Factors Affecting Survival of *Pseudomonas cepacia* in Decongestant Nasal Sprays Containing Thimerosal as Preservative

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Abstract □ Strains of *Pseudomonas cepacia*, isolated from packages of nasal spray preserved with thimerosal, showed a high degree of resistance to the organomercurial, as compared to low and moderate resistance of standard laboratory strains or isolates from water. The product isolates were shown to degrade the thimerosal to metallic mercury which volatilized from the product or assay medium. The addition of organic nutrient was essential for survival of unadapted cells in the product. However, when cells were first grown in diluted product containing added nutrient and then inoculated into the full-strength product, survival and growth occurred even in the absence of added nutrient. The time required for growth to occur was inversely related to the amount of added nutrient. At low nutrient concentrations, ~99.9% of the inoculated cells were killed rapidly, but after a lag time of 7–12 days, the few survivors began to increase in numbers and eventually attained high cell concentrations. These findings should be useful in planning production and testing programs with thimerosal-preserved products.

Keyphrases □ Thimerosal—preservative, factors affecting survival of *Pseudomonas cepacia* in decongestant nasal sprays □ *Pseudomonas cepacia*—factors affecting survival in decongestant nasal sprays containing thimerosal as preservative □ Decongestants—nasal sprays containing thimerosal as preservative, factors affecting survival

Although generally considered nonpathogenic, *Pseudomonas cepacia* can cause severe and sometimes fatal infections, especially when accidently introduced directly from contaminated materials into wounds, blood vessels, or the urinary tract (1–9). Recently, it has been implicated as the cause of contamination of decongestant nasal sprays produced by two different companies. In one case the affected products contained the cationic surfactant benzalkonium chloride as the preservative, while in the other instance, the products contained the organomercurial, thimerosal. The present report describes studies with organisms isolated as contaminants in the latter products.

BACKGROUND

The bacterium, *P. cepacia*, was first reported as the causative agent of yellow rot in onions (10) and was given the species name on the basis of that ability. A taxonomic study of the genus *Pseudomonas* described an organism which was versatile nutritionally (11); based on that characteristic it was named *P. multivorans*. It was suggested that this species, which can utilize over 150 different organic compounds as sources of carbon and energy, had not been described earlier because of its geographic restriction, but it is now known to occur widely in soil and water. The two species, *P. cepacia* and *P. multivorans*, plus a third, *P. kingii*, have since been shown to be identical, and the designation *P. cepacia* has been adopted (12, 13).

In addition to its nutritional versatility, *P. cepacia* possesses the ability to grow in distilled water without added nutrients (14), and most isolates are highly resistant to antibiotics and other antibacterial agents (15, 16). This combination has led to numerous nosocomial problems due to its presence in water reservoirs, hospital aqueous solutions, and disinfectants (17–22). Like some of the other pseudomonads, *P. cepacia* has been isolated from numerous commercial products and rivals *P. aeruginosa* as to its presence and persistance in various pharmaceuticals.

In 1978, pseudomonads were isolated from several lots of both the regular and long-acting formulations of nasal sprays. The regular formulation contains phenylephrine hydrochloride while the long-acting variety contains xylometazoline as the adrenergic component. Both formulations contain 10 ppm of thimerosal as a preservative. Cetylpyridinium chloride also is present in the regular formulation but not in the long-acting brand. A number of separate bacterial isolates from both formulations were identified as *P. cepacia* of several different biotypes[1].

The occurrence of resistance to high levels of thimerosal and other organomercurials has been demonstrated in numerous bacterial species including the pseudomonads, *P. cepacia* and *P. aeruginosa*, and, although the mechanism of resistance varies with the microbial strain and the type of mercurial compound, with organomercurials it normally involves plasmid-determined inducible enzymes which hydrolyze the carbon–mercury linkage and then reduce the released Hg^{+2} to volatile metallic mercury (Hg°) (23, 24).

In this report some physiological characteristics of strains of *P. cepacia* isolated from products containing thimerosal are described. They are compared with strains from other sources, and the conditions under which successful contamination may or may not occur are examined.

EXPERIMENTAL

The bacterial strains employed in these studies were obtained from various sources, as shown in Table I.

Thimerosal sensitivity assays were performed in test tubes containing 7.5 g/liter (one-fourth strength) trypticase soy broth[2] plus various concentrations of thimerosal[3] ranging from 0 to 10,000 ppm in a final volume of 6.0 ml. Tubes were inoculated to cell densities of $\sim 5 \times 10^6$ organisms/ml using 0.05 ml of inocula grown in 7.5 g/liter trypticase soy broth, and were incubated with slow shaking at 30°. The highest concentration yielding growth, as indicated by visible turbidity, was noted. In the thimerosal sensitivity assay, turbidity was always present by Day 5 if growth was to occur. Negative tubes were verified to be free of viable cells by streaking onto trypticase soy agar[2] plates.

Chemical analyses for thimerosal were performed by determining mercury contents using atomic absorption spectrophotometry (25).

Microbiological assays for thimerosal were performed on trypticase soy agar and Mueller–Hinton agar plates using a sensitive strain of *Staphylococcus aureus* as the indicator organism. Filter paper disks were 12.5 mm in diameter. The plates were incubated at 35° and read at 24 hr. Challenge testing of the product was performed by growing test organisms in trypticase soy broth at 30 or 35° and inoculating the product to concentrations ranging from 1.5×10^5 to 2.4×10^7 organisms/ml. In some instances a second challenge was performed with the same organism. Both types of nasal spray were employed and were either freshly prepared or were from commercial lots of different ages.

Tests to determine the ability of the isolates to utilize product components as sole sources of carbon and energy were performed using a mineral salts basal medium. The mineral medium consisted of the following components per liter of distilled water: 80 mg K_2HPO_4, 200 mg NaH_2PO_4, 100 mg $(NH_4)_2SO_4$, 14 mg $MgSO_4 \cdot 7H_2O$, and 2 mg $Fe(NH_4)_2 \cdot 6H_2O$. Organic components of the nasal sprays were tested separately by adding them to the mineral medium to final concentrations of 0.1 and 1.0 g/liter. Thimerosal could not be tested as a carbon and energy source, since in mineral salts medium it was lethal to all strains at concentrations >5 ppm. Inocula for these studies were grown overnight on mineral salts agar containing 1 g/liter monosodium glutamate. Cells were transferred to sterile distilled water and shaken overnight, then

[1] Identified by G. Gilardi, Hospital for Joint Diseases, N.Y.
[2] Baltimore Biological Laboratories, Baltimore, Md.
[3] Sigma Chemical Co., St. Louis, Mo.

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Table I—Thimerosal Sensitivity of Bacterial Strains from Various Sources

Organism		Source	Highest Concentration of Thimerosal-Yielding Growth, ppm	Inoculum Size, cells/ml
S. aureus	19636	ATCC[a]	0.03	5×10^{6}[b]
E. coli	11303	ATCC[a]	1	5×10^{6}
P. fluorescens	12633	ATCC[a]	3	5×10^{6}
P. aeruginosa	10145	ATCC[a]	10	5×10^{6}
P. aeruginosa	9027	ATCC[a]	3	5×10^{6}
P. cepacia	25416	R. Hugh	3	5×10^{6}
P. fluorescens	(2 isolates)	City water entering plant	10	5×10^{6}
P. cepacia	(4 isolates)	City water entering plant	30	5×10^{6}
Pseudomonas sp.	(7 isolates)	City water entering plant	10–100	5×10^{6}
P. cepacia	B7L	Product Isolate	500	5×10^{6}
P. cepacia	CAT	Product Isolate	300	5×10^{6}
P. cepacia	CWJ	Product Isolate	1000	5×10^{6}
P. cepacia	CWO	Product Isolate	1000	5×10^{6}
P. cepacia	CXN	Product Isolate	500	5×10^{6}
P. cepacia	CXN	Product Isolate	300	5×10^{4}
P. cepacia	CXN	Product Isolate	100	5×10^{2}

[a] American Type Culture Collection. [b] Standard.

diluted in distilled water to yield the desired inoculum size, which was calculated to be $\sim 1 \times 10^{2}$ cells/ml. The actual number of cells present at the time of inoculation and at daily intervals for 5 days was determined by spreading samples onto trypticase soy agar plates.

When adapted cultures were employed, they were prepared by growing inocula in a fourfold dilution of product (diluted with deionized water) containing 300 ppm of phytone[2] (soy peptone). In this medium organisms reached visible turbidity in 1–2 days.

In those experiments where nutrient supplementation of the product was employed, individual substances or complex media were prepared at concentrations of 120 g/liter (or lower if solubilities did not permit the higher levels), sterilized by membrane filtration, and added to tubes containing product to yield the desired range of concentrations. As with assays for thimerosal resistance, growth was noted by observing tubes for turbidity. Tubes showing no turbidity were checked periodically for viable cells by streaking or spreading aliquots onto trypticase soy agar plates.

RESULTS AND DISCUSSION

The thimerosal sensitivities of a number of well-characterized bacterial strains, as well as some water and product isolates, are shown in Table I. A difference in resistance is evident between the ATCC strains (growth up to 10 ppm) and the product isolates, which grew in the presence of 300–1000 ppm of thimerosal. No organism was isolated from the product which was not resistant to high levels of thimerosal. Thus, resistance to thimerosal appeared to be essential for survival in the product. Strains of *Pseudomonas* isolated from city water entering the production site had resistance levels intermediate between the laboratory strains and the product isolates. The effect of inoculum size also can be seen in Table I. When strain CXN was inoculated at concentrations 100- and 10,000-fold below the standard level of 5×10^{6} organisms/ml, the highest thimerosal concentration yielding survival and growth fell slightly, but even with the lowest inoculum of 500 cells/ml, the organisms still grew in 100 ppm of thimerosal. A similar effect was noted when strain CWJ was inoculated directly from a contaminated bottle into assay tubes to produce an inoculum level ~100-fold below the standard level (growth was to 500 ppm).

Table II—Microbiological and Chemical Assays[a] for Thimerosal in Uninoculated Samples and in Media Inoculated with Resistant Strains of *P. cepacia*

Sample	Initial Thimerosal Concentration, ppm	Microbial Assay Zone Diameter, mm	Chemical Assay, ppm
Thimerosal in deionized water	1	19	NA[b]
	10	26	NA
	100	36	NA
Thimerosal in uninoculated medium[c]	0	No zone	0
	1	18	0.8
	10	26	10
	100	36	107
Thimerosal in medium + strain CXN	0	No zone	0
	1	No zone	Trace
	10	No zone	0.5
	100	No zone	3.3
Thimerosal in medium + strain B7L	0	NA	0
	1	NA	Trace
	3	NA	Trace
	10	NA	Trace
	30	NA	4.5
	100	NA	2.4

[a] Both uninoculated and inoculated samples were assayed after 4-days incubation at 30°. [b] Not assayed. [c] Trypticase soy broth.

Table II shows the results of chemical and microbial assays for thimerosal using various solutions containing thimerosal, both uninoculated and after growth of two resistant strains. Comparison of thimerosal in deionized water with 4-day-old uninoculated trypticase soy–thimerosal tubes, using the microbiological assay, showed that the one-fourth strength trypticase soy medium had no neutralizing effect on thimerosal. Tubes inoculated with *P. cepacia* strain CXN showed the absence of detectable thimerosal by microbial assay, even when the initial concentration was 100 ppm and even though this assay easily detected 1-ppm concentration in the uninoculated deionized water and in trypticase soy broth controls. Chemical analyses for thimerosal mercury confirmed the inactivation of thimerosal by strains CXN and B7L. Since the chemical assay determines mercury levels, it can be assumed that strains B7L and CXN are resistant to thimerosal by virtue of enzymes which liberate metallic mercury from the media. This also was observed by chemical determination of mercury levels in numerous contaminated and uncontaminated packages of nasal spray. The analyses showed that 1-year-old uncontaminated packages all contained the expected levels of 6.8–7.4 ppm of thimerosal, whereas contaminated packages from the same lots and cases contained 0.1–1.5 ppm. Thus, when the contaminating organisms survived and grew in the product, the thimerosal was degraded.

When strains CWO and CXN were used to challenge both the regular and the long-acting product at levels of 1.5×10^{5}–2.4×10^{7} organisms/ml, no viable organisms could be recovered after 1 hr. In all, over 70 challenge tests were performed with uniformly negative results. This was true whether the products were freshly prepared in the laboratory or were uncontaminated packages taken from cases that contained one or more contaminated packages. Thus, no differences were noted between old and fresh product or between regular and long-acting product with regard to survival of *P. cepacia* strains isolated from the product. Since the long-acting product does not contain cetylpyridinium chloride, while the regular product does, the absence of cetylpyridinium chloride did not allow for survival of the challenge organisms.

It was assumed that the contaminating strains of *P. cepacia* were capable of utilizing some component(s) of the nasal sprays as a source of carbon and energy. To explore this assumption, each component was individually tested for its ability to support growth of *P. cepacia* strains CWO, B7L, and 25416. Within 72 hr, inoculum levels increased from between 40 and 200 organisms/ml to $\sim 2 \times 10^{6}$ organisms/ml in the presence of each tested component. However, the same increase in cell

Table III—Effect of Organic Nutrients on Survival and Growth of *P. cepacia* in Nasal Spray Containing Thimerosal

Nutrient, ppm	Nutrient Source: Trypticase soy broth	Trypticase	Yeast extract	Phytone	Dextrose	Glutamine	Proteose peptone	Acetate	Aspartate	Pyruvate	Benzoate	Monosodium glutamate	Starch	Maltose	Asparagine
20,000	+[a]				+					−[b]	−	+	−		
17,000					+					−	−	−	−		
15,000						−		−	−						−
13,000					+					−	−	−	−		
10,000	+				−				−						
9,000	+				−					−	−	−	−		
7,500						−		−	−						−
6,000					−							−			
5,000	+	+	+	+			+							−	
4,600					−					−	−	−	−		
4,100	+	+	+	+			+							−	
3,800						−		−	−						−
3,200	+	+	+	+	−		+					−		−	
2,200	−	+	+	+	−		−			−	−	−	−	−	
1,800			−			−		−	−						−
1,200	−	−	−	+	−		−		−	−	−	−	−	−	
900						−		−						−	
600	−	−	−	−			−							−	

[a] Growth to $>10^7$ cells/ml. [b] No viable cells present.

population occurred in the mineral salts medium without added substances. When acid-washed glassware and ultrapure reagents were employed, cell yields between 9×10^5 and 1.2×10^6 organisms/ml still were obtained both in mineral salts alone and with product components. When cells were passed repeatedly in sterile distilled water obtained from a quartz element glass still-fed from a deionizer unit, cell densities between 4 and 5×10^5 were maintained. Thus, the addition to water of usable organic nutrient is not necessary for the survival and growth of the product isolates. Growth of *P. cepacia* previously has been reported in mist therapy unit water and commercial distilled water (14).

An explanation was needed for the apparent contradiction between the observed high degree of thimerosal resistance of the product isolates as measured by tube or plate assay in trypticase soy media and the failure of the isolates to survive when introduced back into products similar to those from which they were originally isolated. Since none of the product ingredients stimulated growth of the isolates in minimal media, and since the absence of cetylpyridinium chloride did not enhance survival, two other factors were studied for their effect on survival of the isolates in the product: the addition of nutrient and adaptation of the isolates to the product.

Since the product isolates all showed high levels of thimerosal resistance in the trypticase soy tube assays, this complex medium was employed as a source of nutrient. As shown in Table III, when trypticase soy broth was added to the regular nasal spray formulation at concentrations ≥3200 ppm, survival and growth to visible turbidity was observed. When the organic components of trypticase soy broth (trypticase, phytone, dextrose) were tested individually, phytone (soy peptone) was most effective and supported growth in the regular product at concentrations ≥1200 ppm. Since the concentrations of nutrient required to support growth were higher than the nutrient levels present in the treated water or other components used in formulating the product, organic substances other than those present in trypticase soy broth were also tested in an attempt to find a more efficacious stimulator of successful contamination. The test substrates included the amino acids glutamine, glutamate, asparagine, and aspartate; the organic acids acetate, pyruvate, and benzoate; proteose peptone and yeast extract as complex nutrients; and starch and maltose as nonutilizable organics (for *P. cepacia*). As shown in Table III, none of these compounds was as effective as phytone in allowing survival of *P. cepacia* strain CWO. Of the single substrates, only dextrose and glutamate supported contamination of the product and neither of these was effective in concentrations that were comparable to those yielding growth when the complex nutrients were used. Since the tested organic substrates spanned a variety of utilizable and nonutilizable compounds, it seemed unlikely that a more effective supporter of survival would be found.

Since an alternative explanation was necessary to account for the survival of the contaminant organisms in the product in the absence of high quantities of nutrient, the effect of cultivating strain CWO in diluted product and then inoculating this adapted culture into undiluted commercial product containing a range of phytone concentrations was determined.

The effect of adaptation on the ability of thimerosal-resistant organisms to survive in the product is shown in Table IV. The inocula for these studies were grown in a fourfold dilution of product (regular formulation) containing 300 ppm of phytone, as contrasted with the unadapted inocula employed for the data shown in Table III, which were grown in trypticase soy broth. Table IV shows three significant effects. First, the adapted cells were able to survive and proliferate in the product in the absence of any added nutrient. Second, the time required for growth and turbidity to be evident was inversely related to the added nutrient concentration. Third, at low nutrient levels (0–120 ppm) the number of viable cells declined dramatically from the initial concentration of 1×10^6 cells/ml to between 1 and 15×10^2 cells/ml and remained at the lower levels for up to 2 weeks before an increase in viable cells was noted. The third effect was observed by periodically plating calibrated loops (0.01 ml) of culture from those tubes which showed no turbidity on day 5. These were the tubes containing the four lowest concentrations of phytone. Samples were plated on days 5, 7, and 12. The numbers of viable cells were low and remained stable for at least 7 days in the tubes containing the three lowest concentrations of nutrients (0, 60, and 120 ppm of phytone) (Table IV). The tube containing 240 ppm of phytone, which was not yet turbid on day 5, contained $\sim 10^5$ cells/ml at the time and became turbid on day 7. In the two tubes that became turbid on days 14 and 17 (0 and 120 ppm), increases in cell numbers were evident on day 12. The tube that became turbid on day 19 (60 ppm of phytone) still showed no increase in viable cells on day 12. Thus, inoculation into the product containing low concentrations of extraneous organic matter led to the death of most resistant cells, but the survivors persisted and eventually grew. A similar effect was noted by Pinney when a strain of *P. aeruginosa* resistant to thimerosal was inoculated into a minimal medium containing glucose as the energy source (26).

As expected, the turbidy attained in each tube was related to the level of nutrient present; when survival and growth occurred in the absence of added organic nutrient, the cultures were barely turbid. Similar levels were observed when strain CWO was inoculated into laboratory deionized water containing no added components. Thus, none of the organic components of the nasal spray need serve as a nutrient source in order to allow survival and growth of certain bacteria.

The data shown in Table IV were obtained using the product taken

Table IV—Persistence and Growth of *P. cepacia*[a] in Nasal Spray with Various Concentrations of Nutrient

Days Post Inoculation	Concentration of Soy Peptone, ppm: 0	60	120	240	360	480
5	1[b]	6	15	~1000	TNTC[c]	TNTC
7	0	6	10	TNTC	TNTC	TNTC
12	200	3	260	TNTC	TNTC	TNTC
Day on which culture became turbid	17	19	14	7	3	2

[a] Inoculum was adapted in diluted product (see text) and was adjusted to yield initial concentrations of 1×10^6 cells/ml. [b] Number of colonies present on trypticase soy agar after plating 0.01 ml of product. [c] Too numerous to count.

from commercial polyethylene containers. That product contained thimerosal levels of 8.0–8.5 ppm, which is the usual concentration present several months after manufacture due to the absorption of some thimerosal by the container. When similar challenges were performed with product formulated in glass containers containing thimerosal at 10 ppm, the same inverse relationship between nutrient concentrations and lag time was observed, except that with 10 ppm of thimerosal, cultures in the product containing low nutrient levels took up to 37 days to become turbid. Under these circumstances, low numbers of cells persisted for as long as 1 month before growth occurred. It is likely that during these long lag periods, small numbers of surviving cells were producing thimerosal-inactivating enzymes, which then reduced the thimerosal concentration to a level which allowed growth. The greater enhancement of survival and growth of resistant cells by peptone mixtures as compared with single carbohydrates or amino acids probably was due to the utilization of the peptone amino acids for efficient and rapid synthesis of the thimerosal-inactivating enzymes.

When the product containing thimerosal levels >10 ppm was tested, strain CWO survived and grew only when nutrient was added. The required nutrient levels increased with increasing thimerosal, *e.g.*, at 60 ppm of thimerosal; survival and subsequent growth of adapted cells required the presence of 1000 ppm of phytone. It is suspected that in the presence of thimerosal, the levels of inactivating enzymes are related to the amount of phytone present. Thus, high phytone levels are necessary for survival at higher thimerosal concentrations. At lower thimerosal concentrations, higher phytone levels and, consequently, greater levels of inactivating enzymes more quickly reduce thimerosal below the inhibitory level with the resultant shorter lag time.

One concern emanating from this study results from the long lag period which may precede growth. Thus, samples of the product which are tested microbiologically shortly after manufacture may contain only small numbers of viable organisms, yet if those organisms are resistant strains, eventual growth to high levels may occur. Some measures that would reduce the risk of such an occurrence are as follows:

1. Composite samples must not be made except in neutralizing solutions, since reduction or elimination of resistant cells can occur upon mixing with additional product.
2. Vegetative cells isolated from the product, even in very small numbers, should be checked for resistance to the preservative if the isolates are of a suspect species, especially one of the Gram-negative nonfermentative bacilli.
3. If low counts of an organism are obtained and the organism is shown to be resistant to the preservative by tube or plate testing, the product should be retested at intervals to determine whether counts are increasing. It is preferable to encode and hold individual packages for this purpose. With thimerosal preserved products, if viable vegetative cells are recovered after several days, eventual increases in counts are likely.
4. Challenge testing of product with suspected contaminants may give false assurance if the inocula for the challenges are grown in the absence of preservative. A 10- or 20-fold dilution of the product in water is a useful medium for growth of challenge organisms. In many cases it is not necessary to add additional nutrient to allow growth of Gram-negative organisms. Survival of adapted organisms in the full-strength product then can be compared with unadapted inocula grown in the usual culture media.

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