Laboratory replication of filtration procedures associated with *Serratia marcescens* bloodstream infections in patients receiving compounded amino acid solutions

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Abstract

Purpose.—Specific deviations from *United States Pharmacopeia* standards were analyzed to investigate the factors allowing an outbreak of *Serratia marcescens* bloodstream infections in patients receiving compounded amino acid solutions.

Methods.—Filter challenge experiments using the outbreak strain of *S. marcescens* were compared with those that used the filter challenge organism recommended by ASTM International (*Brevundimonas diminuta* ATCC 19162) to determine the frequency and degree of organism breakthrough. Disk and capsule filters (0.22- and 0.2-μm nominal pore size, respectively) were challenged with either the outbreak strain of *S. marcescens* or *B. diminuta* ATCC 19162. The following variables were compared: culture conditions in which organisms were grown overnight or cultured in sterile water (starved), solution type (15% amino acid solution or sterile water), and filtration with or without a 0.5-μm prefilter.

Results.—Small-scale, syringe-driven, disk-filtration experiments of starved bacterial cultures indicated that approximately 1 in every 1,000 starved *S. marcescens* cells (0.12%) was able to pass

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through a 0.22-μm nominal pore-size filter, and about 1 in every 1,000,000 cells was able to pass through a 0.1-μm nominal pore-size filter. No passage of the *B. diminuta* ATCC 19162 cells was observed with either filter. In full-scale experiments, breakthrough was observed only when 0.2-μm capsule filters were challenged with starved *S. marcescens* in 15% amino acid solution without a 0.5-μm prefiltration step.

Conclusion.—Laboratory simulation testing revealed that under certain conditions, bacteria can pass through 0.22- and 0.2-μm filters intended for sterilization of an amino acid solution. Bacteria did not pass through 0.2-μm filters when a 0.5-μm prefilter was used.

In March 2011, an outbreak of *Serratia marcescens* bloodstream infections with a 47% fatality rate was identified among patients who had received total parenteral nutrition (TPN) at six Alabama hospitals.[1] A national drug shortage of 15% amino acid solution prompted a compounding pharmacy (called *pharmacy A* here) to prepare and filter-sterilize its own supply of 15% amino acid solution.[2] A site visit to pharmacy A by personnel from the Alabama Department of Public Health and the Centers for Disease Control and Prevention revealed that dietary grade bulk powders were used as ingredients for the amino acid solution.[1] The use of nonsterile ingredients in products intended for intravenous administration is defined as a high-risk compounding procedure by chapter 797 of the *United States Pharmacopeia* (*USP*).[3] These amino acid powders were mixed with sterile water in a mixing container, which had previously been cleaned with tap water, and then passed through a 0.2-μm filter; this pore size is generally considered sufficient for terminal sterilization.

Several deviations from *USP* chapter 797 standards were noted during the investigation:

1. The compounded solution was allowed to sit for up to 48 hours before filtration.

2. There was no use of a "prefilter" upstream of the sterilizing filter, despite excessive particulate matter in the solution.

3. Sterilizing filters were changed during filtration slowdown or clogging without replacing the entire tubing set.

4. Insufficient volumes of postfiltration solution were used during sterility testing. [3–5]

However, the contribution of each of these factors to contamination of the final solution was not clear, including whether creating a break in the sterile system to change the sterilizing filter could contribute to contamination. Strain typing by pulsed-field gel electrophoresis revealed that isolates of *S. marcescens* obtained from patients were indistinguishable from strains isolated from the unused TPN solutions and compounded 15% amino acid solutions from pharmacy A and from the mixing container and an anteroom tap water faucet in pharmacy A.

While rare, contamination of compounded products by filterable bacteria is likely to increase in frequency as ongoing drug shortages prompt pharmacies to attempt highrisk compounding.[3,4] *Serratia*[6–9] and other water-associated gram-negative organisms— including *Sphingomonas paucimobilis*[10] and *Burkholderia contaminans*[11] in fentanyl,

Pseudomonas fluorescens in heparin,[12] *Pseudomonas putida*[13] and *Burkholderia cepacia*[14] in intravenous flushes, and *Pseudomonas aeruginosa* in trypan blue ophthalmic solution[15]— have caused healthcare-associated infections in patients receiving locally prepared or repackaged injected medications from hospital pharmacies and compounding pharmacies. Parenteral admixtures also have been subject to contamination with gram-negative bacteria, [16] such as *Enterobacter hormaechei,*[17] *Serratia odorifera,*[18] and *Pantoea* species.[19] Often, the exact source of the intrinsic contamination of these compounded or repackaged injected drugs was undetermined.

The association between the Alabama *S. marcescens* outbreak and the amino acid solution compounding process provided an opportunity for further laboratory investigation of the effectiveness of the filter sterilization process used in pharmacy A. Simulation of pharmacy A's filter-sterilization process was conducted in the laboratory; several variables were removed during the full-scale filtration experiments. The amino acid solution was premixed no sooner than two hours before filtration, only a single filter was used for each run, and approximately 10% of the filtered product was tested per *USP* recommendations.[5] In contrast, the field investigation found that when preparing lots of amino acid solutions as large as 100 L, pharmacy A allowed amino acids to mix in water up to one to two days before filtration, changed the filter four or five times during the filtration step, and sampled only 25 mL of the final solution.[1] We compared the behavior of the outbreak strain of *S. marcescens* to that of *Brevundimonas diminuta* ATCC 19162, a bacterium known to penetrate 0.45-μm filters and used for the validation of sterile filtration processes, such as ASTM F838–05.[20] We also compared the performance of overnight cultures of organisms with nutrient-deprived (starved) organisms and challenged filters using both a 15% amino acid solution and autoclaved reverse osmosis water.

Methods

Preparation of cultures.

Frozen stock cultures of *S. marcescens* from the blood of one of the Alabama patients and of *B. diminuta* ATCC 19162 were cultured on tryptic soy agar plates[a] and incubated overnight at 30 °C. Cells were scraped off the plate and suspended in 9 mL of Butterfield's buffer[a] (BB) by mixing in a Vortex mixer for 2 minutes in 10-second intervals. The entire volume was added to a 1-L bottle of tryptic soy broth[a] and incubated overnight at 30 °C, shaking at 150 rpm with the lid loosened for air exchange. The culture was then washed twice in phosphate-buffered saline using a centrifuge speed of $3500 \times g$ for 15 minutes. The culture was washed a third time using sterile cellculture grade water.[b] The final pellet was resuspended in 100 mL of sterile water and then added to 900 mL of sterile water and shaken for 1 minute to mix the contents. The container was stored at ambient room temperature (20–24 °C) with the lid loosened for air exchange. A week later, the culture was centrifuged at $3500 \times g$ for 15 minutes, and the supernatant was discarded. The pellet was washed three times in cell culture–grade sterile water to remove potential debris and nutrients from lysed cells and resuspended in 1 L (total volume) of sterile water.

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[a]BD, Franklin Lakes, NJ.
[b]CellGro, Manassas, VA.

Periodically, samples were removed, serially diluted in BB, and plated for enumeration. These starved cultures were stored for approximately 120 days before beginning any experiment. Nonstarved cultures of *S. marcescens* and *B. diminuta* ATCC 19162 were prepared in a manner similar to that described in ASTM F838–05, the standard test method for determining bacterial retention of membrane filters. Briefly, 10 mL of tryptic soy broth was inoculated with the stock culture and incubated overnight at 30 °C. After incubation, 2 mL of this culture was then added to a bottle containing 1 L of tryptic soy broth and incubated overnight at 30 °C with shaking on a platform at 150 rpm. Cultures were then centrifuged and washed in sterile phosphate-buffered saline three times.

Preparation of amino acid solution.

Individual amino acids[c] were measured and added to sterile autoclaved reverse-osmosis water to obtain a 100-mL volume of 15% amino acid solution based on the formulation of Aminosyn II (Hospira, Lake Forest, IL). Starved cultures of both *S. marcescens* and *B. diminuta* were added to 100-mL aliquots of prepared 15% amino acid solution at a viable cell density of 10^4 colony-forming units (CFU) per milliliter. The containers were stored at room temperature for 48 hours. Samples were periodically removed, serially diluted in BB, and plated for enumeration on tryptic soy agar to establish whether the solution had any inhibitory or growth-promoting effects. All plates were incubated at 30 °C for up to 48 hours.

Syringe-driven disk-membrane filter challenge.

A 2-mL portion of each starved culture was passed through syringe-driven disk-membrane filters (pore sizes, 0.22 and 0.1 μm).[d] The filtrate was serially diluted in BB and plated in triplicate on tryptic soy agar.

Full-scale filtration challenge.

To replicate the conditions found during the outbreak investigation, the interior of a 100-L carboy was sanitized with 70% alcohol (ethanol) and then rinsed with sterile water. The measurement sizes used to prepare the amino acid solution were scaled up to a volume of 20 L. A stirring agitator[e] with a 3-inch diameter propeller driven at 350 rpm was used to mix the solution for a minimum of 2 hours before the experiment to ensure dispersion of the amino acids. Sterile Masterflex silicone tubing[f] was attached to the spigot and threaded through a peristaltic pump.[g] A brass pressure gauge was attached to the line with a sterilized polyvinyl chloride T-joint to ensure that fluid pressure did not exceed 50 psi. Silicone tubing attached the gauge to a 0.2-μm pore-size capsule filter with a surface area of 1000 cm^3.[h] The filter was primed with the test solution, and a bubble test was performed to ensure filter integrity. The challenge organism was added at a total count of 10^8 total CFU for starved cultures and 10^{10} total CFU for nonstarved cultures. The solution was stirred for five

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[c]Pure Bulk, Roseburg, OR.
[d]Pall Life Sciences, Ann Arbor, MI.
[e]Grovhac, Brookfield, WI.
[f]0.5-inch i.d., Cole-Parmer, Vernon Hills, IL.
[g]Geotech, Denver, CO.
[h]Baxa, Englewood, CO.

minutes before sample collection. Filtrate fractions were collected in 1-L increments in sterile wide-mouth bottles. A 10-mL portion of the remaining stirred, unfiltered solution was serially diluted and plated to confirm inoculum density. Fractions 1, 2, 4, 8, and 16 of filtrate were evaluated by membrane filtration using gridded 0.45-μm pore-size filter funnel cups.[d] For each of the five fractions, triplicate volumes of 10 and 100 mL of filtrate, and a single volume of 250 mL, were filtered (for a total volume of 2.9 L, or 14.5% of the batch volume of 20 L), placed onto tryptic soy agar plates, and incubated for up to 48 hours before counting. Several variables were tested: organism (*S. marcescens* versus *B. diminuta* ATCC 19162*)*, growth status (overnight versus starved), and solution type (amino acid versus sterile water). An additional prefiltration experiment used an Opticap 600 XL system[i] comprised of a 0.5-μm prefilter attached to the 0.2-μm capsule filter and challenged with 15% amino acid solution spiked with starved *S. marcescens*. All experiments were performed in triplicate.

Results

At the time of filter challenge testing, the mean ± S.D. inoculum densities of *S. marcescens* and *B. diminuta* ATCC 19162 held in sterile water were $(7.2 \pm 0.12) \times 10^7$ CFU/mL and $(1.9 \pm 0.09) \times 10^7$ CFU/mL, respectively. Cultures starved in water for 120 days were inhibited by exposure to the amino acid solution during a 48-hour period. Cell counts of starved cultures of *B. diminuta* ATCC 19162 were reduced by a mean ± S.D. of (1.6 ± 0.04) $\log_{10}$ (data not shown), while cell counts of starved *S. marcescens* culture declined by (3.1 ± 0.10) $\log_{10}$.

Small-scale, syringe-driven, disk-filtration experiments of starved bacterial cultures indicated that approximately 1 in every 1,000 starved *S. marcescens* cells (0.12%) was able to pass through a 0.22-μm nominal pore-size filter (Table 1) and about 1 in every 1,000,000 cells was able to pass through a 0.1-μm nominal pore-size filter. No passage of the *B. diminuta* ATCC 19162 cells was observed with either filter. The mean ± S.D. width of *S. marcescens* cells from overnight cultures averaged 0.86 ± 0.11 μm by transmission electron microscope (TEM) imaging ($n = 20$; Figure 1), whereas cells from a one-year-old (starved) culture averaged 0.60 ± 0.09 μm in width ($n = 12$). A later comparison of the 0.45-μm filters used to recover and enumerate organisms revealed that these filters retained 86% of the organisms that 0.22-μm membrane filters retained.

In the full-scale experiments, no breakthrough of organisms was observed except when filters were challenged using starved *S. marcescens* in 15% amino acid solution without prefiltration, as was the procedure in the outbreak investigation (Table 2). Two of these three experiments detected *S. marcescens* in the first collected fraction only, the numbers of which varied by two orders of magnitude (3 $\log_{10}$ when including the third replicate with no breakthrough).

[i]Millipore, Billerica, MA.

Discussion

Simulation of pharmacy A's amino acid compounding process in the laboratory demonstrated that under certain conditions, breakthrough of *S. marcescens* was possible. Certain factors, such as the organism type and its nutritional state, characteristics of the solution, and the filtration setup, likely contributed to the bacterial penetration of the 0.22- and 0.2-μm filters. In this investigation, breakthrough occurred only when using starved *S. marcescens* in amino acid solution; however, breakthrough did not occur when the same experiment was repeated in full scale with the addition of a 0.5-μm prefilter.

Paradoxically, no breakthrough was observed for *B. diminuta* ATCC 19162. Similarly, filter validation studies of a drug solution demonstrated that *Ralstonia pickettii* exposed to a drug solution for 24 hours penetrated 0.2-μm filters whereas *B. diminuta*, the standard challenge organism, did not.[21] The size and morphology of nutrient-depleted organisms from the environment, such as those *S. marcescens* encountered in the tap water, may not be the same as those of the *B. diminuta* produced by the cell paste method of preparing consistently sized cell stock for validation of processes that use 0.2-μm filters for terminal sterilization.[22–24] Bacteria can shrink to form ultrami-crocells when stressed by extremes in pH, temperature, osmolarity, or nutrient deprivation or other unfavorable environmental factors.[25] Ultrami-crocells are generally studied in the context of oligotrophic environments such as marine environments, drinking water systems, and bottled water.[26–28] Subpopulations of shrunken *Escherichia coli* and *Legionella* species cultivated in sterilized river water were capable of passing through a 0.2-μm syringe filter in percentages comparable to those observed for the starved *S. marcescens* cells used in this study.[29] The starved *S. marcescens* observed by TEM imaging averaged 0.6 μm in width, approximately twice the width observed for *B. diminuta*.[29] Sundaram et al.[23] demonstrated that bacteria up to 0.68 μm in width can penetrate filters with a nominal 0.2μm pore size and that the width of the bacteria, instead of the length, determined their ability to penetrate filter pores. Other clinically relevant organisms, including *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, *Pseudomonas putida*,[23] *B. contaminans*,[11] and *R. pickettii*,[30] can also penetrate 0.2-μm filters.

The filtered solution's hydrophobicity, osmolarity, and pH may reduce filter efficacy if they are not chemically and physically compatible with the filter.[3] Similarly, these factors may increase the elasticity of bacteria, enhancing their ability to penetrate filters in a way not predicted by the nominal pore size alone.[31] Particulate matter may interfere with the efficiency of filters by increasing the pressure across parts of the filter membrane that are not blocked.[32] Although the onsite investigation raised concern about changing filters—because doing so creates a break in the sterile system[1]—our experiments demonstrated that breakthrough events can occur in the absence of filter changes. Pharmacy A's practice of storing the amino acid solution for up to 48 hours likely did not enrich any *S. marcescens* that were present.[33] However, any viable cells may have resumed growth once the amino acid solution was compounded into TPN, which represents a more permissive environment in terms of nutrients, pH,[34] and temperature.

Detection of microbial breakthrough is dependent on sample timing and volume. In this study, *S. marcescens* was detected in the first collected liter of filtrate only. Similarly, an investigation of 0.2μm filter challenges with *R. pickettii* cultured for a week in 0.9% sodium chloride solution documented the greatest filter penetration in the earliest collected filtrate fractions.[30] Our detection of breakthrough required a minimum filtrate volume of 100 mL. On the basis of our testing, it is likely that the volumes of compounded amino acid solution submitted by pharmacy A[1] to laboratories for sterility testing were insufficient for the detection of small numbers of heterogeneously distributed organisms, whereas the 10% volume recommended in *USP* chapter 71[5] increases the likelihood of detecting bacterial contamination and more closely approximates the volumes examined in our study.

These experiments likely underestimate the degree and frequency of breakthrough events in filtration and thus represent conservative findings. The bacterial burden for the filter challenges using starved cultures was lower than the recommended number of challenge organisms (10^{10} CFU).[20] The membrane filters used for enumeration of breakthrough organisms had pore sizes of 0.45 μm, which may have allowed some ultra-microcells to pass through.

The nearly 3-$\log_{10}$ range in the number of *S. marcescens* recovered downstream of 15% amino acid filtration suggests that the failure conditions are at the threshold of a breakthrough event. Sufficient volumes of product must be tested as described in *USP* chapter 71 to detect low numbers of penetrating microorganisms. The inclusion of the prefiltration step as recommended by *USP* chapter 797[3] restored the integrity of the filtration process in one of the filter challenges with starved *S. marcescens* in amino acid solution. The use of a prefilter upstream of the sterilizing filter provides an additional layer of protection by reducing both the microbial burden of the solution and removing particulate matter in solution that could cause fluctuations in hydrostatic pressure encountered by individual pores of the 0.2-μm filter. Alternatively, a 0.1μm filter may be used for filtration of high-risk compounding products.[21,23]

Filters cannot be expected to retain bacteria on the basis of pore size alone and must also be tested experimentally for their specific application.[22] For example, pharmacy A had previously compounded cardioplegia solution using the same batch size and mixing and filtration practices used for the 15% amino acid solution but without any known contamination.[1] A standardized challenge organism cultivated in nutrient-rich solution may not be adequate to address the variety of conditions encountered during filtration processes. In addition, the universal use of a prefiltration step or of 0.1-μm filters for terminal sterilization would be cost prohibitive. Thus, empirical evaluations of specific drug solutions and bioburden organisms that may be introduced by the ingredients and the production process would be necessary to determine the filtration needs of any particular compounding process. Adherence to these recommendations by compounding pharmacies is vital to maintaining patient health in the face of ongoing drug shortages.[35–37]

Conclusion

Laboratory simulation testing revealed that under certain conditions, bacteria can pass through 0.2- and 0.22-μm filters intended for sterilization of an amino acid solution. Bacteria did not pass through 0.2-μm filters when a 0.5-μm prefilter was used.

Acknowledgments

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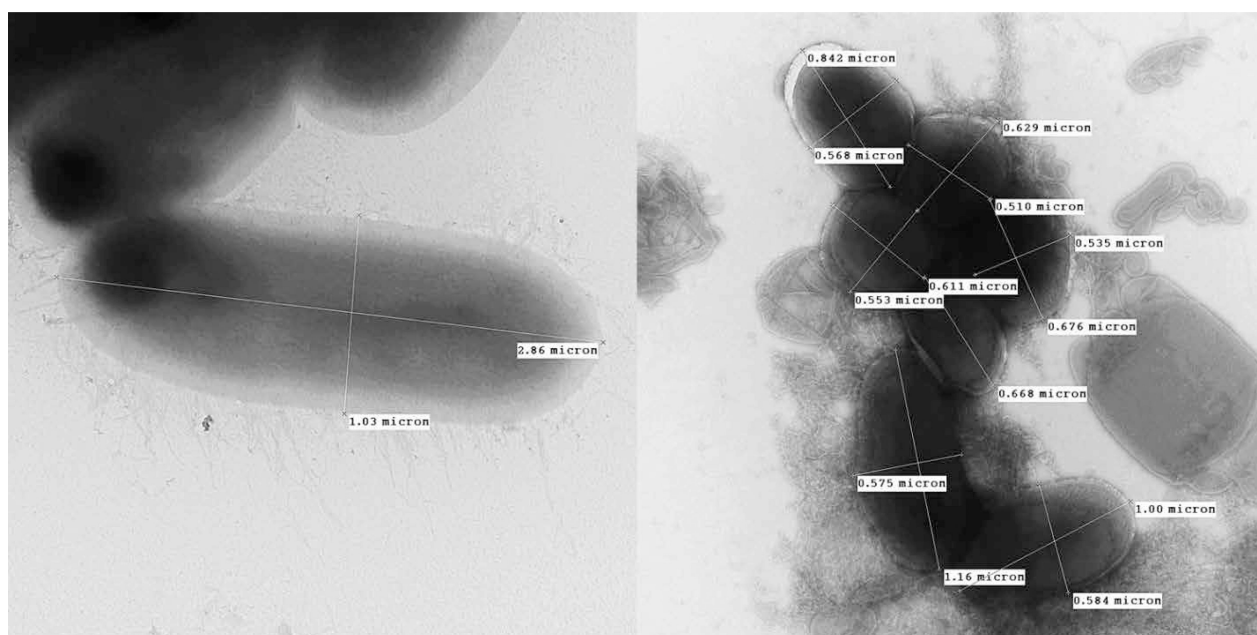
Figure 1.
Transmission electron microscopy image (9300× magnification) showing dimensions of a strain of *Serratia marcescens* cultured overnight (left) and starved in sterile water for one year (right).

Table 1.

Reduction by Syringe-Driven Disk Filters of Bacterial Counts for Organisms Starved for 120 Days in Sterile Water[a]

Organism	Bacterial Count Before Filtration (Mean ± S.D. CFU/mL)	0.22-μm Filter		0.1-μm Filter	
		Mean Bacterial Count After Filtration (CFU/mL)	Log Reduction	Mean Bacterial Count After Filtration (CFU/mL)	Log Reduction
Serratia marcescens outbreak strain	$(6.6 \pm 0.6) \times 10^7$	7.6×10^4	2.94	70	5.97
Brevundimonas diminuta ATCC 19162	$(2.0 \pm 0.1) \times 10^7$	0	7.3	0	7.3

[a] $n = 3$ for all experiments. CFU = colony-forming units.

Table 2.

Reduction by 0.2-μm Capsule Filters of Bacterial Counts for Starved and Overnight Cultures of *Serratia marcescens* and *Brevundimonas diminuta* ATCC 19162[a]

Organism and Culture Condition	Bacterial top Count (Mean ± S.D. CFU)	
	Before Filtration	After Filtration[b]
S. marcescens, starved		
In water	$(5.4 \pm 0.4) \times 10^8$	0
In amino acids	$(6.2 \pm 1.1) \times 10^8$	Replicate 1[c]: $(3.5 \pm 0.5) \times 10^3$
		Replicate 2[c]: $(5.7 \pm 2.3) \times 10^1$
		Replicate 3: 0
In amino acids with 0.5-μm prefiltration	$(2.3 \pm 0.03) \times 10^9$	0
S. marcescens, overnight		
In water	$(1.7 \pm 0.7) \times 10^{10}$	0
In amino acids	$(3.9 \pm 0.2) \times 10^{10}$	0
B. diminuta ATCC 19162, starved		
In water	$(3.9 \pm 0.2) \times 10^{10}$	0
In amino acids	$(1.4 \pm 0.3) \times 10^{10}$	0
B. diminuta ATCC 19162, overnight		
In water	$(2.5 \pm 0.2) \times 10^9$	0
In amino acids	$(5.0 \pm 0.3) \times 10^9$	0

[a] n = 3 for all experiments. CFU = colony-forming units.

[b]Unless otherwise noted, a value of 0 indicates no recovery of bacteria from any of the three experiment replicates.

[c]Bacteria were recovered only from the first fraction collected.