/mL, for 10 min at room temperature in darkness and then were rinsed, and the slide was air-dried again before being observed microscopically.

***FISH controls and baseline (blank) samples.*** One of the vaginal-wash *S. aureus* cultures isolated in the present study served as the positive control sample, and various non–*S. aureus* cultures were used as negative control samples, for all FISH assessments of clinical specimens. To obtain baseline blanks for determination of autofluorescent characteristics of the processed specimens, subsamples of tampon and vaginal-wash specimens were prepared and hybridized as described above, but with no probes and counterstain added to the hybridization incubation.

Additional positive controls for all FISH analyses of tampon fibers involved unused tampon fibers that were first soaked in ethanol and that then were rinsed in filter-sterilized, deionized

**Table 3. Comparison of results of culturing, fluorescent in situ hybridization (FISH), and polymerase chain reaction (PCR), for detection of *Staphylococcus aureus* in clinical tampon and clinical vaginal-wash specimens.**

| | Culturing | | FISH | | PCR | |
|---|---|---|---|---|---|---|
| Woman (visit [specimen]) | Tampon result | Vaginal-wash result | Tampon result | Vaginal-wash result | Tampon result | Vaginal-wash result |
| 1 (C [217]) | − | + | + (B) | + (NB) | NA | + |
| 1 (D [240]) | + | + | + (B) | + (B) | NA | + |
| 2 (C [105]) | − | + | + (B) | + (B) | NA | + |
| 2 (D [246]) | + | + | + (NB) | + (B) | NA | + |
| 3 (C [207]) | + | + | + (B) | + (B) | NA | + |
| 4 (C [128]) | − | + | + (B) | + (B) | NA | + |
| 5 (C [102]) | + | + | + (B) | + (B) | + | − |
| 5 (D [253]) | − | − | + (B) | + (B) | NA | + |
| 6 (C [135]) | − | + | + (B) | + (NB) | NA | + |
| 6 (D [254]) | − | − | + (B) | + (B) | NA | + |
| 7 (C [214]) | − | − | + (B) | + (B) | NA | + |
| 8 (C [176]) | + | + | + (B) | + (B) | NA | + |
| 9 (C [115]) | − | − | + (NB) | + (B) | NA | + |
| 10 (C [231]) | − | − | + (B) | + (B) | NA | + |
| 11 (C [232]) | − | − | + (B) | + (NB) | NA | + |
| 12 (C [157]) | − | − | + (B) | + (B) | NA | + |
| 13 (C [151]) | − | − | + (B) | + (B) | NA | + |
| 14 (C [119]) | − | − | + (B) | + (B) | NA | + |
| 15 (C [161]) | − | − | + (B) | + (NB) | NA | + |
| 16 (C [140]) | − | − | + (NB) | + (B) | NA | + |
| 17 (C [159]) | − | − | + (B) | + (B) | NA | + |
| 18 (C [168]) | − | − | + (B) | + (B) | NA | + |

**NOTE.** +, *S. aureus* detected; −, *S. aureus* below level of detection; (B), *S. aureus*–biofilm cells observed; (NB), *S. aureus*–biofilm cells not observed; NA, not analyzed.

water for 10 min. The control fibers were then transferred into 3 mL of tryptic soy broth, were inoculated with *S. aureus*, and were incubated overnight at 37°C without being shaken. After being incubated, the fibers received 2 20-min washes in 50% ethanol before being stored in 80% ethanol and hybridized as has been described for the clinical tampon samples. All vaginal-wash specimens were reanalyzed in a separate location by a second technician using similar FISH procedures, to confirm the presence of *S. aureus*.

***Microscopy.*** Samples hybridized with FITC-labeled probes were mounted in 1 of the following antifade mounting solutions: ProLong antifade (Molecular Probes), VECTASHIELD mounting medium (Vector Laboratories), or component C of a LIVE/DEAD *Bac*Light bacterial-viability kit (Molecular Probes). Microscopic observations of the hybridized vaginal-wash and tampon samples were conducted by use of several different research-grade, epifluorescent microscopes, all equipped with high-numerical-aperture 100× and 60× oil-objective lenses, a charge-coupled device, and filter sets allowing separate viewing of DAPI, FITC, Cy3, and Cy5 color-emission ranges. For each field of view, a bright-field image, as well as 1 fluorescent image from each of the 4 filter sets, was observed. Because each sample was labeled with only 3 of the 4 color-emission ranges used, the 1 remaining color/filter set (i.e., DAPI [blue] or FITC [green]) was used to check for broad-spectrum autofluorescence.

***FISH image-interpretation criteria for clinical specimens.*** Three criteria were used for identifying an individual object as an *S. aureus* cell: (1) the autofluorescence check resulted in a blank image; (2) the object had a coccoid cell shape during bright-field observation; and (3) the object was positive for $\geqslant 2$ of 3 possible fluorescent signals (i.e., counterstain, Saur327 probe, and Saur72 probe). In addition, a given hybridization was first validated on the basis of its having met the following 3 criteria: (1) similar objects were absent from the baseline blank sample; (2) the negative control culture produced negligible fluorescence; and (3) the positive control sample displayed significant probe-specific signals.

Each sample received replicate assays. Specimens were

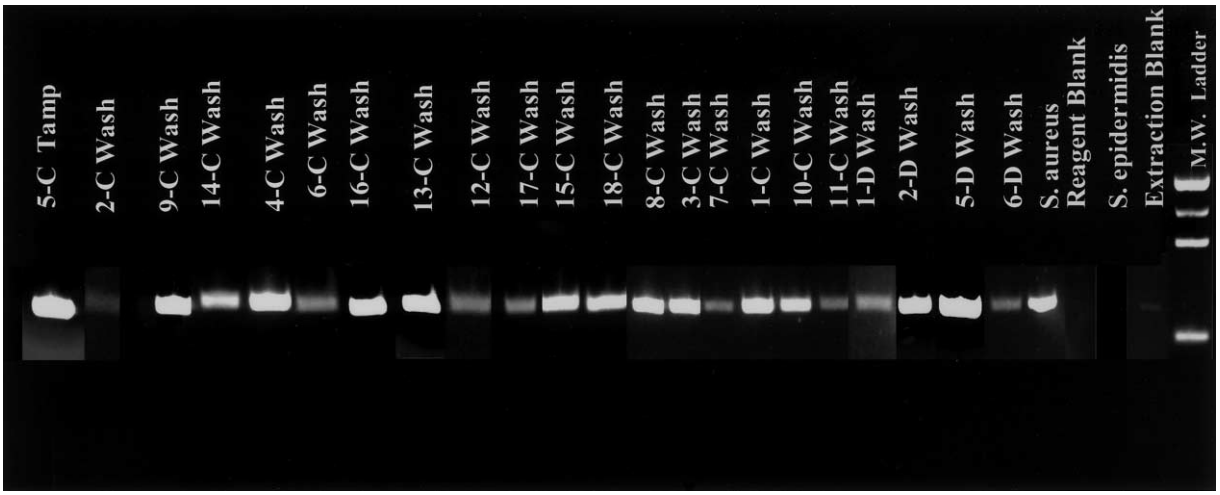



**Figure 1.** Polymerase–chain-reaction amplicons of the *Staphylococcus aureus*–specific *nucA* gene obtained from clinical specimens and from control and baseline samples (ethidium-bromide stained). Individual bands are labeled with a woman identification number and visit letter, corresponding to details provided in table 1. At the far right is a molecular-weight ladder, indicating locations for 200-, 500-, 700-, and 1000-bp bands.

deemed positive for *S. aureus* if it was detected in ⩾3 fields of view, according to the above criteria; specimens were deemed negative for *S. aureus* if no cells meeting the aforementioned criteria were observed after 4 valid replicate hybridizations were examined at high magnification.

The arbitrary criterion established for identification of *S. aureus* biofilm was an observation of ⩾7 *S. aureus* cells clustered in a surface-attached structure. In contrast, *S. aureus*–biofilm flocs were defined as unattached *S. aureus* cell clusters. Individual unattached, unclustered *S. aureus* cells were considered to be planktonic.

***PCR confirmation protocol.*** To confirm the presence of *S. aureus* in specimens from the 18 participants in the present study, PCR was used to detect an *S. aureus*–specific DNA sequence in the thermostable nuclease (*nucA*) gene [31]. The uniqueness of this *nucA* gene to *S. aureus* has been established both in previous research, by comparisons of DNA sequences versus those in the existing bacterial-genomic database (GenBank), and in the present study, by testing of 2 relevant staphylococcal species as negative controls. All 22 vaginal-wash samples, 1 tampon sample, and positive (*S. aureus*) and negative (*Staphylococcus saprophyticus* and *Staphylococcus epidermidis*) control cultures grown overnight at 37°C on blood agar were analyzed by PCR.

The selection of PCR primers was based on a published assay shown, by PCR amplification of the *nucA* gene, to be highly specific for *S. aureus* detection [31]. The forward (nucA1) and reverse (nucA2) primers, which were custom synthesized and

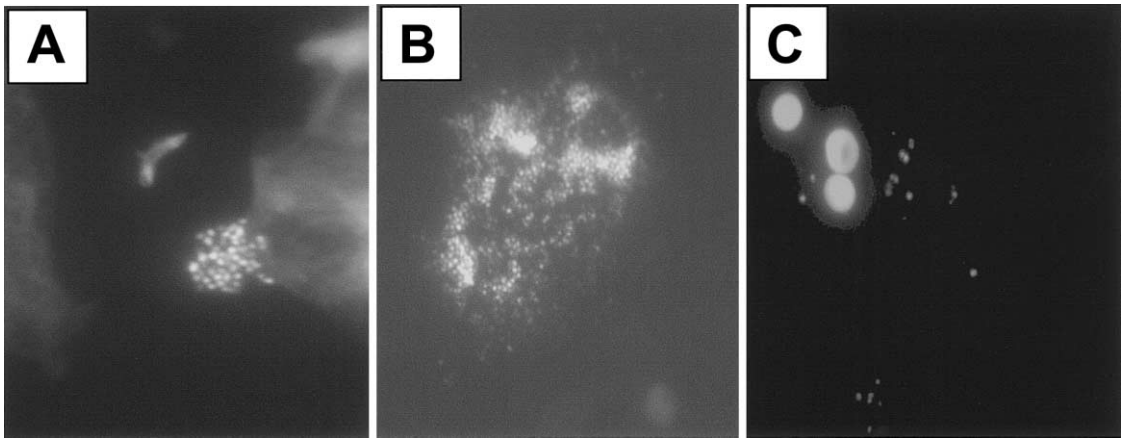



**Figure 2.** Monochromatic epifluorescent photomicrographs of clinical vaginal-wash specimens probed with Saur327-Cy3 and viewed with a 100× oil-objective lens and a Cy3-specific filter: *(A), Staphylococcus aureus* biofilm attached to an epithelial cell; *(B), S. aureus*–biofilm floc/cluster, and *(C)* planktonic *S. aureus* cells near red blood cells.

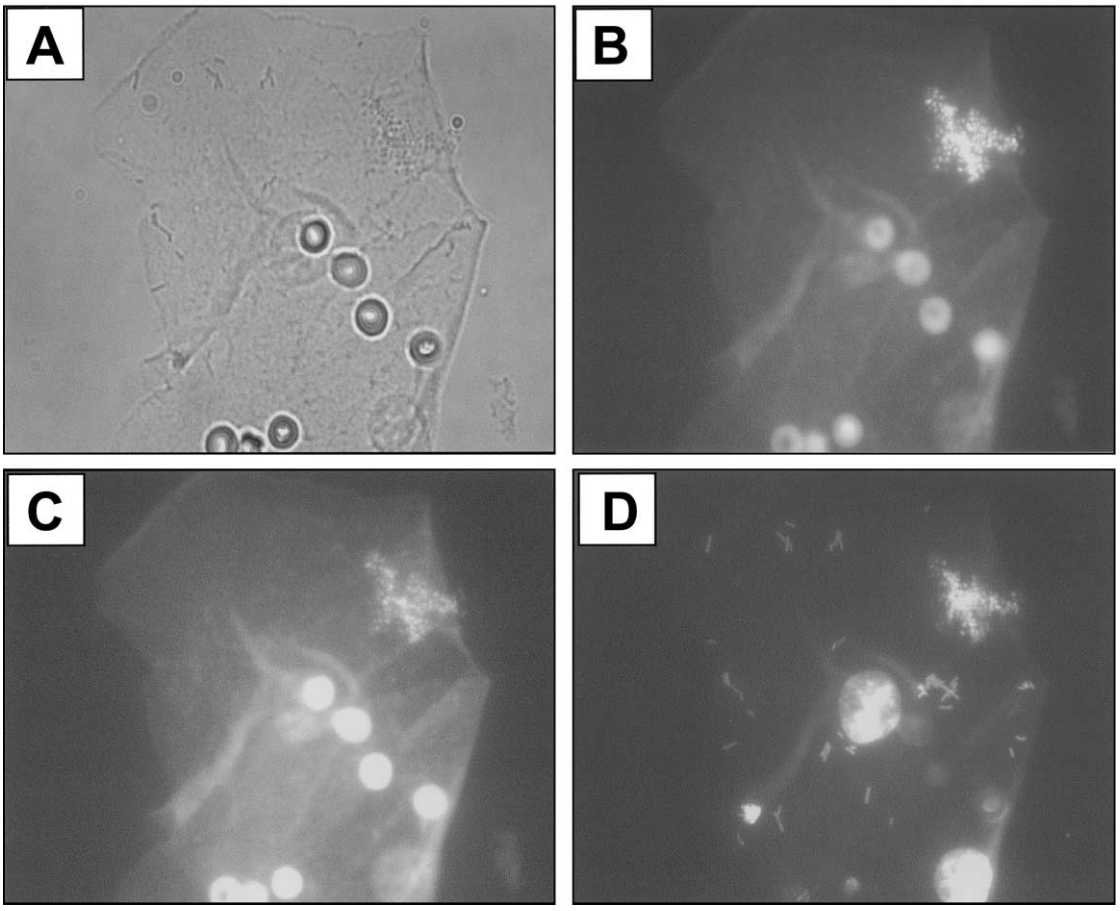



**Figure 3.** Monochromatic epifluorescent photomicrographs of a clinical vaginal-wash sample probed with Saur327-Cy3 and Saur72-Cy5 and counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI): *(A),* bright-field image; *(B)* cocci emitting Cy5 probe–conferred signal; *(C)* cocci emitting Cy3 probe–conferred signal; and *(D),* image of DAPI counterstain. Rods visible in the bright-field and counterstained images are not seen in the images of probe-conferred fluorescence; however, *Staphylococcus aureus*–biofilm cells *(upper right)* are visible in the Cy3 and Cy5 images as well as in the bright-field and counterstained images.

purified (Integrated DNA Technologies), comprised, respectively, nucleotides 48–70 (5′-GCGATTGATGGTGATACGGTT-3′) and nucleotides 303–328 (5′-AGCCAAGCCTTGACGAAC-TAAAGC-3′) of the 447-bp *nucA* gene.

DNA was extracted from the control cultures, from the 22 vaginal-wash samples, and from 1 tampon sample that previously had been analyzed by FISH. Before the DNA was extracted, the specimens were centrifuged at 10,000 *g* for 15 min at 4°C, to pellet the bacterial cells. The supernatant was discarded, and the pellet was washed with 500 mL of 10 mmol Tris-HCl buffer (pH 8) and was centrifuged at 10,000 *g* for 5 min, and the resulting supernatant was decanted. This procedure was repeated with an additional 500 mL of buffer, with the cells being given a final resuspension in a minimal volume of buffer. For specimens forming a large cell pellet, the cells were resuspended in 500 $\mu$L of buffer, a 100-$\mu$L aliquot of which was used for DNA extraction; for specimens with a small pellet, the entire pellet was used for DNA extraction. Genomic DNA was extracted by use of an UltraClean DNA blood-spin kit (MO BIO Laboratories).

PCR was performed on an MJ Research DNA thermocycler (engine model PTC-225) using 5 $\mu$L of template DNA, as described above. A Platinum Quantitative PCR SuperMix containing 1.5 U of *Taq* DNA polymerase, 200 $\mu$mol of each dNTP, 20 mmol Tris-HCl (pH 8.4), 50 mmol KCl, and 3 mmol $MgCl_2$ was combined with 0.4 $\mu$mol of each primer, in a 50-$\mu$L reaction. After an initial denaturation step for 2 min at 95°C, cycling conditions were as follows: 40 cycles of 30 s at 95°C, 1 min at 62°C, and 1 min at 72°C. After cycling was completed, there was a holding period of 15 min at 72°C. The resulting amplified PCR product was run on 2% agarose E-gels (Invitrogen Life Technologies), for 30 min at 60 V. The resulting ethidium bromide–stained DNA bands were visualized by use of a Fluor-S Imaging System (Bio-Rad Laboratories), under standardized exposure settings. A specimen was considered positive if a single band of ~279 bp was observed. Images of PCR-

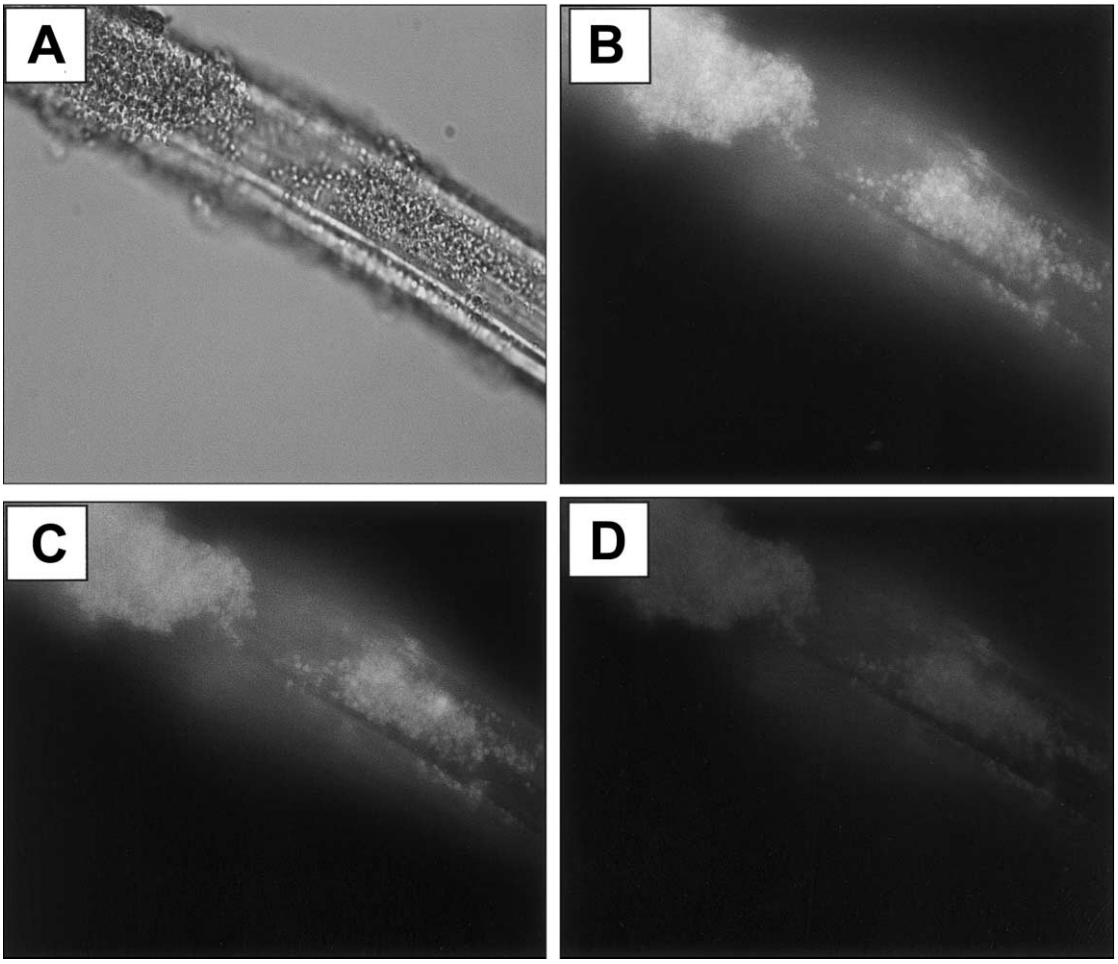



**Figure 4.** Monochromatic epifluorescent photomicrographs (100× oil-objective lens) of fibers from a clinical tampon specimen probed with Saur327-Cy5 and counterprobed with EUB338-FITC and viewed with a 100× objective lens: *(A),* bright-field image; *(B), Staphylococcus aureus* cells emitting Cy5 probe–conferred signal; *(C),* image of EUB338-FITC counterprobe signal; and *(D),* autofluorescence check using Cy3 filter set (unallocated color range).

amplicon bands were then combined into a single image by Adobe Photoshop 4.0 software.

For confirmation of the correct band sequence, a representative PCR amplicon was prepared for DNA capillary sequencing using a Beckman Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter) and the original PCR primers. The resultant PCR products were then analyzed by use of a CEQ2000XL capillary DNA sequencer (Beckman Coulter). To confirm its identity with a sequence in the *nucA* gene, the output sequence was then compared, by a BLAST search, with DNA sequences in GenBank.

## RESULTS

Table 1 summarizes the culture-testing results for the 3 visits in which either only vaginal swabs were used for culturing (visit B) or both tampon and vaginal-wash samples were sampled for culturing (visits C and D). The culturing results from visit B ensured that both culture-positive and culture-negative women were included in the study. Interestingly, of the 8 women who were culture positive at visit B, 6 were culture negative for the tampon samples taken during visit C, and 2 of these 6 were also culture negative for vaginal-wash samples taken during visit C. Conversely, 1 woman who was culture negative during visit B was culture positive for both tampon and vaginal-wash samples taken during visit C. Although only 4 women were sampled again during visit D, again the culturing results were inconsistent; for example, 2 women who were culture negative for the tampon samples taken during visit C were culture positive for the tampon samples taken during visit D, and 1 woman who was culture positive for both tampon and vaginal-wash samples taken at visit C was culture negative for both tampon and vaginal-wash samples taken during visit D.

Results of validation testing of the 23S rRNA–targeted probe, Saur327, designed in the present study are presented in table 2. The bacteria-domain probe, EUB338, consistently produced

strong fluorescent signals and thus performed well as a positive control in all validation hybridizations. Also, the nonsense probe, anti-Saur835, served as an adequate negative control for nonspecific binding, since it failed to produce a significant fluorescent signal in any of the species tested. Hybridizations with the 23S rRNA–targeted, *S. aureus*–specific probe, Saur327, designed in the present study resulted in easily observed fluorescent signals from all 5 strains of *S. aureus* tested. In addition, the Saur327 probe yielded no false-positive signals when tested against the other staphylococcal species and genera included in the present study.

FISH analysis identified *S. aureus* in all 22 clinical tampon specimens and in all 22 vaginal-wash specimens (table 3). A second independent set of FISH experiments were further confirmed by PCR amplification of the *S. aureus*–specific *nucA* gene in 21 of the 22 vaginal washes (figure 1 and table 3); and, when the tampon specimen associated with the 1 vaginal-wash specimen in which *S. aureus* was not detected by PCR of the *nucA* gene was itself evaluated by PCR, it was positive. Sequencing of the PCR product from several vaginal-wash samples revealed a single sequence (not shown), and comparison of this sequence with that in the GenBank database confirmed detection of the *nucA* gene. Interestingly, whereas only 14 of the 44 samples were identified as *S. aureus* positive by culturing techniques, all 44 samples were so identified by FISH. In addition, PCR confirmed the presence of *S. aureus* in 22 of the clinical specimens (1 tampon specimen and 21 vaginal-wash specimens), representing all 18 women. Table 3 summarizes the results of culturing, FISH, and PCR analyses performed on the clinical specimens and provides details regarding the presence of *S. aureus* biofilm.

It is important to note that, in clinical specimens, only bacterial cocci were detected by the *S. aureus*–specific FISH probes (figure 2*B* and 2*C*), whereas bacterial rods and eukaryotic cells were also present and could be observed in counterstained and bright-field images (figure 3). Figure 3 depicts the efficacy of these probes to distinguish *S. aureus* cells in complex bacterial populations in vaginal-wash specimens. This figure also shows typical monochromatic images of a vaginal-wash sample probed with Saur327 and Saur72 and counterstained with DAPI; although erythrocytes displayed some autofluorescence, *S. aureus* biofilm is still easily identified in the upper-right corner of all 4 images.

In the present study, "biofilm growth" was defined as $\geqslant$7 bacterial cells clustered to form a surface-attached structure (figures 2*A,* 3, and 4); and it was the only parameter used to evaluate tampon fibers. In contrast, bacterial "flocs" were defined as including both detached portions of biofilm and possible intact cell clusters not necessarily originating from a surface-attached biofilm (figure 2*B*); the term was used only in the assessment of the vaginal-wash specimens, not in the evaluation of tampon fibers or fiber-wash samples. Individual random cells without structure were considered to be planktonic (figure 2*C*); indeed, planktonic *S. aureus* cells were observed in many of the vaginal-wash samples. Although direct staining of biofilm exopolysaccharide was not performed, shrouds around bacterial clusters attached to surfaces or bacterial flocs could be visualized in most images, suggesting the presence of EPS. Biofilm consisting of FISH-identified *S. aureus* cells was observed in 19 of the 22 clinical tampon samples and in 18 of the 22 vaginal-wash specimens (table 3).

Figure 4 shows by far the most extensive *S. aureus*–biofilm cluster observed during the present study. This large clump of *S. aureus* biofilm was seen attached to a tampon fiber and is not considered representative of the *S. aureus* biofilms typically seen in the present study. However, it does demonstrate the degree to which *S. aureus* biofilm can, albeit only occasionally, develop within such specimens.

## DISCUSSION

In the present study, the presence of a clinically relevant bacterial species, *S. aureus,* was assessed in healthy, menstruating women, by culture-independent molecular techniques (i.e., FISH and PCR), to detect possible biofilm growth on tampons and menses components. The study design also allowed these molecular techniques to be compared with traditional culturing methods, with regard to detection of *S. aureus* from the vaginal environment. FISH detected *S. aureus* in all 44 tampon and vaginal-wash specimens, including those from women shown to be culture negative. These results suggest that vaginal *S. aureus* carriage may occur at rates higher than those reported in previous studies, which used only culture-dependent techniques [3, 4, 32]. The FISH results were also confirmed by PCR analysis of samples from all subjects, providing additional support for the notion that *S. aureus* carriage may go undetected by culturing techniques. This type of result is not unique to the present study. For example, in 2 other reports, molecular techniques (e.g., reverse-transcriptase–PCR, PCR, and DNA sequencing) confirmed (1) the presence of active *Haemophilus influenzae* in culture-negative samples from patients with otitis media [33] and (2) the presence of *Staphylococcus* species and *Streptococcus* species in 2 cases of culture-negative patients with endocarditis [34]. Indeed, for at least the past decade, the term "viable but nonculturable" (VBNC) has seen increased usage in the literature, as a way to describe living bacteria that will not grow on either solid or liquid culturing media. Other bacterial species with relevance as human pathogens have also been shown to exist in the VBNC state. For example, in 1 study, *Escherichia coli, Enterococcus faecalis, Salmonella typhimurium,* and *Yersinia enterocolitica* remained in the VBNC state 56 days after in situ exposure to a polar marine environment [35], and, in other experiments, *Vibrio cholerae* was capable of transforming into a VBNC state for $\geqslant$1 year [36]. In the former study, percentages of respiring

*E. coli* and *Salmonella typhimurium* increased significantly after addition of nutrients, at all temperatures tested, indicating that nutrient availability, rather than temperature, limited enteric bacterial activity in this very cold environment. In the latter study, the VBNC state could also be induced in previously culturable cells by alteration of the growth conditions. Examination of the ultrastructure of these cells by transmission and scanning-electron microscopy clearly showed that the cells became smaller in size and changed from a larger, rod-shaped, to an ovoid or coccoid morphology, an observation indicative of the VBNC state, with the central region of the cells becoming compressed and surrounded by denser cytoplasm. Although VBNC has been associated predominantly with Gram-negative bacteria, traditional culturing techniques have documented both poor growth and isolation of subpopulations of *S. aureus,* known as "small colony variants" (SCVs) [37]. SCVs are purported to have defective electron-transport systems and to exhibit atypical phenotypic characteristics such as slow growth, decreased pigment formation, low coagulase, reduced hemolytic capabilities, alpha-toxin production, and less tissue damage [38, 39]. These studies convey the importance of understanding both how environmental factors may alter the physiology of colonizing bacterial species and how culturing techniques may mask the presence of viable pathogenic microbes. The inconsistencies in the culturing data from the present study (presented in table 1) also support this conclusion.

The vaginal environment represents a unique and complex niche in which pathogenic and commensal microbes are certainly exposed to cyclical changes in the human host [4, 10, 11]. Because bacteria may exist in different physiological or phenotypic states dictated by environmental factors [13], we must now realize the limitations that standard culturing techniques have in assessment of the presence of specific bacteria in environments within the human body. For example, bacterial growth in biofilms has been shown to complicate culture-dependent diagnosis and treatment of urinary-tract infections [14]. Also, it has been observed that *S. aureus* can demonstrate variable growth when cultured from mucosal surfaces. *S. aureus* was isolated from ~33% of mucosal cultures after incubation for 24–48 h; however, the number of positive cultures can be increased to ~60% after incubation for 2–3 weeks (P. M. Schlievert, personal communication). A possible explanation may be that the normal microflora, including alpha-hemolytic streptococci and lactobacilli, could have been inhibiting *S. aureus* growth on the agar plates initially but that, because they died during extended retention of the plate, the *S. aureus* present in a VBNC state were allowed to start growing. Thus, consideration must always be given to the possible effects of a selective culturing medium such as the mannitol salt agar used in the present study. Although it may promote selective growth and subsequent isolation of *S. aureus* from certain environments, it also may impose additional stress on cells relocated from other environments, thereby reducing its sensitivity. The findings of the present study support the use of culture-independent techniques, such as FISH and/or PCR, to detect *S. aureus* cells in clinical specimens, since we found a broad discrepancy between culturable and FISH-detectable staphylococci (table 3).

The results of the present study also demonstrate that the Saur327 probe can identify *S. aureus* cells within a microbial consortium in the complex matrices of clinical specimens. Sporadic bacterial autofluorescence was problematic in the early stages of probe testing, occluding observation of fluorescent hybridization signals. This periodically observed autofluorescence spanned the entire visible spectrum and resulted in protocol modifications such as repeated washing of the specimen and additional use of the Cy5 fluorophore, which has an emission-wavelength range above the visible spectrum.

Although in situ identification of specific bacterial cells within complex matrices and microbial consortia is an obvious goal of ecological studies [40–42], the benefits of using FISH to achieve this are not always recognized. One of the most significant advantages afforded by FISH is that target-group specificity is controllable; that is, probes can be designed along a hierarchy of phylogenetic groupings, ranging from broad phyla to individual species [23]. In addition, researchers can minimize nonspecific binding, since the stringency of the hybridization is easily controlled by conditions of heat, salt, and denaturing chemicals (e.g., formamide); because no blocking steps are required, this results in a relatively quick and simple protocol, compared with many immunohistochemical procedures. The fact that probes can now be purchased from many supply companies specializing in routine DNA synthesis has resulted in inexpensive and easily accessible products of consistently high quality. Since current culturing methods are time-consuming, often requiring days for positive bacterial identification, the FISH technique, because of its speed, accuracy, and ability to detect nonculturable cells, is being further explored as a routine clinical diagnostic tool for the detection of pathogenic bacteria [43–46].

The results of the present study support previous observations of cell-adherent bacterial biofilm in vaginal-wash specimens [47], demonstrating that *S. aureus* can be a component of such biofilms, both on vaginal tissues and on tampon fibers. The fact that *S. aureus* biofilm or cell clusters could be observed in the majority (37 of 44) of rigorously explored tampon and vaginal-wash specimens from healthy, menstruating women suggests that such a mode of growth may be common for *S. aureus,* although this organism was neither dominant nor ubiquitous in these types of samples. Human vaginal biofilms, even in the presence of potential pathogens such as *S. aureus,* should be considered as quite natural microbial communities. It is not the mere presence of a potential bacterial pathogen that is the

most important or relevant consideration for the understanding of vaginal diseases—rather, it is how environmental and ecological factors control expression of virulence in pathogenic organisms. Our demonstration that *S. aureus* biofilm is common in menstrual vaginal specimens leaves open the possibility that factors involving biofilm-related–virulence expression may be relevant to vaginal diseases involving *S. aureus.*


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## APPENDIX

## EXCLUSION CRITERIA

1. Immunosuppressive drugs taken during the past 6 weeks
2. Currently taking chemotherapeutic drugs
3. Infection of the genital area, requiring physician's treatment, during the past 6 weeks.
4. Clinically diagnosed as ever having had toxic-shock syndrome
5. Currently pregnant (self-declared)
6. Diagnosed with a condition affecting the vaginal microflora (e.g., AIDS [human-immunodeficiency-virus positive], diabetes, kidney failure, or hepatitis)
7. Currently participating in another menstrual study
8. Use of medications, including either systemic antimicrobial drugs or antimicrobial drugs used to treat a vaginal infection during the past 30 days
9. Bathing or showering <2 h before scheduled visit
10. Use of douching substances, vaginal medications, suppositories, feminine sprays, genital wipes, or contraceptive spermacides <48 h before scheduled visit
11. Sexual intercourse <24 h before scheduled visit

## References


1. Casewell MW, Hill RL. The carrier state: methicillin-resistant *Staphylococcus aureus.* J Antimicrob Chemother **1986**; 18(Suppl A):1-12.
2. Lansdell LW, Taplin D, Aldrich TE. Recovery of *Staphylococcus aureus* from multiple body sites in menstruating women. J Clin Microbiol **1984**; 20:307–10.
3. Guinan ME, Dan BB, Guidotti RJ, et al. Vaginal colonization with *Staphylococcus aureus* in healthy women: a review of four studies. Ann Intern Med **1982**; 96:944–7.
4. Smith CB, Noble V, Bensch R, Ahlin PA, Jacobson JA, Latham RH. Bacterial flora of the vagina during the menstrual cycle: findings in users of tampons, napkins, and sea sponges. Ann Intern Med **1982**; 96:948–51.
5. Dickgiesser N, Brombacher A, Wiest W. Toxic shock syndrome toxin-1 producing strains of *Staphylococcus aureus* in vaginal smears [in German]. Geburtshilfe Frauenheilkd **1987**; 47:104–6.
6. Lowy FD. *Staphylococcus aureus* infections. N Engl J Med **1998**; 339: 520–32.
7. Linnemann CC Jr, Staneck JL, Hornstein S, et al. The epidemiology of genital colonization with *Staphylococcus aureus.* Ann Intern Med **1982**; 96:940–4.
8. Chesney PJ, Davis JP, Purdy WK, Wand PJ, Chesney RW. Clinical manifestations of toxic shock syndrome. JAMA **1981**; 246:741–8.
9. Elsner P, Maibach HI. Microbiology of specialized skin: the vulva. Semin Dermatol **1990**; 9:300–4.
10. Wagner G, Ottesen B. Vaginal physiology during menstruation. Ann Intern Med **1982**; 96:921–3.
11. Brown WJ. Variations in the vaginal bacterial flora: a preliminary report. Ann Intern Med **1982**; 96:931–4.
12. Yarwood, JM, Schlievert PM. Oxygen and carbon dioxide regulation of toxic shock syndrome toxin 1 production by *Staphylococcus aureus* MN8. J Clin Microbiol **2000**; 38:1797–803.
13. Sauer K, Camper AK. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. J Bacteriol **2001**; 183:6579–89.
14. Nickel JC, Costerton JW, McLean RJ, Olson M. Bacterial biofilms: influence on the pathogenesis, diagnosis and treatment of urinary tract infections. J Antimicrob Chemother **1994**; 33(Suppl A):31–41.
15. Ji G, Beavis RC, Novick RP. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc Natl Acad Sci USA **1995**; 92:12055–9.
16. Larsen B, Monif GR. Understanding the bacterial flora of the female genital tract. Clin Infect Dis **2001**; 32:e69–77.
17. Mobarry BK, Wagner M, Urbain V, Rittmann BE, Stahl DA. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl Environ Microbiol **1996**; 62:2156–62.
18. Schramm A, Larsen LH, Revsbech NP, Ramsing NB, Amann R, Schleifer K-H. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Appl Environ Microbiol **1996**; 62:4641–7.
19. Ovreas L, Forney L, Daae FL, Torsvik V. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol **1997**; 63:3367–73.
20. Murray AE, Preston CM, Massana R, et al. Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers Island, Antarctica. Appl Environ Microbiol **1998**; 64:2585–95.
21. Alfreider A, Pernthaler J, Amann R, et al. Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization. Appl Environ Microbiol **1996**; 62:2138–44.
22. O'Donnell AG, Whiteley AS. Fluorescent in situ hybridization and the analysis of the single cell. In: Edwards C, ed. Environmental monitoring of bacteria. Vol 12. Totowa, NJ: Humana Press, **1999**:221–35.
23. Woese CR. Bacterial evolution. Microbiol Rev **1987**; 51:221–71.
24. Ward DM, Bateson MM, Weller R, Ruff-Roberts AL. Ribosomal RNA analysis of microorganisms as they occur in nature. In: Marshall KC, ed. Advances in microbial ecology. Vol 12. New York: Plenum Press, **1992**:219–86.

25. Amann RI. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev **1995**; 59:143–69.
26. Lipski A, Friedrich U, Altendork K. Application of rRNA-targeted oligonucleotide probes in biotechnology. Appl Microbiol Biotechnol **2001**; 56: 40–57.
27. Bentley RW, Harland NM, Leigh JA, Collins MD. A *Staphylococcus aureus*-specific oligonucleotide probe derived from 16S rRNA gene sequences. Lett Appl Microbiol **1993**; 16:203–6.
28. Ausubel F, Dracopoli NC. Informatics for molecular biologists. In: Ausubel FM, Brent R, Kingston RE, et al., eds. Current protocols in molecular biology. Vol 3. Chichester, United Kingdom: John Wiley & Sons, **1996**:19.1.1–3.38.
29. Manz W, Szewzyk U, Ericsson P, Amann R, Schleifer K-H, Stenstrom T-A. In situ identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA–directed fluorescent oligonucleotide probes. Appl Environ Microbiol **1993**; 59:2293–8.
30. Neef A, Zaglauer A, Meier H, Amann R, Lemmer H, Schleifer KH. Population analysis in a denitrifying sand filter: conventional and in situ identification of *Paracoccus* spp. in methanol-fed biofilms. Appl Environ Microbiol **1996**; 62:4329–39.
31. Brakstad OG, Aasbakk K, Maeland JA. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. J Clin Microbiol **1992**; 30:1654–60.
32. DuBois A, Osterling W, Delaney M, et al. Prevalence of *Staphylococcus aureus* and toxic shock syndrome toxin 1 (TSST-1) recovered from vaginal, anal, and nasal samples [abstract D-47]. In: Abstracts of the 100th general meeting of the American Society for Microbiology (Los Angeles). Washington, DC: American Society for Microbiology, **2003**: 236.
33. Rayner MG, Zhang Y, Gorry MC, Chen Y, Post JC, Ehrlich GD. Evidence of bacterial metabolic activity in culture-negative otitis media with effusion. JAMA **1998**; 279:296–9.
34. Wilck MA, Wu Y, Howe JG, Crouch JY, Edberg SC. Endocarditis caused by culture-negative organisms visible by Brown and Brenn staining: utility of PCR and DNA sequencing for diagnosis. J Clin Microbiol **2001**; 39: 2025–7.
35. Smith JJ, Howington JP, McFeters GA. Survival, physiological response, and recovery of enteric bacteria exposed to a polar marine environment. Appl Environ Microbiol **1994**; 60:2977–84
36. Chaiyanan S, Chaiyanan S, Huq A, Maugel T, Colwell RR. Viability of the nonculturable Vibrio cholerae O1 and O139. Syst Appl Microbiol **2001**; 24:331–41.
37. Looney WJ. Small-colony variants of *Staphylococcus aureus.* Br J Biomed Sci **2000**; 57:317–22.
38. von Eiff C, Heilman C, Proctor RA, Woltz C, Peters G, Gotz F. A site-directed *Staphylococcus aureus* hem B mutant is a small-colony variant which persists intercellularly. J Bacteriol **1997**; 179:4706–12.
39. Chuard C, Vaudaux PE, Proctor RA, Lew DP. Decreased susceptibility to antibiotic killing of a stable small colony variant of *Staphylococcus aureus* in fluid phase and on fibronectin-coated surfaces. J Antimicrob Chemother **1997**; 39:603–8.
40. Lawrence JR, Neu TR, Swerhone GDW. Application of multiple parameter imaging for the quantification of algal, bacterial and exopolymer components of microbial biofilms. J Microbiol Methods **1998**; 32:253–61.
41. Sanford BA, de Feijter AW, Wade MH, Thomas VL. A dual fluorescence technique for visualization of *Staphylococcus epidermidis* biofilm using scanning confocal laser microscopy. J Ind Microbiol **1996**; 16:48–56.
42. Sorensen AH, Torsvik VL, Torsvik T, Poulsen LK, Ahring BK. Whole-cell hybridization of *Methanosarcina* cells with two new oligonucleotide probes. Appl Environ Microbiol **1997**; 63:3043–50.
43. Oliveira K, Procop GW, Wilson D, Coull J, Stender H. Rapid identification of *Staphylococcus aureus* directly from blood cultures by fluorescence in situ hybridization with peptide nucleic acid probes. J Clin Microbiol **2002**; 40:247–251.
44. Jansen, GJ, Mooibroek M, Idema J, Harmsen HJM, Welling GW, Degener JE. Rapid identification of bacteria in blood cultures by using fluorescently labeled oligonucleotide probes. J Clin Microbiol **2000**; 38: 814–7.
45. Hogardt M, Trebesius K, Geiger AM, Hornef M, Rosenecker J, Heesemann J. Specific and rapid detection by fluorescent in situ hybridization of bacteria in clinical samples obtained from cystic fibrosis patients. J Clin Microbiol **2000**; 38:818–25.
46. Regnault B, Martin-Delautre S, Grimont PA. Problems associated with the direct viable count procedure applied to gram-positive bacteria. Int J Food Microbiol **2000**; 55:281–4.
47. Domingue PA, Sadhu K, Costerton JW, Bartlett K, Chow AW. The human vagina: normal flora considered as an in situ tissue-associated, adherent biofilm. Genitourin Med **1991**; 67:226–31.