Nationwide Epidemic of Septicemia Caused by Contaminated Intravenous Products: Mechanisms of Intrinsic Contamination

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Between 1 July 1970 and April 1971, in many hospitals in this country, there were outbreaks of nosocomial septicemia caused by *Enterobacter cloacae* or *E. agglomerans* (formerly *Erwinia, herbicola-lathyri*). All of these hospitals used infusion products manufactured by one company, Abbott Laboratories, and all affected patients had onset of septicemia while receiving the company's infusion products. Septicemia was epidemiologically and microbiologically traced to intrinsic contamination of the company's screw-cap closure for infusion bottles which was sealed with a newly introduced elastomer liner. Epidemic organisms were isolated from these closures. Investigations both in the laboratory and in the manufacturing plant into the mechanism of contamination of these products revealed the following. (i) Epidemic strains were present in numerous areas throughout the manufacturing plants. (ii) Viable microorganisms gained access to the interior of screw-cap closures after the autoclave step of production. (iii) Cooling closures actively drew moisture through the thread interstices into the inner-most depths of the closure. (iv) Transfer of contaminants from closures to fluid was easily effected by simple manipulations duplicating normal in-hospital use. (v) The red-rubber liner used in the company's screw-cap closures before the introduction of elastomer contained a broad-spectrum antimicrobial inhibitor. The findings from this epidemic and the associated studies show that the screw-cap closure as it is now designed cannot be considered secure for products that must remain sterile.

Between 1 July 1970 and April 1971, many hospitals in this country that routinely used infusion products made by one manufacturer, Abbott Laboratories, experienced outbreaks of septicemia with *Enterobacter cloacae* or *Enterobacter agglomerans* (formerly called *Erwinia, herbicola-lathyri* group [10]) (3-5; D. G. Maki, F. S. Rhame, D. C. Mackel, and J. V. Bennett, *Am. J. Med.*, in press). Epidemiological and microbiological investigations showed that the epidemic was caused by intrinsic microbial contamination of a newly introduced screw-cap closure with an elastomer liner (Maki et al., *Am. J. Med.*, in press). The new elastomer-lined cap replaced caps incorporating shellacked paper (Gilsonite) and red rubber liners beginning in March 1970 (Fig. 1).

Studies were undertaken in late February 1971 to determine the mechanisms by which the product became contaminated during manufacture. Investigations were conducted at both of Abbott's two manufacturing plants in March 1971 at the invitation and with the cooperation

of the company. The studies reported here are based only on investigations at the Rocky Mount, N.C., plant. Screw-cap closures with elastomer liners were in use on all intravenous fluids produced by this plant at the time of these investigations. Additional experiments to determine the means by which closures and fluids became contaminated were conducted at the Center for Disease Control, Atlanta, Ga.

MATERIALS AND METHODS

The manufacturing process. The steps in production of infusion fluids in Abbott's Rocky Mount plant during the epidemic are depicted in Fig. 2. Bottles, after being inspected and washed, moved through the various production steps on a stainless steel conveyer belt. Caps were machine assembled from individual components within the plant each day, but assembled caps were routinely stored for several months before being used on bottles. Bottles were filled, loaded onto autoclave racks, autoclaved, unloaded to the conveyer belt, and rinsed, all by machine. They were then hand inspected, labeled and oversealed by machine, and the metal strap

GILSONITE

ELASTOMER

FIG. 1. Structures of Gilsonite and elastomer liners.

FIG. 2. Diagram of production sequence.

used for hanging the bottles was applied by hand. Finally, the bottles were reinspected and packaged by hand into cartons which were machine sealed and sent to the warehouse. Bottle breakage occurred along the conveyer belt, especially after autoclaving. This consistently averaged several percent of all bottles produced and the contents of these broken bottles, frequently containing glucose, spilled onto the floor. Loads that passed sterility testing usually remained in the warehouse for approximately 4 weeks before being shipped to storage depots or hospitals. There was, on the average, a 2- to 3-month interval between production and use of fluid in hospitals at the time of the outbreak.

Abbott conducted microbiological sampling of every autoclave load using the USP standard procedure (17) as required by law. Biological indicators were not used to assess autoclave function. Ten

bottles were selected for testing from every autoclave load. These bottles were manually removed from the autoclave. A 10-ml amount of fluid from each bottle was inoculated into thioglycolate broth and incubated for 7 days while the autoclave load of approximately 5,500 bottles was held in quarantine pending the outcome of sterility tests. Microbial growth in any of the 10 tubes designated the load a "tentative failure"; 20 additional bottles from the same load were immediately resampled by the same method. Growth in any of the second set of samples was considered a basis by the company for rejection and destruction of the entire load. Fluid contaminants were morphologically identified by Gram stain, but complete bacteriological identification was not routinely performed.

In 1969, 7 of about 2,200 autoclave loads showed growth on initial testing; however, only 1 of the 7 was ultimately rejected. In 1970, 31 of about 3,850

autoclave loads showed initial growth and 10 were ultimately rejected; 28 of these 31 autoclave loads occurred during the 9-month period from April through November 1970. This marked rise in positivity during 1970 coincided temporally with the introduction and progressive increase in use of elastomer-lined closures at the plant, which began in March 1970. Increased rates of lots failing the initial sterility test occurred during this time with autoclave loads of Gilsonite and elastomer, when compared with baseline 1969 rates (solely Gilsonite).

Besides the required microbiological quality control sampling of every autoclave load, Abbott carried out an environmental contamination surveillance program. Multiple water samples from the production line at various stages of the manufacturing process, especially from the autoclave, were sampled daily. The plant received potable municipal water from the town of Rocky Mount, which was 6 miles (about 9.6 km) away. Chlorine residuals in this water were consistently less than 0.1 part per million (ppm), and bacteriological testing had shown frequent contamination in the past. During 1970, high bacterial counts were occasionally recorded in water samples from the autoclave during the cooling cycle; however, organisms had not been microbiologically identified. Each autoclave load was monitored by thermo-couple testing. Because of the relatively low chlorine residuals in city water received in the plant, water used in the autoclave cooling cycle was either selectively chlorinated or passed through an ultraviolet decontaminator. After the sterilization step of the autoclave cycle, the load was slowly cooled by the autoclave condensate and chlorinated, or ultraviolet-treated water was progressively introduced. The autoclave coolant temperature immediately prior to removal of the load was approximately 25 C, and the temperature of the bottles at this point was about 40 C.

Microbiological methods. The enrichment broth used in all studies, both for field investigations and laboratory studies, was brain heart infusion broth enriched with 0.5% beef extract (EBHIB). Various amounts and concentrations of broth were used depending upon the final dilution resulting from the samples and sampling procedures used.

Intravenous fluid was cultured by the addition of concentrated EBHIB directly into the bottle (Maki et al., *Am. J. Med.*, in press). Studies on 107 one-liter bottles of fluid had compared this technique to membrane filtration under strict aseptic conditions in a laminar flow hood. One-half of each bottle was passed through a bacteriological membrane filtration system (0.45 μ m pore size, Millipore Corp.). The filter was then placed onto Trypticase soy agar containing 5% sheep blood with its surface premoistened with 0.2 ml of EBHIB. Samples were incubated for 48 h at 35 to 37 C and then at room temperature for 5 days. The remaining 500 ml in each bottle was cultured by adding an equal volume of double-strength EBHIB. These were incubated at 35 to 37 C and discarded at 7 days if no growth appeared. Contaminants in the fluid, including the epidemic strains, were isolated in a substantially

higher frequency from fluid that was sampled by the direct addition of concentrated broth (Table 1).

Unless stated otherwise, all cultures were incubated at 35 to 37 C for 18 to 24 h and then examined for growth. All cultures having no growth were incubated at room temperature for at least 5 more days and reexamined daily. All broth cultures showing growth were streaked to Trypticase soy agar containing 5.0% sheep blood (TSAB) and to MacConkey agar. Representatives of all distinct microbial colonies were picked and inoculated to triple sugar iron agar unless colonial morphology and Gram stain suggested the use of a different medium.

Standard procedures were used for the definitive identification of isolated organisms. Susceptibility of isolates to antibiotics was determined by a standardized disk testing technique (1).

RESULTS

A. Environmental studies at the production facility. The environmental studies at the production facility were designed to determine if the epidemic strains were present in the inanimate environment of the plant and, if so, where and in what numbers.

Samples were collected during normal production. Surface samples were taken with cotton swabs premoistened in EBHIB; these were rubbed over the sampling area and broken off into a tube containing 6 ml of broth. Samples of water and liquid quaternary ammonium compound (used as a conveyor belt lubrication) were filtered through a 0.45- μ m membrane filter. Membrane filters used for sampling belt lubricants were washed with an equal volume of EBHIB-containing neutralizers, 0.5% Tween 80, and 0.07% lecithin. In addition, portions of belt lubricant were inoculated directly into neutralizing broth for culture. Air sampling was performed by 10-min exposures of open petri dishes containing TSAB.

Of the 96 environmental samples, 16 (17%)

TABLE 1. *Comparative microbiological results from direct addition of broth and membrane filtration techniques*

	No. of bottles with:	
	Any organisms	Epidemic organisms
Positive only by broth technique	17	5
Positive only by membrane filtration techniques	0	0
Positive by both methods	25	0
Negative by both techniques	65	102

yielded the epidemic organisms *E. cloacae* or *E. agglomerans*. Table 2 shows the various locations in the plant where the epidemic-associated organisms were found; they were from surfaces, air, and materials used in these areas.

Multiple samples from water from various points in the processing plant yielded contamination, most commonly *Bacillus* species; *E. cloacae* and *E. agglomerans* were not isolated from water.

High levels of microbial contamination were detected on 10 exposed settling plates placed in areas used for rinsing and inspecting bottles after autoclaving. *E. agglomerans* was isolated from three samples, *E. cloacae* was isolated from two, and *Klebsiella pneumoniae*, including indole-positive strains, was isolated from seven. Other organisms from these samples included *Pseudomonas* sp., both oxidative and nonoxidative *Acinetobacter calcoaceticus*, *Bacillus* sp., *Corynebacterium* sp., *Planococcus* sp., *Staphylococcus epidermidis*, and fungi.

The antibiotic susceptibility patterns of the environmental epidemic strains were identical to those found in human blood isolates (Maki et al., Am. J. Med., in press).

E. cloacae and *E. agglomerans* were prevalent in areas corresponding to several post-autoclave production stages, particularly those steps immediately postautoclave where bottle breakage was high and the floor was frequently splashed with parenteral solution. The unique

and selective ability of these microorganisms, members of the tribe *Klebsiellae*, to grow readily at room temperature in commercial fluids containing glucose may account for their predominance in these areas (13).

B. Culture studies of closures during normal production. The culture studies of closures during normal production were conducted to ascertain the point or points in the production process where contamination was introduced into the bottle closure.

Unused cap components and assembled caps were pooled (20 components with 10 cap shells or assembled caps per pool) and immersed in flasks containing EBHIB.

Samples of caps and bottle threads were examined immediately after completion of various stages in a normal production run of elastomer-capped 1,000-ml bottles of 5% dextrose in 0.25% normal saline. The elastomer liner was extracted from the cap and cultured as previously described (Maki et al., Am. J. Med., in press). Thread areas were sampled with a swab premoistened in EBHIB immediately after removal of the cap.

In addition, a second group of bottles containing the above solution were specially marked and uncapped, and the mouth of each bottle was covered with a metal test tube closure. These bottles were then sterilized in a laboratory autoclave at 121 C for 60 min and cooled to ambient temperature in the autoclave. Test tube closures were then removed from the bottles, and presterilized elastomer closures were applied on the capping machine. The sterility of these test bottles was confirmed by test of a subsample. Twelve presterilized test bottles were inserted into the production line at the start of nine different steps in production including postautoclave. Immediately after completion of a step, the 12 inserted test bottles and 12 randomly chosen bottles that had also just completed the regular production sequence up to that point were removed from the production line and taken to the laboratory for immediate sampling.

In conjunction with the above, samples were collected from water used to cool the autoclaves for bacteriological examination and testing for available and total chlorine content.

All cultures of stock cap shells and completed caps were negative. One of 13 pools of elastomer liners yielded *Pseudomonas aeruginosa*.

Contamination of elastomer cap liners or thread areas of bottles from the regular production run was found at each stage of production except immediately after autoclaving (Table 3). *E. cloacae* predominated among isolates obtained from samples collected before exposure

TABLE 2. Study A: Environmental cultures of surfaces, air, and materials by production area

Production area sampled	No. of samples	No. positive	
		<i>E. cloacae</i>	<i>E. agglomerans</i>
Preautoclave			
Before filling and capping	42	3	1
Filling and capping	5	0	0
Rack loader	4	1	0
Autoclave	9	0	0
Subtotal preautoclave	60	4	1
Postautoclave			
Rack unloader	8	0	2
Rinse	10	3	1
Inspection	7	0	4
Overseal	4	0	0
Bailing	2	1	0
Casing	5	0	0
Warehouse	0		
Subtotal postautoclave	36	4	7

TABLE 3. Study B: Frequency of contamination of elastomer liner or thread areas from randomly selected bottles at completion of various stages of normal production

Production step immediately preceding sample	Sampled	No. of bottles	
		Contamination in liner or thread area	Contamination with <i>E. cloacae</i> in liner or thread
Preautoclave			
Filling and capping	12	9	9
Rack loader	12	6	4
Autoclave	12	0	0
Postautoclave			
Rack un-loader	12	2	0
Rinse	12	6	0
Inspection	12	3	1
Overseal	0	—	—
Bailing	12	7	0
Casing		2	0
Warehouse (24-h storage)	12	9	0
Postautoclave subtotal	72	29	1

to the autoclave; nearly half of all such samples were positive for this organism. *E. cloacae* was detected on the elastomer liners of 9 of 12 bottles immediately after the filling and capping step, and from the thread areas of 4 of 12 bottles taken from the autoclave loader. Members of the *Acinetobacter* group greatly predominated among contaminants in postautoclave samples and were found only in such samples. Except for one isolate of *E. cloacae* from the threads of a bottle after the inspection step, no isolates of epidemic organisms were recovered from postautoclave samples.

Indole-positive *K. pneumoniae* organisms were isolated from the threads of two bottles immediately after application of the cap overseal, and from both threads and cap liner of another bottle after 1 day in the warehouse.

Moisture was not observed on internal cap components before autoclaving, but moisture was visible in all components inspected immediately after autoclaving and at all subsequent stages of processing, including after 1 day in the warehouse. Moisture throughout the inner cap assembly was noted to persist for at least 2 months after production.

None of the presterilized test bottles inserted into individual stages of the manufacturing process showed moisture in the closure, except those used to assess the autoclave step. Also, none showed contamination with *E. cloacae* or *E. agglomerans*. However, three were contaminated with other organisms—two closures examined after the inspection step and one closure examined after 1 day in the warehouse.

Analysis of water for available and total chlorine showed fluctuation throughout each day and from day to day in samples of autoclave cooling water taken during routine production, ranging from 0.0 to 0.1 ppm and 0.0 to 0.2 ppm, respectively. However, during the above studies, levels in the autoclave coolant were observed to be considerably higher than previously observed, 0.3 and 0.4 ppm for available and 0.6 and 0.9 ppm for total chlorine.

Only *Bacillus* species were identified in the cooling water.

Contamination was found in about 40% of closures taken from bottles randomly selected at the various production steps after autoclaving. However, the number of samples obtained at completion of the various steps in postautoclave production was insufficient to reliably establish the relative contribution of each step as a source of contamination.

Only 3 of 108 presterilized test bottles inserted at sequential steps were found to have contaminants in the elastomer liners or threads. This frequency of contamination was markedly less than found in normal production products sampled simultaneously. This dichotomy is probably best explained by temperature differences in the closure of the two groups which are characterized more fully by studies described below.

The unexplained and inordinately high chlorine levels in autoclave cooling water during the above experiments may also have influenced the frequency of contamination found.

C. Salt studies. The salt studies were conducted to ascertain whether coolant water could enter the inner portions of the cap assembly during the cooling cycle of the autoclave.

Salt added to autoclave coolant during production provided a convenient means of "labeling" water from this source. To prevent low-level sodium contamination of intravenous (i.v.) solution used in the test, the i.v. solution tank was rinsed repeatedly with distilled water until flame and silver nitrate tests were negative for sodium. Solution bottles were filled with 5% dextrose and water, capped with elastomer-lined closures, and loaded in the autoclave in a routine manner.

A control group of 20 bottles was not autoclaved and not exposed to salt-laden coolant.

In addition, a rubber finger cot was placed over the entire closure of a second control group of 20 production bottles which were positioned throughout the autoclave load in a random fashion.

The bottles in the autoclave were then sterilized in routine production fashion. As the temperature approached 100 C during the cooling cycle, approximately 700 lb (about 318 kg) of commercial bulk salt was rapidly introduced into the cooling water line at the autoclave pump. In the second test, the entire experiment was repeated, except salt was introduced when the temperature reached 60 C. In both tests closures were exposed for 10 to 20 min to coolant containing salt.

At the end of the cooling cycle, bottles were removed from the autoclave and immediately taken to the laboratory for analysis of sodium in the inner cap assembly components. The top of the cap was carefully removed with a sharp knife without altering the relationship of the cap to the bottle thread area. The technologist tested for sodium chloride by adding minute droplets of 0.1% silver nitrate to the various areas and looking for formation of white silver chloride precipitate. The presence of sodium within the intrinsic moisture found in various portions of the cap assembly was also tested for by probing with a clean platinum wire loop which was then flamed.

The salt concentration of the cooling water in the two experiments was 1.0 and 0.6%, respectively. In both experiments, whether salt was added at the beginning or the middle of the cooling cycle, sodium chloride was repeatedly detected in the interstices of the thread areas all the way to the elastomer disk of every closure tested (Fig. 1). Salt was also found in the space between the metal slip disk and the elastomer liner. No sodium chloride was detected on the inner surface of the elastomer disk that faces fluid or within the fluid itself.

No sodium chloride was detected in the 40 caps covered with rubber finger cots during the two experiments or in the control bottle caps from nonautoclaved bottles.

The above observations indicated that autoclave cooling water, if contaminated with microorganisms, could have served as a vehicle by which the inner cap assembly became contaminated. The absence of fluid in closures with an impervious rubber "overseal" suggested a way to prevent access of contaminated fluid to the closure during production.

D. Fluorescein dye studies of cap assemblies. Fluorescein dye was utilized as a marker

to study factors influencing migration of environmental moisture into the closure.

Twelve commercially obtained 1-liter bottles of D5/W with elastomer-lined caps were studied. Approximately 0.25 lb (about 113 g) of water-soluble uranine (sodium fluorescein) was dissolved in tap water in deep stainless steel pans at room temperature. Upright bottles were then exposed to the dye in three different ways.

In experiment D-1, four bottles, totally immersed in the dye solution, were heated to 100 C, atmospheric pressure, for 40 min. The bottles were then allowed to cool in the dye solution to approximately 65 to 70 C before removal and examination.

Experiment D-2 was similar to experiment D-1 except that four bottles were immersed in the dye solution only up to a line approximately 1 cm below the lower lip of the cap, i.e., at the lowermost thread of the neck.

In experiment D-3, four bottles were placed in a pan containing sufficient water without dye to create a fluid level at the middle of the upright bottles. The bottles were then heated to 100 C in an autoclave and allowed to cool to a temperature of approximately 75 to 80 C; at this time warmed dye solution (65 to 70 C) was poured from above onto the tops of the caps.

Four control bottles were handled exactly as the respective test bottles in each experiment except they were not heated.

Each bottle was thoroughly wiped dry with a clean Cel-Fiber paper towel immediately upon removal from the dye solution. When the bottle reached room temperature, the top of the cap was removed with a clean knife immediately above the plastic slip disk. Each inner component of the cap assembly was then carefully removed with clean hemostats and placed in separate petri dishes. The presence of dye on each individual component was tested using white and long- and short-wave ultraviolet light.

The control (nonheated) bottles showed no fluorescence beyond the threaded area in any of the experiments. In contrast, bottles which had been heated and allowed to cool had dye within the innermost portions of the closure, for example, between the slip disk and liner, in all three experiments.

Similar results were obtained when an equal number of bottles capped with Gilsonite-lined closures were subjected to identical experimental conditions.

This laboratory experiment confirmed the earlier studies (study B) which showed that movement of moisture into the closure depended upon a falling cap temperature. This

was not the sole mechanism of cap contamination, however, because control bottles at ambient temperature in study B also manifested contamination, although in a greatly reduced frequency.

E. Matched study comparing elastomer and Gilsonite closures. As previously noted (Maki et al., Am. J. Med., in press), no cases of septicemia were traced to Gilsonite-capped fluids during the nationwide outbreak. To determine the relative frequency and nature of contamination of closures and fluid, elastomer- and Gilsonite-capped fluids were processed in the same production load under comparable conditions of manufacture.

Approximately 1,400 bottles of D5/1/4 normal saline capped with Gilsonite closures were produced in the middle of a 20,000-bottle elastomer-capped production run. Both production autoclaves were employed to assess the differential risk of contamination between the two autoclaves in the plant. Immediately upon reaching the warehouse, random cases of bottles with each type of closure were selected and sent to the Center for Disease Control for microbiological testing of caps and fluid.

High-frequency contamination of both types of closures was observed (Table 4). However, Gilsonite liners were significantly less frequently contaminated (26.9%) than elastomer liners (85.0%) ($\chi^2 = 85.6$, $P < 0.005$). Furthermore, *E. cloacae* and *E. agglomerans* were isolated from 8.3% of closures lined with elastomer but from none of those lined with Gilsonite ($P = 0.001$, Fisher's exact test). Of 120 fluids from bottles with each type of closure, 64.1% of the elastomer-lined closures and 23.3% of the Gilsonite-lined closures were contaminated. Epidemic organisms were found in eight elastomer fluids, but in no fluids from Gilsonite-capped bottles. Both Gilsonite and elastomer closures sampled within 2 weeks of manufacture were uniformly noted to contain visible moisture at the time they were opened for culture.

Cultures from elastomer caps yielded nine different microorganisms, including the two

epidemic species, whereas only three species were recovered from Gilsonite caps (Table 5), all of which were also isolated from elastomer liners. No fermentative organisms were isolated from Gilsonite closures.

No significant differences were noted between the two autoclaves in contamination rates of cap liners or fluids.

The extraordinarily high frequency of contamination of elastomer closures compared with cultures of these closures from stock supplies (Maki et al., Am. J. Med., in press) or study B may have been related to a lower free chlorine content of autoclave cooling water during the matched study (0.1 ppm, compared with 0.3 to 0.4 ppm in study B). Also, the lower frequency of contamination of stock supplies compared with the present study may have been related to the freshness of contamination; virtually all stock supplies were cultured after being stored for 1 or more months in the Abbott warehouse and the hospital which provided time for the cap interior to dry.

The lower frequency of overall contamination of Gilsonite compared with elastomer did not seem to be explained by a lesser vulnerability of the former to ingress of contaminated

TABLE 5. Study E: Microorganisms isolated from cap enclosures

Elastomer	Gilsonite
<i>Enterobacter cloacae</i>	<i>Pseudomonas aeruginosa</i>
<i>Enterobacter agglomerans</i> (aerogenic and anaerogenic)	<i>Acinetobacter calcoaceticus</i> (oxidative and nonoxidative)
<i>Klebsiella pneumoniae</i>	
<i>Citrobacter freundii</i>	
<i>Bacillus</i> sp.	
Nonfermenter (unidentified)	
<i>Pseudomonas aeruginosa</i>	
<i>Acinetobacter calcoaceticus</i> (oxidative and non-oxidative)	

TABLE 4. Study E: Comparative study of elastomer and Gilsonite closure and subsequent fluid contamination during production—matched study

Determination	Type of closure		Fluid	
	Elastomer	Gilsonite	Elastomer	Gilsonite
No. sampled	120	115	120	120
No. contaminated	102 (85.0%)	31 (26.9%)	77 (64.1%)	28 (23.3%)
No. contaminated with <i>E. cloacae</i> or <i>E. agglomerans</i>	10 (8.3%)	0 (0.0%)	8 (6.6%)	0 (0.0%)

moisture, since closures of both types uniformly demonstrated moisture and behaved similarly in study D with fluorescein dye. Therefore, the lower frequency of overall contamination and the absence of the epidemic strains in Gilsonite caps suggested the possibility of a microbial inhibitor in this closure.

F. Inhibitory properties of cap components. The following studies were undertaken to investigate the possibility of an inhibitor in the Gilsonite closure.

Agar and broth diffusion studies were performed to detect possible inhibitory substances from the components of Gilsonite and elastomer closures. Agar plate studies utilized inocula identical to those used in standardized antimicrobial disk susceptibility tests (1) and Mueller-Hinton agar. Broth studies employed EBHIB and inocula of either 10^3 or 10^6 test organisms. Organisms used for these studies were isolated from either elastomer or Gilsonite closures except for the ATCC 25923 *Staphylococcus aureus* strain and a urinary isolate of *Serratia marcescens*. Other organisms tested included two strains of *E. cloacae*, both aerogenic and anaerogenic *E. agglomerans*, one strain each of indole-positive and indole-negative *K. pneumoniae*, and one strain each of oxidative and nonoxidative *A. calcoaceticus*, *Bacillus* sp., *P. maltophilia*, nonfermenter Ve, and a yeast species.

Components were removed from assembled closures furnished by the company and from closures of bottles from the matched study described above; they were autoclave-sterilized before use in the following tests.

Agar plate diffusion studies showed that the red rubber disk from the Gilsonite closure inhibited surface growth of *E. cloacae* and *E. agglomerans*. Zones of inhibition of 2 to 4 mm were noted around the disk, and no growth occurred under the disk. No inhibition occurred around or under Gilsonite paper, elastomer liners, or other components of either cap.

One thousand organisms of both epidemic strains inoculated into 6 ml of broth containing a single red rubber liner showed no growth after 48 h of incubation at 35 to 37 C. Inoculated broth alone as well as broth containing Gilsonite paper or elastomer disks consistently demonstrated expected turbidity within 24 h. Epidemic strains were consistently inhibited in more than 40 experiments using different red rubber liners. Using an inoculum of 10^6 organisms, minimal growth of the epidemic strains occurred after overnight incubation, but turbidity was slight compared with growth in control tubes (1+ compared to 4+ on a 0 to 4+ scale).

Similar experiments were conducted on other test strains using an inoculum of 10^3 in 6 ml of broth to which a single liner had been added. Again, the red rubber liner completely inhibited the test strains of *Serratia*, *Klebsiella*, *Staphylococcus*, *Acinetobacter*, *Bacillus*, and yeast, but only for 24 h; of all tested organisms, only *P. aeruginosa* was not inhibited.

These experiments were repeated with red rubber liners from Gilsonite closures taken from bottles that had been stored in a warehouse for at least 2 years after production; no demonstrable inhibitory activity remained at this time. However, red rubber liners from closures frozen at -10 C for 2 years retained full inhibitory activity.

The above experiments indicated the presence of an antimicrobial inhibitor with broad-spectrum activity in the red rubber liner of the Gilsonite closure; activity appeared to diminish with time at room temperature. This property probably best explains the significantly lower frequency of contamination of Gilsonite closures and the failure to identify clinical infections traced to these products. Epidemic strains were more sensitive to the inhibitor than other tested organisms.

G. Contamination transfer from closures to fluid. When frequent contamination with epidemic organisms of the elastomer closures of stock products was recognized (Maki et al., Am. J. Med., in press), studies were conducted to determine if microorganisms in cap liners could be transferred to fluid.

A number of serial manipulations were performed on bottles with elastomer closures from various 1-liter lots of solutions containing dextrose. Several of the experimental manipulations were specifically designed to simulate as closely as possible normal in-hospital practices in infusion therapy.

Only the elastomer liners in the caps were cultured; these and fluids were sampled by methods previously described (Maki et al., Am. J. Med., in press).

All sampling procedures were performed in laminar flow hoods by technologists wearing sterile gloves and clean laboratory overgarments. Open petri dishes containing TSAB were exposed in the hoods to monitor the sterile air environment.

In experiment G-1, the side of the cap of 59 bottles was struck gently on a bench edge, after which the cap was carefully unscrewed and lifted just above the bottle lip. The cap was then immediately replaced and tightened and each bottle was briefly shaken. (These actions simulated the removal of a tight cap and the addition to and mixing of an additive into the

solution.) All bottles were left at room temperature for 48 h before both closures and fluids were cultured. The 59 bottles were from five different lots.

In experiment G-2, the closure on each of 18 test bottles was immediately replaced after removal without prior striking. Each bottle was briefly shaken and then kept at room temperature for 48 h before caps and fluids were cultured. As controls, 18 bottles from the same lot were directly sampled without any manipulation, i.e., the cap was removed and the fluid was sampled immediately.

Exposed TSAB plates used for monitoring air in the laminar flow hoods were consistently negative for microbial growth.

Cultures of cap liners in experiment G-1 showed microorganisms in 33 (57%) of 59 samples, and microorganisms were detected in 28 of 59 (48%) solutions examined (Table 6). Fluid within the bottle contained the same organism as found on its cap liner in 17 instances (Table 7). A total of five fluids were found contaminated with two microorganisms. In only three bottles in which the fluids were contaminated were the caps sterile. The remaining 25 positive fluids occurred among 33 bottles with contaminated caps.

TABLE 6. Study G: Transfer of contaminants from cap to fluid by bottle manipulating

Expt	Conditions	No. of bottles	% Contaminated	
			Caps	Fluid
G-1 ^a	Hit on bench, open and close, shake	59	56.9	47.5
G-2 ^b	Open and close, shake	18	61.1	72.2
	Unmanipulated	18	77.8	5.6

^a Bottles were derived from five different lots.

^b All bottles were from the same lot.

In experiment G-2, contamination was found in 11 of the 18 examined caps, and in 13 of the 18 fluids (Table 6). In six instances, the same contaminants were found in caps and fluids (Table 7). One of these matched samples yielded both *E. cloacae* and *E. agglomerans*. Fluids were contaminated, but caps were sterile in four bottles. The remaining nine positive fluids had caps that were contaminated.

The frequency of cap contamination in controls (unmanipulated bottles) of experiment G-2 (78%) did not differ significantly from that obtained for caps from the manipulated bottles in experiments G-1 and G-2. However, the frequency of fluid contamination in the control bottles (1 of 18) was statistically significantly less than the manipulated test bottles ($P = 0.00004$ and $P = 0.00008$, respectively, by Fisher's test).

The frequency with which systems with contaminated caps had matching isolates from fluid (Table 7) was significantly greater in manipulated bottles of experiment G-1 and G-2 than controls ($P = 0.0038$ and $P = 0.014$, respectively, by Fisher's test). The likelihood of finding a fluid contaminated with organisms isolated from a contaminated cap was more than seven times greater after manipulation of the cap and bottle than when systems were sampled directly.

Table 7 also gives the frequency with which matching organisms were present in the cap for all positive fluid cultures. The number of observations on controls (one match of one positive fluid) is insufficient to permit reliable comparisons of these frequencies.

These experiments clearly showed that manipulations of the cap and bottle simulating those commonly observed during normal hospital use significantly increased the rate of fluid contamination with organisms present in contaminated caps.

The presence of contaminated fluid in bottles with sterile caps does not eliminate other cap components as sources of the organisms in fluid. Only the elastomer liner was extracted and cultured, but other cap components and

TABLE 7. Study G: Transfer of contaminants from cap to fluid by bottle manipulating

Expt	Total contaminated caps	Matching organisms in fluid		All contaminated fluids	
		No. of caps	All contaminated caps (%)	Total	Matching organisms in cap (%)
G-1	33	17	51.5	28	60.7
G-2	11	6	54.5	13	46.2
Test controls (unmanipulated)	14	1	7.1	1	100.0

thread areas may have been important sites from which organisms could be transferred.

DISCUSSION

Bacterial species similar to those associated with epidemic cases were frequently isolated from the manufacturing plant environment. These strains possessed characteristic biochemical activities and had multiply-sensitive antimicrobial susceptibility patterns identical to those isolated from cultures of stock infusion products, in-use i.v. fluid, and blood cultures from epidemic cases (3-5; Maki et al., Am. J. Med., in press). Early in the investigation, the in vitro susceptibility of these strains to most tested antimicrobials suggested an extra-hospital, inanimate source of these organisms, unexposed to antibiotics or high concentration of other gram-negative bacteria capable of transferring episomal resistance.

Environmental specimens frequently yielded organisms of genera *Klebsiella*, *Enterobacter*, or *Serratia* (members of the tribe *Klebsiellae*). It is probable that the glucose-rich environment of infusion product manufacturing plants provide members of this tribe with selective growth advantages. We found that 50 of 51 tested strains, all members of this tribe, proliferated luxuriously in commercial D5/W at 25 C, attaining a mean concentration of more than 10^5 organisms per ml in 24 h, whereas 48 of 49 tested randomly selected bacterial strains that were not members of tribe *Klebsiellae* either remained static or died (13). In five of the six outbreaks of epidemic septicemia linked to contaminated infusion fluid (3-9, 12, 14, 15; Maki et al., Am. J. Med., in press), members of tribe *Klebsiellae* have predominated; this is almost certainly related to the selective growth properties of these organisms in glucose-containing fluids.

Large-scale microbiological studies of warehouse- and hospital-stored Abbott stock showed that a high percentage of cap liners, particularly the newly introduced elastomer (about 40%) were contaminated (Maki et al., Am. J. Med., in press). Cultures of unassembled cap components (study B) indicated that the materials were not contaminated with the epidemic strains prior to introduction on the production line; contamination, specifically with the epidemic organisms *E. cloacae* and *E. agglomerans*, occurred after capping, both before and to a lesser extent after autoclaving (Table 2).

The autoclave cycle (112 C, 22 min) appeared to be adequate to destroy frequent preautoclave contamination, and the finding of no contamination immediately after autoclaving sug-

gests that contamination of autoclave coolant or survival of organisms present from preautoclave stages of production did not play a major role in post-sterilization contamination at the time of our study. Most importantly, study B showed that contaminants were readily introduced at multiple stages of postautoclave processing; very frequent contamination (9 of 12 samples of warehouse stock) suggested the cumulative nature of manufacturing contamination. The number of production-line samples obtained in study B was insufficient to permit valid statistical evaluation of the relative risk of contamination of each postautoclave production stage.

It could be assumed that some microorganisms, if present in autoclave cooling water, could be introduced into closures, since study B demonstrated conclusively that autoclave coolant infiltrated all aspects of the inner cap assembly. Gilsonite-lined closures were considerably less frequently contaminated than elastomer-lined ones in surveys of hospital and of warehouse stock (40% versus 8% (Maki et al., Am. J. Med., in press)) and in study E comparing fresh, simultaneously manufactured products. Most interestingly, although the epidemic strains were frequently isolated from elastomer-lined closures, Gilsonite-lined closures did not yield these organisms. In vitro studies revealed a diffusible antibacterial substance in the red rubber liner of the Gilsonite closure which exhibited substantial activity against most tested bacteria, including the epidemic strains (study F). Findings of this study may explain the far-reaching consequences of Abbott's seemingly innocuous switch from the use of Gilsonite to elastomer in its bottle closures in March 1970 and the apparent absence of clinical problems with the Gilsonite screw-cap closure for over 35 years.

The absence of contamination of presterilized controls (study B) which were inserted into the production line at room temperature, rather than the usual 40 C, and study D using fluorescein dye strongly suggested the importance of a falling cap temperature in promoting ingress of environmental contaminants into the closure. A cooling closure actively drew ambient moisture, with any contained microorganisms, through the thread interstices and up into the innermost parts of the cap. This apparently was not the sole mechanism of cap contamination, however, because control bottles at ambient temperature in study B also manifested contamination although in a greatly reduced frequency (Table 2). This phenomenon presumably explains why the

closures of test bottles at room temperature failed to become contaminated after insertion at various stages of production despite heavy contamination of bottles which were taken concurrently from the production line and which were falling in temperature.

The frequency of contamination of both Gilsonite and elastomer closures and fluids from production line stock was extremely high in these plant studies, much higher than culture studies of warehouse or hospital stock (Maki et al., *Am. J. Med.*, in press), possibly reflecting the freshness of samples. Visible moisture was present throughout the closures of these freshly manufactured bottles, and it is possible, but unproven, that storage conditions lead to drying, which secondarily leads to a substantial reduction in the frequency of detectable contamination, especially with gram-negative bacilli.

The chemical constituents of red rubber are numerous and highly complex. Information on the composition of the red rubber and the elastomer liners could not be obtained from the manufacturers. Natural rubber contains about 95% hydrocarbon, 3% protein, 2% resins and fatty acids, and very little mineral matter. During vulcanization and molding, various minerals and chemicals are added, including sulfur, zinc-dimethyldithiocarbonate, and zinc oxide. Depending on the desired final product, other chemicals may also be used, e.g., aromatic chlorinated hydrocarbons, ketones, zinc chloride, mevalonic acid, and isopentenyl pyrophosphate (2). Investigators in Australia identified various chemicals in infusion fluids, such as zinc oxide, traceable to the rubber closures. Unidentified chemicals were believed to be accelerators and "chemicals comprising the secret formulary of the rubber manufacturer" (11). Many of these compounds are known to have antiseptic, astringent, and fungicidal properties (2, 16).

Migration of bacterial contamination from caps to fluid was easily accomplished, *in vitro*, by simple manipulations simulating normal in-hospital use (study G). These procedures produced fluid contamination in more than 50% of bottles with contaminated caps. Although fluid within unopened bottles may have rarely been contaminated, causing almost immediate sepsis at the inception of *i.v.* therapy (Maki et al., *Am. J. Med.*, in press), most septicemias probably arose from the transfer of microorganisms present in the closure to fluid during therapy, effected by routine and expected manipulations of the type simulated experimentally.

U.S.P. microbiological quality-control requirements followed by the company would permit 99.3% of autoclave loads with an intrinsic contamination frequency of 6/1,000, the actual value in samples of Abbott products, to be released for consumer use (Maki et al., *Am. J. Med.*, in press). In addition, U.S.P. did not require the history of previous quality control positives to be taken into account in judging the likely sterility of subsequent autoclave loads. The substantial increase in autoclave loads failing both initial sterility test and retest in 1970 involved both elastomer- and Gilsonite-capped products. Since bottles selected for quality control were sampled immediately after autoclaving, the most likely source of organisms in these bottles was autoclave cooling water. Contamination of the products with the epidemic strains and other organisms was observed to occur at postautoclave stage of production; thus, contaminated autoclave cooling water, if involved, was not the only source of the contamination. Since organisms in positive quality control specimens were not identified, the contribution of autoclave cooling water must necessarily remain speculative. Sequential sampling schemes requiring the cumulative number of positive samples to be plotted against the total number of samples is capable of detecting departures from expected experience. The formal adoption of such sampling plans should be seriously considered by industry and regulatory agencies. Sequential sampling will detect ongoing production problems resulting in systematic, low-level contamination of products. Quality control sampling is not a practical way to reliably detect low-frequency contamination of individual autoclave loads; biological indicators to monitor autoclave function and adherence to good manufacturing practices are additional ways of assuring that infusion products are microbiologically safe.

Our findings show that, in addition to sequential sampling, other improvements in quality control are needed. (i) Finished products should be selected for sampling from newly produced stock when they reach the warehouse, rather than immediately after autoclaving. (ii) The total volume of fluid within bottles should be sampled, rather than fractions. (iii) The direct addition of concentrated broth to the container appears to be a simpler and more sensitive culture technique than membrane filtration. (iv) Culture medium such as enriched brain heart infusion broth is preferred to thioglycolate media. (v) Gram-negative isolates from positive quality-control specimens should be identified through genus and species;

other isolates should be identified through genus.

The identification of gram-negative isolates in positive quality-control samples serves both scientific and public health objectives. Knowing the identity of such organisms helps the assessment of potential clinical seriousness of contamination, permits correlation to be made between product and i.v.-associated human cases if they occur, and provides the manufacturer with information of great value in detecting important implant sources of contamination. Further characterization of such isolates by antibiotic susceptibility and application of other typing systems would enhance the objectives.

These studies raise the question of whether a screw-cap closure in its conventional form can be made totally safe from contamination. At present this type of closure cannot be recommended for use with infusion products or other medications that must remain sterile.

Within individual hospitals the outbreaks were terminated by discontinuing use of infusion products from Abbott Laboratories and nationwide by total product recall (3-5; Maki et al., *Am. J. Med.*, in press). These epidemiological and laboratory investigations were brought to a successful conclusion because each discipline complemented the other. Hypotheses generated by epidemiological observation were subjected to microbiological testing. The results of such tests led to new epidemiological hypotheses and studies that ultimately provided clear insight into the genesis of a complex problem.

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