Effects of Time, Temperature, and Storage Container on the Growth of Fusarium Species

Implications for the Worldwide Fusarium Keratitis Epidemic of 2004-2006

John D. Bullock, MD, MPH, MSc; B. Laurel Elder, PhD; Harry J. Khamis, PhD; Ronald E. Warwar, MD

Objective: To demonstrate the effects of time, temperature, and container properties on the ability of ReNu with MoistureLoc (ReNuML; contains the antimicrobial agent alexidine) to inhibit growth of Fusarium species.

Methods: ReNu with MoistureLoc was stored in its Bausch & Lomb (Rochester, New York) plastic or similarly sized glass containers for 1 and 4 weeks at room temperature, 42°C, and 56°C, and then tested for its ability to inhibit growth of 7 Fusarium isolates.

Results: ReNu with MoistureLoc stored in glass containers for 1 or 4 weeks at all 3 temperatures demonstrated no significant fungistatic deterioration. However, ReNuML stored at 56°C in its Bausch & Lomb plastic container demonstrated a statistically significant fungistatic deterioration compared with room temperature storage in its original plastic container or with glass container storage at any temperature.

Conclusion: When exposed to elevated storage temperature, it appears that an interaction between ReNuML and its Bausch & Lomb plastic container adversely affects the fungistatic properties of ReNuML, which could have contributed to the Fusarium keratitis epidemic of 2004 through 2006.

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In August 2004, Bausch & Lomb (Rochester, New York) introduced a new contact lens solution, ReNu with MoistureLoc (ReNuML), containing the antimicrobial agent alexidine, which was not found in other contact lens solutions. During the next 2 years, hundreds of cases of Fusarium keratitis associated with ReNuML occurred worldwide, and the product was withdrawn from the market in May 2006. Levy et al subsequently reported that Bausch & Lomb investigators had performed "extensive testing" of the ReNuML formula and its components with "extended storage studies" as well as "elevated temperature studies, including impact on biocidal efficacy versus F solani." However, as previously noted, the Bausch & Lomb investigators did not specify the experimental conditions, the temperature(s) used, or the duration(s) of exposure and, to the best of our knowledge, have never reported the results of those studies.

All cases were related to the ReNuML produced only in Bausch & Lomb’s Greenville, South Carolina, plant and, after the Food and Drug Administration’s inspection of the Greenville facility, Bausch & Lomb was cited for inadequacies in temperature control in the production, storage, and transport of their products. The effects of elevated temperature on the antifungal properties of ReNuML and 5 other contact lens solutions have been investigated. ReNu with MoistureLoc demonstrated the greatest decline in fungistatic efficacy after 60°C storage for 4 weeks in its high-density polyethylene plastic container (room temperature [RT]: Fusarium growth in 27 of 84 samples; 60°C: Fusarium growth in 67 of 84 samples; P < .001 [Fisher exact test]). However, when ReNuML was boiled (approximately 100°C) for 10 minutes in a glass tube, there was no degradation of its fungistatic capability compared with RT storage, suggesting that both the plastic container and heat exposure are required for fungistatic failure of ReNuML. The study reported here was undertaken to investigate the effects of time, temperature, and container properties on the ability of ReNuML to inhibit growth of Fusarium.

Author Affiliations: Departments of Community Health (Drs Bullock and Khamis), Mathematics and Statistics (Drs Bullock and Khamis), Ophthalmology (Drs Bullock and Warwar), and Pathology (Dr Elder), and the Statistical Consulting Center (Dr Khamis), Wright State University, Dayton, Ohio.
through 2006, were obtained from the Centers for Disease Control and Prevention (Atlanta, Georgia). These same isolates were used and described in a previous study.10 Three unopened 60-mL plastic containers and 1 unopened 355-mL plastic container of ReNuML were originally procured in the Dayton, Ohio, area. The containers had 4 different lot numbers and expiration dates that were between 13 and 19 months prior to their use in these experiments. Solutions from these containers were combined, and six 46-mL aliquots of the combined solution were returned to the 3 original 60-mL plastic containers and into three 100-mL glass media containers with screw caps (Fisher Scientific, Pittsburgh, Pennsylvania). Each plastic and glass container was then incubated at RT (23°C-26°C), 42°C, or 56°C. Since the precise temperature and duration of exposure required for fungistatic failure were not determined in the original study,11(p126) we chose 2 new test temperatures (42°C and 56°C) and an additional incubation period of 1 week. The 42°C temperature was chosen to establish a midpoint between RT and the previously tested 60°C. The 56°C temperature was chosen to attempt to establish a new upper limit. Each of the 7 Fusarium isolates was plated on potato dextrose agar (Becton Dickinson) and incubated for approximately 7 days or until growth and sporulation were present. Testing was performed by adapting the recommendations of the Clinical and Laboratory Standards Institute document on Susceptibility Testing for Filamentous Fungi.12 After 1 week and 4 weeks, 21 mL of ReNuML was withdrawn from each plastic or glass container and used to make serial dilutions of each of the 6 ReNuML aliquots (RT, 42°C, and 56°C in original plastic or glass containers) in sterile 48-well tissue culture plates using Hanks balanced salt solution. A suspension of each Fusarium isolate was prepared in RPMI-1640 medium with L-glutamine ([RPMI] Lonza, Inc, Allendale, New Jersey) to a density equivalent to a McFarland 0.5 standard and then diluted 1:50 in RPMI.13 A 0.5-mL aliquot of this 1:50 dilution was then diluted with growth were plated on trypticase soy agar with 5% sheep blood (Becton Dickinson) and incubated for 48 hours to verify purity of Fusarium species. A growth control consisting of 0.5 mL of the 1:50 diluted Fusarium species suspension and 0.5 mL of RPMI was performed for all isolates.

P values were calculated using the Kruskal-Wallis and Wilcoxon rank sum tests to compare inhibitory titers for various combinations of temperatures and containers for 1- and 4-week incubation periods. An outcome value was assigned to each inhibitory titer so that whole numbers could be used for statistical analysis. The outcome values were 1 for an inhibitory titer of less than 1:2, 2 for 1:2, 4 for 1:4, 8 for 1:8, and 16 for 1:16 or greater. Statistical analysis was performed on 21 outcome values for each condition tested (7 Fusarium species performed in triplicate). The Kruskal-Wallis test was used to determine whether inhibitory activity differed significantly among the 6 experimental conditions (21 for each experimental condition [temperature-container combinations]). The Wilcoxon rank sum test was used for specific a priori comparisons. The 7 Fusarium isolates were included in the experiment so that the scope of the conclusions was the broadest possible. All calculations were performed using the Statistical Analysis System software (version 9.2; SAS Institute, Inc, Cary, North Carolina). The sequentially rejective Bonferroni procedure was used to ensure that the overall type 1 error rate was bounded by 0.05.13

SPECTROSCOPIC ANALYSIS

The interior surface of a single, separate, unheated ReNuML 60-mL plastic container was examined using Fourier transform infrared spectroscopy (performed by TEI Analytical, Inc, Niles, Illinois) to identify its composition.

RESULTS

Results for the 7 Fusarium isolates are shown in Table 2 and Table 3. The Kruskal-Wallis test showed that the inhibitory activity differed among the 6 temperature-container combinations presented in Tables 2 (P < .001) and 3 (P < .001). The results of individual comparisons are given in Table 4. Variables studied were time, temperature, and storage container (plastic vs glass). Fungal growth was observed in all samples tested in which the ReNuML dilution was 1:16; therefore, there were no inhibitory titers of 1:16 or greater. A combination of both elevated temperature and storage in its Bausch & Lomb plastic container was necessary for ReNuML to develop a statistically significant loss of inhibitory activity. Thus, comparison of storage at RT in a glass container vs RT in a plastic container did not show a loss of inhibitory activity, indicating that the storage container alone was insufficient to produce a significant difference in inhibitory activity (P = .61 [1 week]; P = .24 [4 weeks]). Storage in a glass container at 56°C vs RT did not result in a loss of inhibitory activity, indicating that elevated temperature alone was also insuf-

Table 1. Definition of Inhibitory Titer and Outcome Value

<table>
<thead>
<tr>
<th>Dilution of ReNuML</th>
<th>1:16</th>
<th>1:8</th>
<th>1:4</th>
<th>1:2</th>
<th>Inhibitory titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Outcome value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:16</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>≥1:16</td>
<td>16</td>
</tr>
<tr>
<td>1:8</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>1:8</td>
<td>8</td>
</tr>
<tr>
<td>1:4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>1:4</td>
<td>4</td>
</tr>
<tr>
<td>1:2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1:2</td>
<td>2</td>
</tr>
</tbody>
</table>

Abbreviations: ReNuML, ReNu with MoistureLoc; 0, no visible growth; +, visible fungal growth.

<sup>a</sup>The inhibitory titer was defined as the highest dilution of ReNuML contact lens solution that was able to inhibit fungal growth.

<sup>b</sup>An outcome value was assigned to each inhibitory titer so that whole numbers could be used for statistical analysis.
ficient to produce a significant difference ($P = .72$ [1 week]; $P = .52$ [4 weeks]). However, storage of ReNuML in its plastic container at 56°C vs glass container at 56°C or plastic container at RT resulted in significantly less inhibitory activity (plastic at 56°C vs glass at 56°C: $P = .003$ [1 week]; $P < .001$ [4 weeks]; plastic at 56°C vs plastic at RT: $P = .003$ [1 week]; $P < .001$ [4 weeks]). All inferences drawn from Table 4 were made at an overall significance level of .05, using the sequentially rejective Bonferroni procedure.

The results with 42°C incubation are less clear. Significant loss of inhibitory activity was seen after 1 week of incubation at 42°C in the original plastic container vs RT storage in the original plastic container ($P = .001$), but this was not demonstrated after 4 weeks of incubation.
(P = .47). Thus, it appears that with 42°C incubation, the results are inconsistent. Fungal growth was observed in the control plates for all isolates.

Fourier transform infrared spectroscopy revealed that the ReNuML container was polyethylene plastic.

The temperature-dependent decline in the fungistatic capability of ReNuML in its original plastic container, which was previously reported,10 was reproduced nearly identically in the study reported here. The ReNuML used in the present experiment was past its expiration date; however, when stored in a glass container at all 3 temperatures or at RT in its Bausch & Lomb plastic container, it was still very effective at inhibiting growth of *Fusarium* species and performed nearly identically to the unexpired ReNuML product under similar conditions.10 Furthermore, the expired ReNuML stored in its plastic container at 56°C (present study) performed nearly identically to the unexpired ReNuML in its plastic container stored at 60°C in a previous study.10 In both instances, there was a significant temperature-dependent decline in its fungistatic capability.

Based on the results of the study reported here, it appears that the temperature and incubation period required for fungistatic failure of ReNuML in its plastic container is greater than 42°C and 56°C or less for 1 week or less. However, when placed in a glass tube, ReNuML did not lose fungistatic efficacy after being boiled for 10 minutes10 or when incubated in a glass container at 56°C for 1 or 4 weeks (present study). These results strongly suggest that, in the presence of elevated temperature, there is an interaction between ReNuML’s antimicrobial agent, alexidine, and some component of the high-density polyethylene plastic Bausch & Lomb container. From 2004 to 2006, ReNuML was marketed by Bausch & Lomb in a plastic container with the Society of the Plastics Industry, Inc, Resin Identification Code “2,” indicating high-density polyethylene plastic.14 Fourier transform infrared spectroscopy in the present study confirmed that the ReNuML container was, in fact, composed of polyethylene. Whether the interaction between the plastic container and alexidine involves migration into the ReNuML solution of any contaminant from the container, which inactivates alexidine, or whether some other mechanism is involved remains to be determined. This study suggests that plastics may have a negative effect on human health in a way not previously considered.

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Correspondence: John D. Bullock, MD, MPH, MSc, Center for Global Health Systems, Management, and Policy, Wright State University, Boonshoft School of Medicine, 3123 Research Blvd, Ste 200, Dayton, OH 45420-4006 (johndbullock@aol.com).

Author Contributions: Dr Bullock had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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References