APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1985, p. 1343–1348
0099-2240/85/121343-06$02.00/0
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Vol. 50, No. 6

Factors Associated with *Pseudomonas pickettii* Intrinsic Contamination of Commercial Respiratory Therapy Solutions Marketed as Sterile

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Received 10 May 1985/Accepted 25 August 1985

Laboratory investigations were conducted to study the growth dynamics of *Pseudomonas pickettii* in commercial 0.9% sodium chloride solution under various environmental conditions and to determine the retention of these organisms after challenge through a 0.2-μm cartridge filter system. Low numbers of *P. pickettii* (1 to 10 CFU/ml of test solution) inoculated into commercial vials containing 5 ml of 0.9% sodium chloride solution and 500-ml volumes of 0.9% sodium chloride solution were shown to proliferate over a 168-h incubation period. These organisms demonstrated growth over a wide range of temperatures (15 to 42°C) in this salt solution, and survival studies at 50, 55, and 60°C indicated that this strain was not unusually resistant to heat (with the times required at a given temperature to reduce the surviving microbial population 10-fold [D-values] being 26.0, 1.9, and 0.7 min, respectively). A challenge test demonstrated that *P. pickettii* organisms were not completely retained by a 0.2-μm cartridge filter. The number of organisms detected increased from 1 CFU/liter of effluent at 1 to 2 min to a maximum of 176 CFU/liter at 4 to 5 min. Our results indicate that *P. pickettii* can penetrate a 0.2-μm filtration system and that the passage of organisms and subsequent microbial growth in the filter effluent probably are the mechanisms by which these organisms were recovered from "sterile" commercial 0.9% sodium chloride solution.

Recent epidemiologic and microbiologic investigations have shown that commercially prepared plastic vials containing 5 ml of 0.9% sodium chloride solution (saline), Modu-Dose (Becton Dickinson Respiratory Systems, Lincoln Park, N.J.), were intrinsically contaminated with *Pseudomonas pickettii* and were the source of these organisms that colonized the respiratory tracts of neonates (2). Once the manufacturer confirmed the bacterial contamination of saline vials with *P. pickettii*, a voluntary product recall was initiated by the producing company, and a recall notice was issued by the Food and Drug Administration (5). Our investigation of the processing line of the manufacturing plant that produced these 5-ml vials of saline demonstrated recovery of *P. pickettii* from deionized water and from sampling points further down the processing line (R. L. Anderson, M. M. McNeil, B. J. Davis, M. S. Favero, D. C. Mackel, and L. A. Bland, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, Q96, p. 220). This is the second reported incident of intrinsic contamination of a sterile solution with *P. pickettii*. The first outbreak, which occurred in Great Britain (15), involved contaminated water that was purified in the hospital pharmacy. The intrinsically contaminated product resulted in patient colonization and infection with *Pseudomonas thomasii* (which is a biovar of *P. pickettii*), and improper autoclaving procedures were identified as the major contributory cause of nonsterility.

We initiated laboratory studies to better understand the growth dynamics and the presence of *P. pickettii* in finished products. In the laboratory, we tested hypotheses to account for the contamination of these 0.9% saline solutions with high numbers of *P. pickettii* after the sterile-fill process of the manufacturing plant. We studied *P. pickettii* organisms under a variety of environmental conditions, including their growth in small and large volumes of commercial 0.9% saline, the effect of temperature on their growth in saline, their heat tolerance, electron microscopic examination of cells from the contaminated product, and 0.2-μm filter microbial challenge.

MATERIALS AND METHODS

Growth studies in a small volume of 0.9% saline. *P. pickettii* originally recovered from contaminated 5-ml Modu-Dose vials containing 0.9% sodium chloride solution was used to inoculate 5-ml uncontaminated vials of saline for growth studies. Two final inocula were used in the test solutions: low (1 to 10 CFU/ml) and high (10^2 to 10^3 CFU/ml). For the inocula, 0.1 ml was taken from the appropriate 10-fold dilution and added to each test vial with a 1-ml sterile tuberculin syringe with attached needle. Vials were mixed; incubated at 25°C; and sampled at 0, 6, 24, 30, 48, 72, and 168 h.

At the time of sampling, 5-ml test vials were thoroughly mixed, and two 0.2-ml fractions were removed by syringe and needle; one fraction was diluted 10-fold (10^{-1} to 10^{-3}). The undiluted 0.2-ml suspension and 0.2 ml from each test dilution were then separately added to the surface of Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy agar containing 5% sheep erythrocytes (TSAB) plates. Test fractions were evenly spread over the agar surface with individual sterile, bent-glass L-rods, and plates were incubated at 35 to 37°C for 48 h. After incubation, colonies on TSAB plates were enumerated and recorded as CFU/ml.

Growth studies in a large volume of 0.9% saline. Two 500-ml quantities of sterile 0.9% sodium chloride solution were inoculated with *P. pickettii* recovered from contami-

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nated Modu-Dose 5-ml vials: (i) pooled saline from 100 5-ml Modu-Dose vials that were from a different lot and that were not contaminated and (ii) one 500-ml bottle of commercial 0.9% saline for injection (Cutter Laboratories, Inc., Berkeley, Calif.). Desired inocula per milliliter of test solution were 1 to 10 CFU. Incubation temperatures and designated sampling times were as described above for growth studies with a small volume of 0.9% saline.

Sampling was performed by the membrane filter technique. Undiluted test fractions of 1.0 ml were added to 9.0-ml sterile water dilution blanks and 1.0 ml of the undiluted test suspensions and 1.0 ml from each of four 10-fold dilutions (10^{-1} to 10^{-4}) were sampled. The entire sample was passed through sterile sampling tubes attached to bacteriologic 0.45-μm membrane field monitor filters (Millipore Corp., Bedford, Mass.). Filters were aseptically removed and placed on the surface of TSAB plates; plates were incubated at 35 to 37°C for 48 h.

Effect of temperature on the growth of *P. pickettii*. Six 500-ml bottles of commercial 0.9% sodium chloride solution for injection were used to study the effect of temperature on the growth of *P. pickettii*. Inocula were prepared from contaminated Modu-Dose 5-ml vials; a final concentration of approximately 1 to 10 CFU/ml of test saline was desired. The appropriate inoculum was determined from 10-fold dilutions and added to each of the six test bottles. Bottles were mixed, and one bottle each was incubated at 15, 22, 25, 30, 35, and 42°C. Samples were removed at 0, 6, 24, 30, 48, 72, and 168 h and assayed by the membrane filter technique as described above.

Heat resistance studies. Experiments were performed to determine the heat resistivity of *P. pickettii* in 0.9% sodium chloride solution. Organisms were grown in 0.9% saline to a level of 10^4 to 10^5 CFU/ml. Fractions (10.0 ml) were removed, placed in sterile screw-cap tubes (15 by 125 mm), and separately exposed to water bath temperatures of 50, 55, and 60°C. Sample fractions were removed from the 50°C exposure at 0, 15, 30, 45, 60, 75, and 90 min; from the 55°C exposure at 0, 3, 6, 9, 12, 15, and 18 min; and from the 60°C exposure at 1-min intervals through 8 min to determine the number of surviving organisms. A temperature come-up time of 3 min was previously determined and used to adjust exposure time in all test systems.

Two 1-ml fractions were removed at each sampling time: one fraction was added to a 9.0-ml sterile water dilution blank and the other was used to make four 10-fold dilutions (10^{-1} to 10^{-4}). All of the samples were membrane filtered, and the filters were plated as previously described.

Temperature survival data with the time required at a given temperature to reduce the surviving microbial population 10-fold (D value) and the number of degrees of temperature change required for the D value to change by a factor of 10 (Z value) were calculated by methods previously described by Pflug and Holcomb (14).

Preparation of *P. pickettii* for electron microscopy. *P. pickettii* grown in 3,000 ml of 0.9% sodium chloride solution (10^6 CFU/ml) and organisms pooled from 240 5-ml vials (1,200 ml) of Modu-Dose (4.5×10^3 CFU/ml) were separately filtered to concentrate microbial cells with a 0.4-μm membrane filter (Nuclepore Corp., Pleasanton, Calif.). Each filter was then ultrasonicated for 1.5 min in 5 ml of commercially prepared sterile water, and the resulting fluid was centrifuged for 15 min at 6,500 × *g* in a microcentrifuge (Brinkmann Instruments, Inc., Westbury, N.Y.). The centrifugate was removed, and the pellet was suspended in 0.1 ml of commercially prepared sterile water for injection (Cutter Laboratories). Samples for electron microscopy were prepared from this microbial suspension.

Copper grids, covered with carbon-coated Parlodion (Mallinckrodt, Inc., St. Louis, Mo.), were inverted on a drop of 0.5% aqueous bovine serum albumin. The grid was then inverted on a drop of microbial suspension, blotted dry, and stained with 1% aqueous uranyl acetate. Specimens were examined under 100 kV in an RCA-EMU4 electron microscope.

Filter challenge studies. Two 0.2-μm cartridge filter elements (Ultipor AB; Pall Corp., Glen Cove, N.Y.), identical to and produced by the same manufacturer as the sterilizing filter used in the manufacturing plant, were purchased, along with an appropriate filter housing. Before testing, the filter was placed in the filter housing, and the entire assembly was sterilized with ethylene oxide according to the recommendation of the manufacturer. The sterile filter assembly was then placed in a closed-loop circulation system consisting of a pump, a flow meter, filter influent and effluent sampling ports, and a 20-liter fluid reservoir. All components in the fluid circuit, except the sterilized filter assembly, were steam sterilized. The circulating fluid was sterile normal saline (0.9% NaCl).

To approximate the product flow rate at the manufacturing plant, the sterile saline was recirculated through the closed-loop system at 1 liter/min. Prior to microbial challenge of the filter, 1-liter samples of the circulating sterile saline were collected from the filter's influent and effluent ports for microbial analysis. The filter was subsequently challenged by removing the pump inlet tube from the sterile saline reservoir and placing it in a 4-liter flask of normal saline inoculated 5 days previously with *P. pickettii* and containing 6.0×10^5 CFU/ml. The inocula of *P. pickettii* were obtained from contaminated 5-ml Modu-Dose product vials by emptying their contents directly into a carboy of sterile 0.9% sodium chloride solution.

The 4-liter challenge of *P. pickettii*-contaminated saline was then pumped through the filter at the same flow rate (1 liter/min). A total challenge of 2.4×10^9 organisms was given to the filter. Immediately after the 4 liters of contaminated saline had been pumped through the filter, the pump inlet tube was placed back into the sterile saline reservoir, and circulation was continued at 1 liter/min. The filter effluent was collected in 1-liter samples at 0, 1, 2, 3, 4, 5, 6, 10, 15, 30, and 60 min after the start of the bacterial challenge.

All samples were assayed by the standard membrane filter technique. Membrane filters (0.45-μm) were placed on TSAB plates and incubated at 30°C, and colonies were counted at 48 h. A 0.45-μm membrane filter was used for the bacterial assays because it has been shown to provide a significantly higher recovery of naturally occurring water microorganisms when compared with a 0.22-μm membrane filter (1).

RESULTS

Growth of *P. pickettii* in small and large volumes of 0.9% sodium chloride solution. Low numbers of *P. pickettii* (5 to 50 CFU), when inoculated into Modu-Dose vials containing 5 ml of 0.9% sodium chloride solution, were shown to proliferate over a 168-h incubation period (Fig. 1). The following log increases in CFU per milliliter were observed: 1 log unit at 24 h, 2 log units at 48 h, and 4 log units at 72 h. Modu-Dose 5-ml vials inoculated with large numbers of *P. pickettii* (10^3) demonstrated logarithmic growth and reached a level of 10^6 CFU/ml at 168 h.

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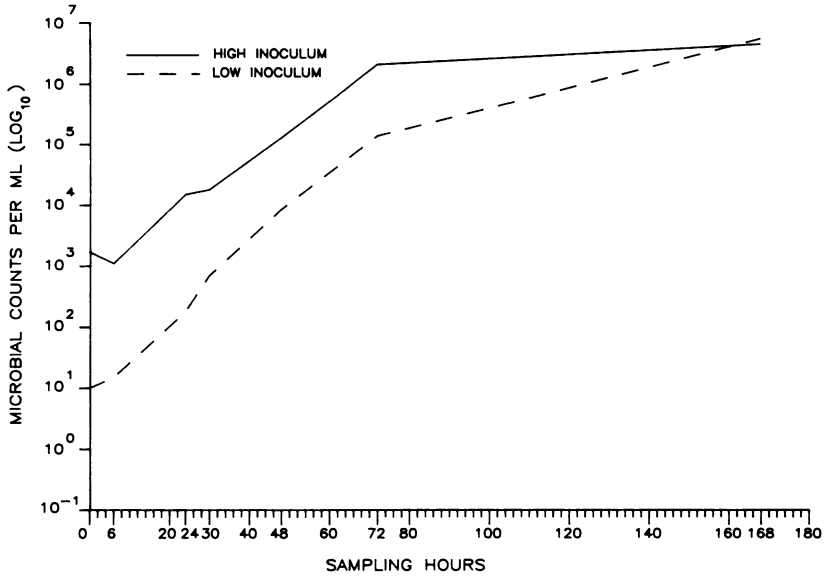

FIG. 1. Growth of *P. pickettii* in 5-ml Modu-Dose 0.9% saline vials at 25°C.

Growth of *P. pickettii* was also observed in 500-ml volumes of commercial 0.9% sodium chloride solution that were examined (Fig. 2). Low inocula of less than 1 CFU/ml were added to the 500-ml test solutions and shown to proliferate to high numbers (10^6 CFU/ml) during the 168-h study period.

Growth of *P. pickettii* at various temperatures. The growth of *P. pickettii* in 0.9% sodium chloride solution at various temperatures is shown in Fig. 3 and 4. Organisms demonstrated excellent growth at incubation temperatures of 22, 25, 30, 35, and 42°C. Growth was slower at 15°C, and a 1-log increase was observed at 168 h.

Effect of heat on *P. pickettii*. The effect of temperature on the survival of *P. pickettii* grown in 0.9% sodium chloride solution is illustrated in Table 1. The following D values were observed: at 50°C, 26.0 min; at 55°C, 1.9 min; at 60°C, 0.7 min. A Z value of 6.3°C was determined from the calculated D values.

Examination of *P. pickettii* by electron microscopy. *P. pickettii* organisms grown in commercial 0.9% saline were more bacillus shaped and consistent in morphology. Bacterial cells recovered from intrinsically contaminated 5-ml Modu-Dose saline vials were smaller, more spherical, and slightly pleomorphic. The average length of cells recovered from Modu-Dose vials was 1.33 μm, whereas the length of cells grown in 0.9% saline was 2.03 μm; the average width of the cells was 0.88 and 0.81 μm, respectively.

Challenge of *P. pickettii* to a 0.2-μm cartridge filter. Microbial assay of the circulating 0.9% saline prior to the filter challenge revealed no growth of *P. pickettii* and indicated that a sterile fluid circulating system had been achieved.

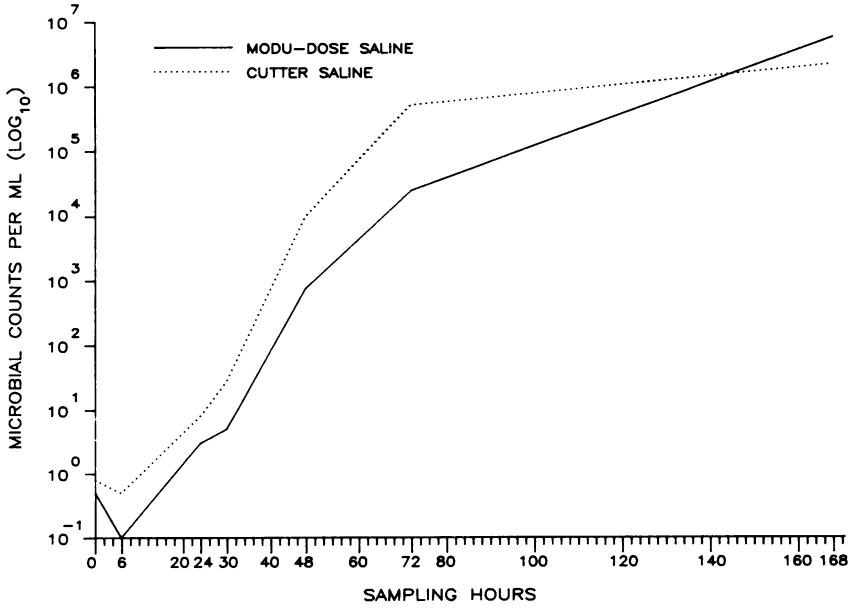

FIG. 2. Growth of *P. pickettii* in large-volume commercial 0.9% saline at 25°C.

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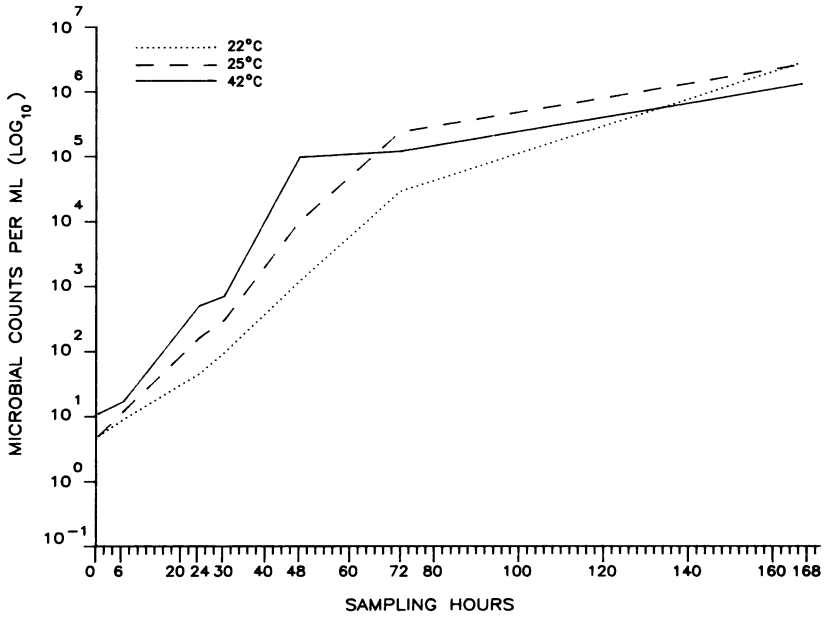

FIG. 3. Growth of *P. pickettii* in large-volume commercial 0.9% saline at various temperatures.

Within 1 to 2 min after the bacterial challenge to the filter was initiated, *P. pickettii* organisms were detected in the effluent of the filter. The number of organisms detected increased from 1 CFU/liter at 1 to 2 min to a maximum of 176 CFU/liter at 4 to 5 min (Fig. 5). Bacterial counts then gradually decreased to 6 CFU/liter at 30 min and to 2 CFU/liter at 60 min.

DISCUSSION

P. pickettii is an aerobic, nonfermentative, gram-negative bacillus that has been recovered from clinical specimens and from other environmental sources such as water distribution systems (10, 15, 16, 18, 21). Correct identification of this organism depends on reactions to a battery of conventional biochemical tests (8, 11, 16–18, 23), and accurate identification may not always be possible when an automated system is used (3). Biochemical reactions of *P. pickettii* are very similar to those for *Achromobacter xylosoxidans*. *P. pickettii*, including biovars Va-1, Va-2, and *P. thomasii*, are thought to be a low virulence, but there have been isolated reports of bacteremia and meningitis with *P. pickettii* (4, 6). *P. thomasii* has also been responsible for an outbreak of hospital-acquired colonization and infection related to contamination of water purified in a hospital pharmacy by improper autoclaving procedures (15). More recently, six cases of septicemia occurred in cardiac intensive care unit

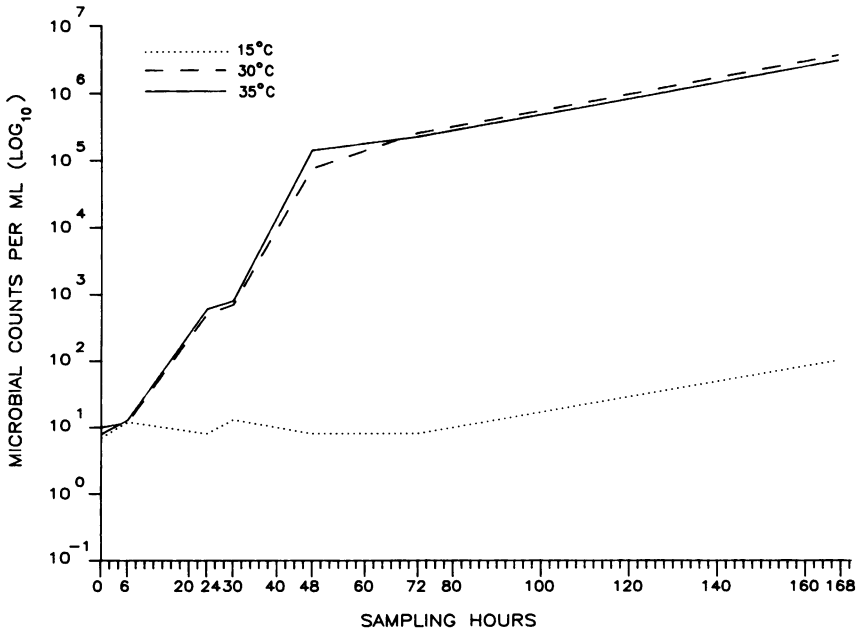

FIG. 4. Growth of *P. pickettii* in large-volume commercial 0.9% saline at various temperatures.

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TABLE 1. Effect of temperature on the survival of *P. pickettii* grown in 0.9% sodium chloride solution

Exposure temp (°C [°F])	D value (min)[a]	Z value (°C [°F])[b]
50 (122)	26.0	
55 (131)	1.9	
60 (140)	0.7	6.3 (43.3)

[a] The time required at a given temperature to reduce the surviving microbial population 10-fold.
[b] The number of degrees of temperature change required for the D value to change by a factor of 10.

patients with *P. pickettii* of biovar Va-1 (10). The associated reservoir of infection with this nosocomial outbreak was a 0.05% aqueous solution of chlorhexidine prepared with contaminated bidistilled water. In addition, an outbreak of pseudobacteremia caused by *P. pickettii* (Va-1) has been reported (21). The common source was aqueous chlorhexidine antiseptic solution that was prepared by the hospital pharmacy with contaminated distilled water.

The laboratory studies reported here were designed to help explain why a 0.9% saline solution marketed as sterile was contaminated with *P. pickettii*. An on-site inspection of the manufacturing plant producing this saline product provided information on the actual processing steps (M. M. McNeil, S. L. Solomon, R. L. Anderson, B. J. Davis, R. F. Spengler, B. E. Reisberg, and W. J. Martone, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, L14, p. 309), which led to the development of rationale for the laboratory investigations described here (11a). The primary manufacturing steps for this product include the treatment of city water (softening, deionization, and distillation), retention of distilled water in a 180°F (82.2°C) holding tank (holding in this 1,000-gallon [3,785-liter] tank had been bypassed when the contaminated lots of 0.9% saline were produced), cooling of water in a heat exchanger, product mixing and 0.2-μm filtration, and, finally, storage of the filtered saline product in a second holding tank prior to a final 0.2-μm filtration (in a separate, automated, sterile-fill process room) and packaging (11a). Based on these observed manufacturing practices, the following areas were investigated: effect of temperature on the growth of *P. pickettii* in 0.9% saline, heat resistivity of this organism, electron microscopic examination of *P. pickettii*, and filter retention studies.

P. pickettii organisms were shown to proliferate in small (i.e., in 5-ml Modu-Dose vials) and large (500-ml) volumes of 0.9% commercial saline (Fig. 1 through 4). This finding strengthens the hypothesis that growth of this organism in saline could have occurred at different production stages along the processing line (e.g., in the product mixing and holding tanks or in the finished saline). This organism also was shown to be able to grow in 0.9% saline over a wide range of incubation temperatures, including 15 and 42°C. Most certainly the range of temperatures that existed along the processing line at the manufacturing plant was extensive.

During production of the contaminated lots of 0.9% saline, the step of placing water distilled at 82.2°C in a 1,000-gallon (3,785-liter) holding tank was bypassed. Results of our laboratory studies on the heat resistivity of *P. pickettii* to different temperatures (i.e., 50, 55, and 60°C; Table 1) show that the normal temperature of the water in the holding tank (82.2°C) would have been lethal to the contaminating strain. However, because the high-temperature holding tank was bypassed, *P. pickettii* organisms in the process water could have contaminated or colonized product reservoirs and distribution lines downstream of the high-temperature holding tank and established the potential for growth in subsequently produced saline solutions.

Electron micrographs of *P. pickettii* organisms in contaminated commercial 5-ml saline vials and of those grown in large volumes of commercial saline in our laboratory showed differences in cellular morphology. *P. pickettii* recovered from intrinsically contaminated 5-ml Modu-Dose saline vials were smaller and more round or oval; freshly grown organisms in large volumes of saline were more elongated and consistent in morphology. These microscopic findings may be relevant to the intrinsic contamination in the 5-ml saline vials. In a recent study, coccoidal, spheroplast-like forms

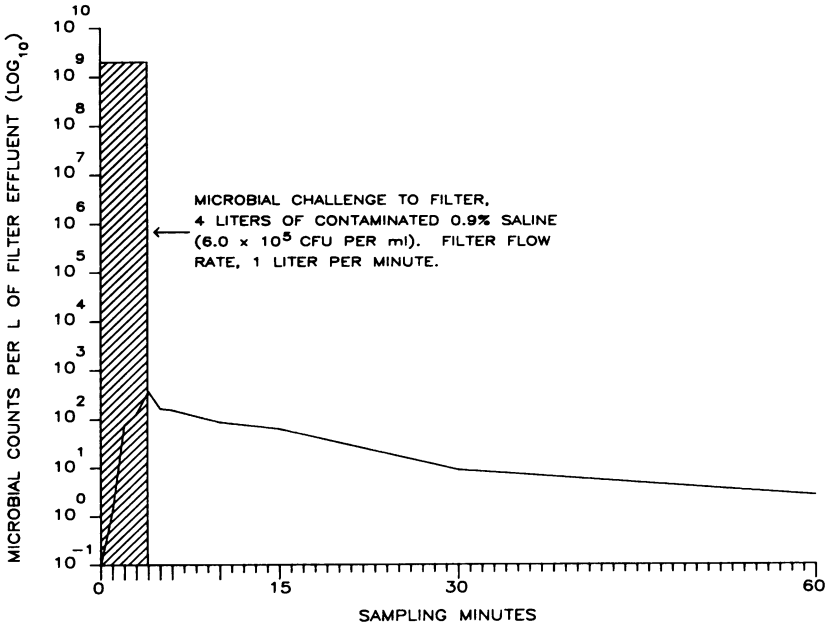

FIG. 5. *P. pickettii* challenge of a 0.2-μm absolute cartridge filter.

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(i.e., cell wall-defective bacteria) of *P. pickettii* Va-2 were shown to pass through a 0.2-μm filter (13). The presence of *P. pickettii* organisms in the finished product (0.9% saline) could suggest the ability of this organism to pass through a 0.2-μm filter and indicates that these *P. pickettii* are deformable and capable of squeezing or growing through a small filter pore size. Investigations have recently demonstrated that *Pseudomonas diminuta* can grow through polyvinylidene difluoride and nylon 0.2-μm cartridge and membrane filters (19); in addition, persistent passage of *P. diminuta* through 0.45-μm membranes is well documented. A transmissible agent capable of passing through a 0.2-μm system into saline could be *P. pickettii* organisms that have defective cell walls.

Since *P. pickettii* organisms were recovered from supposedly sterile 0.9% saline, passage of this organism through the final-sterilizing 0.2-μm filtering system into the filter effluent (i.e., the filtrate) must have occurred. The 0.2-μm filtration system in the aseptic fill room was the final production step used to ensure sterility of the 0.9% saline. Our laboratory studies demonstrate that *P. pickettii* organisms (adapted to a fluid environment) could penetrate a 0.2-μm filtration system and colonize the effluent side. Other investigations also have demonstrated the passage of bacteria (most often species indigenous to water or small degenerate bacterial forms) through a 0.2-μm filtration system with resultant contamination of the "sterile" filtrate (7, 22). In addition, studies have documented the passage of 0.5-μm latex spheres through a 0.2-μm filter system (12), and a variety of other physical and biologic factors associated with filter efficiency have been described (12, 20). More recently, in-house manufacturing validation studies have demonstrated the passage of *P. pickettii* through 0.2- and 0.1-μm filters used for sterility testing (9).

Once the passage of *P. pickettii* organisms into the filtrate had occurred, they probably grew to large numbers in the 0.9% saline in the 5-ml Modu-Dose vials. Based on the results of these laboratory studies, caution should be used by manufacturing personnel when 0.2-μm filtration is used as the final sterilization step, and consideration should be given to using smaller porosity filters (e.g., 0.1-μm) whenever filter passage of water bacteria is suspected or in those cases in which high bioburdens (i.e., large numbers of bacteria) may exist along the production line.

ACKNOWLEDGMENTS

We thank Betty W. Holland, Janice K. Carr, and Sonia M. Aguero for technical assistance during these laboratory investigations. In addition, we greatly appreciate the consultation provided by Walter W. Bond concerning temperature survival curve data and the typing assistance provided by Nancy N. Sarratt and Peggy C. Hutton.

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