Prolonged Survival of *Serratia marcescens* in Chlorhexidine

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During an outbreak of *Serratia marcescens* infections at our hospital, we discovered widespread contamination of the 2% chlorhexidine hand-washing solution by *S. marcescens*. Examination by electron microscopy of the sides of bottles in which this solution was stored revealed that microorganisms were embedded in a fibrous matrix. Bacteria, free in the liquid, were morphologically abnormal, showing cell wall disruption or cytoplasmic changes. Furthermore, bacteria adherent to the walls of the storage jugs and embedded in this fibrous matrix also had morphologically abnormal cytoplasm. Despite these changes, viable *S. marcescens* organisms were recovered from the fluid during a storage period of 27 months. The concentration of chlorhexidine required to inhibit these strains of *Serratia* was 1,024 μg/ml; however, the organism could survive in concentrations of up to 20,000 μg/ml. Additional studies are needed to define the mechanism(s) that allows such bacteria to contaminate and survive in disinfectants.

MATERIALS AND METHODS

Survival of organisms in stock solutions of Hibitane. Six 4-liter plastic stock bottles containing 2% chlorhexidine (Hibitane) from different wards were stored in the laboratory. Colony counts were performed on samples from each stock bottle at approximately monthly intervals for 27 months. Organisms were identified according to the criteria of Edwards and Ewing (6).

Determination of MICs. (i) Organisms tested. Twenty-five isolates of *S. marcescens* from contaminated chlorhexidine and five strains of *S. marcescens* isolated from various clinical sources (three from urine, one from sputum, and one from blood) were tested. Twenty isolates of other bacteria from clinical sources, including *Escherichia coli* (8), *Enterobacter* spp. (3), *Klebsiella* spp. (2), *Pseudomonas aeruginosa* (4), *Proteus* spp. (1), and *Staphylococcus aureus* (2), were collected 6 months after disposal of the contaminated Hibitane.

(ii) Antimicrobial agents. Amikacin powder was obtained from Bristol Laboratories, Montreal, Quebec, Canada, and gentamicin was obtained from Schering Laboratories, Pointe Claire, Quebec, Canada.

Serial twofold dilutions of amikacin and gentamicin were incorporated into Mueller-Hinton agar. Chlorhexidine gluconate (20%; Ayerst Laboratories) was diluted and incorporated into agar in serial twofold concentrations from 0.5 to 2,048 μg/ml.

(iii) MIC. Organisms to be tested were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) for 6 h and diluted to match the density of a 0.5 McFarland standard (1). A Steers replicator (14) was used to inoculate the surfaces of the agar plates, and the plates were incubated for 18 h at 35°C.
The minimal inhibitory concentration (MIC) was the concentration which completely inhibited growth or resulted in a light haze.

A control strain of *Staphylococcus aureus* and of *Escherichia coli* with known chlorhexidine MICs were obtained from Harold Baker, Ayerst Laboratories, Montreal, Quebec, Canada. In addition, a strain of *Pseudomonas aeruginosa* with known amikacin and gentamicin MICs was also used as a control strain.

**Electron microscopy studies.** (i) Scanning electron microscopy. A 3- by 2-cm piece of plastic was cut from the side of each of the five bottles containing contaminated chlorhexidine. The bottle containing chlorhexidine that was negative on culture served as a control. In addition, a plastic bottle containing 4% chlorhexidine (as soon as it was received in the pharmacy from the manufacturer) was examined. In all instances, the plastic samples were taken from the sides of the bottles at 2 to 3 cm below the top of the fluid level.

The first piece of plastic was examined after the contaminated chlorhexidine had been stored in the laboratory for 20 months. The remaining pieces were examined after 26 months of storage.

The inner surface of the plastic was sampled just before the fixation procedure, using a cotton swab. The swab was inoculated onto MacConkey agar, and the organisms were identified as outlined above. The pieces of plastic were fixed for 24 h at 20°C with a fixative solution consisting of 5% glutaraldehyde and cacodylate buffer (0.067 M; pH 6.2) with 0.15% ruthenium red. Each piece of plastic was then "metallized" with osmium tetroxide and thioacarbobxide (10), dehydrated in ethanol and Freon-113 before critical point drying (5), and examined with a Hitachi S450 scanning electron microscope (Hitachi, Rexdale, Ontario, Canada).

(ii) Transmission electron microscopy. (a) Plastic. Another piece of plastic from the storage jug, containing only *Serratia marcescens* on culture, was fixed as detailed above, washed five times in the buffer, postfixed in 2% OsO₄ in buffer, washed five more times in buffer, and dehydrated through a series of acetone washes. All of the solutions used in processing the specimen, after the glutaraldehyde fixation to dehydration with the 70% acetone solution, contained 0.05% ruthenium red (ruthenium red was omitted from the 90 and 100% acetone solutions because of its limited solubility in these solutions). After further dehydration in propylene oxide, the specimen was embedded in Vestopal (Ladd Industries, Burlington, Vt.), sectioned, stained with uranyl acetate and lead citrate (11), reinforced with evaporated carbon, and examined with an electron microscope (AEI Model No. 801; Associated Electronic Industries, Harlow, England) at an acceleration voltage of 60 kV.

(b) Sediment from contaminated chlorhexidine. Ten-milliliter samples of contaminated chlorhexidine from three storage jars (from which only *Serratia marcescens* was isolated) were centrifuged at 300 × g for 10 min. Each sediment was washed three times in phosphate-buffered saline (pH 7.2) and then fixed in 5% glutaraldehyde and cacodylate buffer (0.067 M; pH 6.2) with 0.15% ruthenium red for 2 h at room temperature (~20°C). The remainder of the steps were performed as outlined above.

At the time these samples were collected, the contaminated Hibitane had been stored in the laboratory for 18 months.

(c) Bacteria from contaminated chlorhexidine grown on solid medium. A 0.001-ml amount of contaminated chlorhexidine was streaked onto brain heart infusion agar plates containing Tween 80 (8) and incubated aerobically for 18 h at 37°C. Three colonies were then scraped off the plates, emulsified in phosphate-buffered saline, and centrifuged at 300 × g for 10 min. The remaining steps were performed as outlined above.

(d) Material from the side of storage jug. The chlorhexidine was discarded from storage jug 7BU (only *Serratia marcescens* was isolated from the fluid) after a 27-month period. A reddish precipitate was evident on the sides of the jug. This was scraped off with a scalpel blade. The material was transferred to a test tube, and 5% glutaraldehyde and cacodylate buffer (0.067 M; pH 6.2) with 0.15% ruthenium red was added. The remaining steps were performed as outlined above.

(e) An isolate of *Serratia marcescens* which had never been exposed to chlorhexidine. An isolate of *Serratia marcescens* (from the urine of a catheterized patient) was inoculated to brain heart infusion broth, incubated for 6 h, and then centrifuged at 300 × g for 10 min. The sediment was then fixed as outlined above.

Sudan black stain. A 10-ml sample of contaminated chlorhexidine from each stock bottle was removed and centrifuged at 300 × g for 10 min. The sediment was washed once in phosphate-buffered saline and then smeared onto a glass slide, air dried, and heat fixed. It was then stained with Sudan black B for 10 min, washed, cleared with xylol and counterstained with 0.5% aqueous safranine for 15 s, washed in tap water, blotted dry, and examined under oil immersion with a Zeiss light microscope (4).

**RESULTS**

Chlorhexidine from five of the six stock bottles was contaminated with *Serratia marcescens*, and samples from two of these also grew *Flavobacter* spp. and *Pseudomonas* sp. Chlorhexidine from the sixth container was negative when cultured initially and remained so throughout (Table 1). The concentration of organisms in the five contaminated bottles was 10⁵ colony-forming units per ml initially and declined during the 27-month observation period (Table 1). *Serratia marcescens* was still recovered from one stock bottle (7BU) in concentrations of 10⁴ colony-forming units per ml after 27 months of storage in the laboratory. Three species of organisms, *Serratia marcescens*, *Pseudomonas* sp., and *Flavobacter* sp., were recovered initially from two stock bottles, 4S and 7B. The total number of organisms was similar in both (2.4 × 10⁵ and 3 × 10⁵ colony-forming units per ml, respec-
TABLE 1. Results of serial cultures of stock solutions of chlorhexidine

<table>
<thead>
<tr>
<th>Source of sample</th>
<th>Organism isolated</th>
<th>No. of CFU/ml isolated at indicated month, day, and year</th>
</tr>
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<tbody>
<tr>
<td>10V</td>
<td>Serratia marcescens</td>
<td>1.49 × 10⁸</td>
</tr>
<tr>
<td>4S</td>
<td>Serratia marcescens</td>
<td>3 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas sp.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flavobacter sp.</td>
<td></td>
</tr>
<tr>
<td>7B</td>
<td>Serratia marcescens</td>
<td>2.4 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas sp.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Flavobacter sp.</td>
<td></td>
</tr>
<tr>
<td>7BU</td>
<td>Serratia marcescens</td>
<td>1.6 × 10⁸</td>
</tr>
<tr>
<td>8A</td>
<td>Serratia marcescens</td>
<td>1.3 × 10⁸</td>
</tr>
<tr>
<td>BC</td>
<td>Serratia marcescens</td>
<td>NG</td>
</tr>
</tbody>
</table>

*a* These bottles were held in the laboratory; samples from 10V, 7BU, 8A, and BC were cultured at least monthly.

*b* CFU, Colony-forming units; NG, no growth; ND, not done.

*c* Stock bottle used for electron microscopy studies at this time.

*d* Pseudomonas sp. only.

TABLE 2. Minimal inhibitory concentrations of chlorhexidine gluconate required to inhibit indicated organisms

<table>
<thead>
<tr>
<th>Organism (source)</th>
<th>MIC (μg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>Serratia marcescens (stock solutions of chlorhexidine)</td>
<td>25</td>
</tr>
<tr>
<td>Serratia marcescens (clinical specimens)</td>
<td>5</td>
</tr>
<tr>
<td>Escherichia coli (clinical specimens)</td>
<td>8</td>
</tr>
<tr>
<td>Enterobacter spp. (clinical specimens)</td>
<td>3</td>
</tr>
<tr>
<td>Klebsiella spp. (clinical specimens)</td>
<td>2</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa (clinical specimens)</td>
<td>4</td>
</tr>
<tr>
<td>Proteus sp. (clinical specimens)</td>
<td>1</td>
</tr>
<tr>
<td>Staphylococcus aureus (clinical specimens)</td>
<td>2</td>
</tr>
</tbody>
</table>

FIG. 1. Scanning electron micrograph of the inner surface of a piece of a plastic chlorhexidine stock bottle. The chlorhexidine from this bottle was negative when cultured aerobically. (Note the absence of bacteria and the woven texture of the surface of the plastic.) The bar indicates 5 μm.
was negative on culture. (Note the uneven texture of the surface.)

Scanning electron microscopy of the inner surface of the 10V plastic stock bottle is shown in Fig. 2 through 4. Only *Serratia marcescens* was isolated from the liquid, sediment, and sides of this bottle. Figure 2 is a low-power view showing bacteria embedded in a matrix of fibrous material that, in some areas, is heaped up into distinct masses. Discrete microorganisms are evident adherent to the plastic. In Fig. 3, bacteria are seen within the fibrous matrix, and in Fig. 4, these organisms are seen to be embedded in the fibrous matrix to varying degrees. When sections from the remaining plastic stock bottles were examined (1 year later than the piece of plastic shown in Fig. 2 through 4), bacteria were still evident embedded in a matrix (Fig. 5); however, this material was not as extensive as is shown in Fig. 2 through 4. Figure 6 is a transmission electron micrograph of a bacterium adherent to the surface of a piece of plastic from the 10V stock bottle (sample taken from an area adjacent to the piece shown in Fig. 2 through 4). The ruthenium red-positive nature of the material that covers the bacterium is evident even though this material is radically condensed by dehydration.

A transmission electron micrograph (Fig. 7) of the sediment of contaminated chlorhexidine showed that most of the bacteria free in the fluid were abnormal. Bacteria from all three contaminated stock bottles containing *Serratia* only had the same appearance. As seen in Fig. 7,
Fig. 3. High-magnification scanning electron micrograph of the inner surface of a section of a plastic chlorhexidine stock bottle (10V) contaminated with Serratia marcescens. (Note the association of bacterial cells with the matrix of the fibrous masses.) The bar indicates 1.0 μm.

Fig. 4. High-magnification scanning electron micrograph of the inner surface of a section of a plastic chlorhexidine stock bottle contaminated with Serratia marcescens. Some bacterial cells are completely or partially (arrows) submerged in this confluent mass of fibrous material. The bar indicates 1.0 μm.
Figure 10 is a transmission electron micrograph of material scraped from the surface of storage jug 7BU (Table 1). Only *Serratia marcescens* was isolated from this container. The cell walls of the bacteria in this section are well resolved, but intracellular detail is still poor. There are no discernible ribosomes, and the cytoplasm resembles that of the bacteria seen in Figure 8A. (Note the continuum of the fine fibrous matrix.)

**DISCUSSION**

Contamination of disinfectants in hospitals is not a new problem. In 1967, Burdon and Whitby (3) described contamination of chlorhexidine and savlon (chlorhexidine-cetrimide mixture) by *Pseudomonas* spp. The addition of 4% (vol/vol) isopropyl alcohol to aqueous preparations of these disinfectants reduced the incidence of con-

Figure 5. Scanning electron micrograph of a piece of plastic from a chlorhexidine stock bottle contaminated with *Serratia marcescens* (7BU; Table 1). Many of the bacteria are embedded in a matrix. The woven background of the plastic is not seen in most of the micrograph, suggesting that it is covered by the matrix. The bar indicates 5 μm.

many of the bacteria were disrupted, whereas others contained large vacuoles in their cytoplasm. When examined by light microscopy, these vacuoles were seen to stain with Sudan black. Although the cell walls of some of the bacteria that had been suspended in chlorhexidine for 18 months appeared to be normal, their cytoplasm (Fig. 8A) was never seen to consist of discrete ribosomes but, rather, to consist of uniformly electron-dense masses of various dimensions. Contrast the appearance of these bacterial cells with those of an isolate of *Serratia marcescens* (from the catheterized urinary tract of a patient) that had never been exposed to chlorhexidine (Figure 8B). The ribosomes and nuclear material are well seen. A fibrous capsule is also evident. The cytoplasmic structure of cells of *Serratia marcescens* recovered from the contaminated chlorhexidine and grown on an agar surface is seen in Fig. 9, and this structure is entirely typical of the ultrastructure of bacterial cells.

![Figure 5](image_url)  
Fig. 5. Scanning electron micrograph of a piece of plastic from a chlorhexidine stock bottle contaminated with *Serratia marcescens* (7BU; Table 1). Many of the bacteria are embedded in a matrix. The woven background of the plastic is not seen in most of the micrograph, suggesting that it is covered by the matrix. The bar indicates 5 μm.

![Figure 6](image_url)  
Fig. 6. Transmission electron micrograph of a section of a ruthenium red-stained preparation of a fragment of a plastic chlorhexidine stock bottle (10V; Table 1) contaminated with *Serratia marcescens*. This gram-negative bacterial cell is surrounded by an electron-dense condensed, anionic, ruthenium red-positive material (P). This same electron-dense condensed material (M) is seen to cover much of the inner surface of the plastic container. The bar indicates 0.1 μm.
tamination. *Pseudomonas maltophilia* has been isolated from contaminated savlon (17). *Pseudomonas cepacia* was adapted to a 1:30 dilution of savlon; however, it did not survive in this concentration when the pH was adjusted to 7.2 with hard tap water (2). The *Pseudomonas cepacia* organisms remained viable in tap water for 1 year. We found that the *Serratia marcescens* organisms survived in chlorhexidine for more than 27 months.

The highest concentration of chlorhexidine required to inhibit the strains of *Serratia* isolated from this disinfectant was 1,024 μg/ml; however, the organism could be grown from solutions containing 20,000 μg/ml without using inhibitors of chlorhexidine in the medium. The scanning electron micrographs showed that many of the organisms were embedded in a very extensive fibrous matrix, and transmission electron micrographs (Fig. 6 and 10) showed that this matrix was ruthenium red positive. This material is probably polysaccharide, since ruthenium red, a cationic dye, has an affinity for strongly anionic polymers, most of which are polysaccharides (9). It stains extracellular material associated with the surface of cell walls that is otherwise difficult or impossible to demonstrate. Whether this ruthenium red-positive material interferes with penetration of the chlorhexidine, a cationic agent, to the surface of the cell is speculation only. Indeed, the cells shown in Fig. 10 (scraped from the surface of a chlorhexidine storage jug and surrounded by extensive fibrous matrix) had abnormal cytoplasm and were similar in appearance to some of the cells free in the liquid (Figure 8A), suggesting that the fibrous matrix did not completely prevent the chlorhexidine from interacting with the cell. Chlorhexidine interacts with bacterial cells in several ways (7). It is adsorbed onto the surface of the bacterial cell and damages permeability barriers, resulting in leakage of cell contents (12). At higher concentrations, it precipitates or coagulates cytoplasm.

Stickler and Thomas (15) studied 802 bacterial isolates from patients with urinary tract...
infections and found that none of the 369 isolates of \textit{Escherichia coli} were resistant to 500 \mu g of chlorhexidine per ml, whereas 83 and 45.7\% of their \textit{Providencia stuartii} and \textit{Pseudomonas aeruginosa} isolates, respectively, were so resistant. In a subsequent study (16), they were unable to explain the resistance of \textit{Providencia stuartii} (chlorhexidine MIC, 1,600 \mu g/ml) in terms of differences in lipid content between sensitive and resistant strains. Furthermore, the resistance could not be attributed to reduced adsorption of the antiseptic or its enzymatic degradation. They speculated that there might be a layer in the cell wall of \textit{Providencia stuartii} which absorbs the agent and prevents further penetration through to the cytoplasmic membrane. Our observations would suggest that this is not so for \textit{Serratia marcescens} (Fig. 7, 8A, and 10).

Cytoplasmic vacuoles (Fig. 7) were present in organisms free in the chlorhexidine. These vacuoles stained with Sudan black B and were not present in cells (from the chlorhexidine) that had been grown on a solid medium.

We have described the morphology of \textit{Serratia marcescens} that has survived in chlorhexidine for a prolonged period. Additional studies are needed to elucidate the mechanism of this survival.

We were unable to determine the clinical relevance of the contaminated chlorhexidine. The \textit{Serratia} isolates from contaminated chlorhexidine could not be O serotyped. The antibiotic susceptibility of such isolates (generally gentamicin resistant and amikacin sensitive) resembled the pattern of isolates from the urine of infected patients. With the institution of an appropriate infection control program, many measures, such as cohorting infected or colonized patients, obtaining separate urine measuring containers for each patient, etc., were introduced at about the time the contaminated chlorhexidine was discovered. The \textit{Serratia} infection/colonization rate before our infection control program began was 132/10,500 discharges and, in 1980, when the program had been operational for 3 years, the rate was 62.1/10,000 discharges.

**Fig. 8.** (A) Transmission electron micrograph of the same sediment of contaminated chlorhexidine as that shown in Fig. 7. The cell wall of this bacterium (\textit{Serratia marcescens} on culture) is normal. The cytoplasm is abnormal, however, in that it consists of amorphous, electron-dense masses. In contrast, cells of \textit{Serratia marcescens} grown in BH1 broth which was never exposed to chlorhexidine (B) show easily discernible ribosomes and nuclear material. (Note the fibrous capsule surrounding these bacteria.) The bar indicates 0.1 \mu m.
(P < 0.001) (Marrie et al., submitted for publication).

ACKNOWLEDGMENTS
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LITERATURE CITED
FIG. 10. Transmission electron micrograph of material scraped from the side of a chlorhexidine storage jug (7BU; Table 1) contaminated with Serratia marcescens. The cell wall of these bacteria is well resolved; however, the cytoplasm is amorphous with no discernible ribosomes seen. (Note the extensive fibrous matrix.) The bar indicates 0.1 μm.

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