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Loss of Effectiveness of Preservative Systems of Mascaras with Age

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The preservative systems of unused, anhydrous mascaras were challenged periodically with microorganisms, using a modified membrane procedure. Shelf life of preservative activity against *Pseudomonas aeruginosa* varied for different brands from as little as 1 month to over 36 months. Generally, *P. aeruginosa* grew in mascaras after the mascaras were challenged first with *Staphylococcus epidermidis* or *Candida albicans*.

Eye area cosmetics contaminated with microorganisms during use, particularly mascaras that are applied to the eyelashes with brushes, have been associated with ocular infections (2, 6). The applicator brush may be an instrument of accidental trauma that introduces potentially hazardous microorganisms to the cornea, notably *Pseudomonas aeruginosa* (3, 6). Preservatives, such as parabens, mercurials, and Formalin derivatives, usually are incorporated in mascaras to prevent microbial contamination. Various procedures have been employed for determining the effectiveness of the preservative systems in mascaras, but no standard method for semisolid anhydrous products currently is accepted. McConville et al. (4) emulsified anhydrous cosmetics with Arlacel 80 and Tween 60 for quantitative recovery of microorganisms. Ahearn et al. (1) described a membrane challenge procedure for determining the relative preservative efficacy of semisolid oily mascaras. In this report, we describe a modification of the membrane procedure and report on the deterioration of the preservative systems in unused mascaras with time.

Mascaras for study were purchased retail in lot amounts between 1975 and 1979 and kept in their original containers at room temperature in the laboratory and examined periodically. All mascaras contained preservatives in their formulations. The challenge procedures were adapted from the previously described double-membrane procedure (1, 6). In brief, the mascaras were removed from their containers and packed into sterile glass wells (3 by 13 mm). A 13-mm-diameter membrane (0.8-μm porosity) was placed on the mascara surface and dampened with 0.01 ml of phosphate-buffered saline (PBS; 8.0 g of NaCl, 0.2 g of KCl, 0.12 g of KH_2PO_4, 0.91 g of Na_2HPO_4, 1,000 ml of deionized water [pH 7.3]). The challenge microorganisms, *P. aeruginosa* 3 (ATCC 9027) and *Staphylococcus epidermidis* 4, were grown with agitation in tryptic soy broth (BBL Microbiology Systems) for 15 to 18 h at 35°C. *Candida albicans* 1, initially isolated from a used container of mascara B1, was grown with agitation for 24 h at 35°C in a broth containing 1.0% glucose, 0.5% peptone, and 0.5% yeast extract. Cells were harvested by centrifugation, washed in PBS, and suspended in PBS from a standard curve to approximately 10^6 cells per ml. One milliliter of this suspension was diluted in 5 ml of PBS and blended in a Vortex mixer. The entire 6 ml was drawn through a 0.22-μm-porosity membrane (13-mm diameter) fitted in a syringe-type filter holder (Swinney). The membrane (Millipore Corp.; cellulose ester) was removed from the holder and placed, organism-side up, on top of the 0.8-μm membrane. The test systems were kept in a moist chamber at room temperature. After 1 and 7 days, the upper membrane was removed, placed in a syringe filter holder, and backflushed with 5 ml of PBS. The membrane was removed from the holder and placed in the 5 ml of PBS used for backflushing and blended in a Vortex mixer vigorously for 15 s. This cell suspension was serially diluted (with Vortex blending between each dilution series) to 10^{-8} in Letheen broth (Difco Laboratories). The membrane was incubated in a separate tube of broth. All broth cultures were incubated at room temperature with agitation and examined visually for growth. All tests were performed in triplicate in each of three separate test runs.

The mascaras were challenged singly with *P. aeruginosa* and sequentially with the primary challenge by *S. epidermidis* (for all mascaras except A3 and B1 for which *C. albicans* was used) and the secondary challenge by *P. aeruginosa*. In the sequential challenge, the 0.2-μm membrane holding *P. aeruginosa* was placed in the well on the surface of the 0.8-μm membrane within 2 to 3 h after removal of the first challenge membrane.

Of eight brands of mascara examined, lots of

two brands, A and B, supported the active growth of *P. aeruginosa* on initial challenge within a month of purchase. These two brands were included in the study because we found them to be contaminated frequently in studies of used cosmetics (1, 3, 6). The recovery of *P. aeruginosa* 3 from different lots of these two brands of mascara in a representative challenge test is presented in Table 1. The more recently manufactured lots, indicated by the higher number, were the most inhibitory. Identical results were obtained in the triplicate tests with the dilution procedure. Challenge of the same mascaras with *P. aeruginosa* ATCC 9027 gave similar results; however, with mascara A3 this strain was recovered also at the 10^{-3} dilution after 7 days.

In experiments with a sequential challenge of mascaras A1 through A4 and B1 and B2 with *S. epidermidis* 4 followed by *P. aeruginosa* 3, staphylococci were not recovered from dilutions beyond 10^{-4} after 1 day and none were recovered after 7 days. The recovery of *P. aeruginosa* from the sequential challenge was similar to that presented in Table 1. The only difference was that *P. aeruginosa* grew in dilutions up to 10^{-6} of mascara A3 by day 1. The maximum dilutions cultured were 10^{-8}. However, using the plating procedure of the originally described membrane procedure (1), the densities of *P. aeruginosa* had increased on the surface of the membranes to over 10^{12} on mascara B1 by 1 day and on mascaras A1 and A2 by 7 days. At these concentrations, growth was visible on the membranes.

TABLE 1. *Recovery of P. aeruginosa 3 from different lots of two mascaras after a single challenge*

Mascara	After 1 day	After 7 days
A1	10^{-3}[a]	10^{-8}
A2	10^{-3}	10^{-8}
A3	10^{-3}	—[b]
A4	—	—
B1	10^{-8}	10^{-8}
B2	—	—
Control[c]	10^{-6}	10^{-5}

[a] Highest dilution of Letheen broth giving growth; 10^{-8}, highest dilution tested.
[b] —, No growth from dilution broths or membranes.
[c] White petrolatum.

The mascaras were examined periodically over an extended time (Table 2). The first evidence of loss of inhibitory activity of a mascara was detected after a sequential challenge. These products later supported the growth (membranes yielding at 7 days at least 2 logs more cells than in the inoculum) of *P. aeruginosa* after a single challenge. Lots of six brands of mascara, represented by C1 which, of the six, was studied over the longest time period, never supported growth of bacteria; the membranes usually yielded <1.0% of the inoculum.

Previously, laboratory challenge and in-use studies of brands of mascara implicated in the epidemiology of eye area infections indicated that some mascaras readily supported microbial growth (1, 6). There was incomplete agreement between the retrospective analyses of mascaras used by patients and the results of laboratory challenge studies of the same brand of mascara, probably because of differences in ages of the mascaras. Various lots of mascara differed markedly in the shelf life of their preservative systems. The loss of antimicrobial activity is unexplained, but it is of interest that chemical analysis (performed by the FDA) for preservative content of two lots of the same brand of mascara of different pigmentation—one that readily supported growth and one that did not—indicated similar concentrations of preservatives in both. This further supports the statements of Ramp and Witkowski (5) that preservatives are efficacious in some systems and inactivated in others. Since the inclusion of preservatives in various

TABLE 2. *Recovery of P. aeruginosa 3 from different lots of mascaras of various ages*

Mascara	<1[a]		6		12		24		36		48	
	S[b]	Sq	S	Sq	S	Sq	S	Sq	S	Sq	S	Sq
A1	+[c]	+	+	+	+	ND	+	ND	+	ND	+	ND
A2	−	−	−	−	−	+	+	−	ND	ND	+	+
A3	−	−	−	−	−	−	±	+	ND	ND	ND	ND
A4	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B1	−	+	−	+	+	+	ND	ND	ND	ND	ND	ND
B2	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C1	−	−	−	−	−	−	−	−	−	−	ND	ND

[a] Age in months.
[b] S, Single challenge with *P. aeruginosa*; Sq, sequential challenge with *S. epidermidis* followed by *P. aeruginosa* (except A3 and B1 where first challenge was with *C. albicans*).
[c] +, Recovery of *P. aeruginosa* after 7 days in concentrations at least 2 logs above inoculum; −, no recovery or recovery of <1.0% of inoculum at 7 days; ND, not done; ±, recovery of approximately 50% of inoculum after 7 days.

mascara formulations does not guarantee their activity, laboratory challenge procedures are necessary.

The membrane test, which was designed to detect the effectiveness of preservatives in the moisture film forming on the surface of anhydrous or semisolid mascaras and to permit sequential challenge of the same sample of mascara with different organisms, gave results in a relatively short period. We modified the procedure by omitting the mild sonification steps used to facilitate release of cells from the membranes and by omitting the plating procedures for enumeration of bacteria. The serial dilution in Leethen broth of bacteria flushed from the membrane provides sufficient accuracy for detecting acceptable product preservation, i.e., over 99% reduction in the challenge inoculum within 7 days (5). Culture of the membranes in broth serves to detect small numbers of surviving cells not readily demonstrated by the original plating procedure. A major advantage of the membrane procedure is the rapid detection of a product which supports microbial growth. The omission of the sonification and plating steps reduced the time necessary for an individual to examine two products and controls from about 12 days to 9 days (time for preparation of media is not included). Two of the 9 days were 8-h days, 2 were 3- to 4-h days, and the remaining 5 days required less than an hour per day. The technique requires skill and experience in handling the membranes. The procedure could be simplified further by replacing the wells and depression plate with a sterilizable unit.

The membrane test should be examined further with mascaras of known age and different preservative formulations to determine its sensitivity and predictive values of shelf life.

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