Chemical Destruction of *Mycobacterium bovis* in Milk

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**ABSTRACT**

The tuberculocidal activity of phenol and 1-Stroke Environ was tested using five *Mycobacterium bovis* strains added separately to five samples of untreated cows milk. The tuberculocidal activity of each disinfectant was significantly improved (P = .005) by increasing exposure temperature from 4 to 23°C or by increasing exposure time from 1 to 6 h or by increasing disinfectant concentrations two-fold.

Environ diluted 1:8 or phenol diluted 1:32 killed each of the five strains of *M. bovis* suspended in untreated milk (6 mg/ml) during a 6-h exposure at 23°C. Either disinfectant could be used to destroy *M. bovis* in unsalable milk from tuberculous cows scheduled for slaughter.

*Mycobacterium bovis* in milk from tuberculous cows was a major human health problem before pasteurization of milk and continues to be a threat to the health of animals (1). Disposal of milk from tuberculous cows and continuing to be a threat to the health of animals (1). Disposal of milk from tuberculous cows scheduled for slaughter presents an unusual environmental problem when unsalable milk is discarded.

Chemical treatment appears to be a feasible method for killing *M. bovis* in milk, thereby eliminating the hazard of bacterial contamination of the environment when unsalable milk is discarded.

Although the tuberculocidal activity of several disinfectants has been studied (3), there is currently no standard method for chemical destruction of *M. bovis* in milk. The purpose of this study was to evaluate two disinfectants, phenol and 1-Stroke Environ, for destroying *M. bovis* in milk. Phenol was chosen because it is a reference compound, readily available and proven effective as a tuberculocide (6). Environ was chosen because it is an approved USDA tuberculocide and was superior to other substituted phenolic tuberculocides tested in a preliminary probe.

**MATERIALS AND METHODS**

**Disinfectants**

Two disinfectants were used in the study: 1-Stroke Environ germicidal detergent (o-phenylphenol 10.0%, 0 benzyl-p-chlorophenol 8.5%, p-tertiary-amylphenol 2.0%) (Vestal Laboratories, St. Louis, Mo. 63110), and liquified phenol, (Mallinckrodt Inc., St. Louis, Mo. 63160). Both are hazardous chemicals (4).

**Cultures**

*M. bovis* strains 878 (ATCC 19211), 1042, 1332, 1505, and 1606 were isolated in this laboratory from bovine tissue.

**Milk**

Raw milk was obtained from a healthy Holstein cow approximately 2 h before use in disinfectant tests.

**Procedure**

Each *M. bovis* culture was transferred from a primary colony to 9.9 ml of Dubos broth containing Tween 80 and bovine albumin (DTA) (7) and incubated at 37°C. The optical density (O.D.) was measured daily using a spectrophotometer operated at 525 nm. When the O.D. reached 0.25, mid logarithmic phase (2), the DTA culture was either subcultured or transferred to a suspension containing 6 mg of *M. bovis* culture (wet weight) per ml of milk.

Serial two-fold dilutions of disinfectant were made by mixing equal volumes of disinfectant and milk containing 6 mg of *M. bovis* culture per ml. The approximate effective levels of each disinfectant were determined by preliminary dose responses. All disinfectant tests were done in duplicate at 23 and 4°C.

Following the appropriate exposure time of the primary dilutions (e.g. 1:4, 1:8, 1:16) each disinfectant was further diluted in DTA 10,000 fold to stop the action of the disinfectants. A 1:4 became a 1:40,000 dilution, etc. The higher (final) dilutions were prepared in duplicate and incubated at 37°C. The O.D. of each dilution was measured initially and again at 1.4, 8, 11, 15, 22, 29, and 36 days of incubation. The appearance time of new growth was defined as the reading day at which the O.D. increased by 0.05 or more. Trials in which no increase was detected at 36 days of incubation are identified with an asterisk in Table 1. The 36-day maximum incubation time was established following a preliminary probe in which no final dilution of any *M. bovis* grew in the disinfectant dilutions used here, when exposure was at 23°C for 1 h.

Positive dilution controls were made by suspending 6 mg of each test culture in sufficient DTA to form a 1:10,000 dilution. The suspension was used to determine whether or not sufficient mycobacterial cells remained in the final dilution to produce measurable turbid growth in DTA.

Negative milk controls were made by diluting portions of the untreated milk test samples to 1:100 (five trials) and 1:10,000 (10 trials) in DTA. The purpose of this control was to establish a baseline of O.D. (produced by the normal bacterial flora of milk) for later comparison with O.D. values of the chemically treated milk controls.

Chemically treated milk controls were made by mixing separate samples of raw milk with routine test concentrations of each disinfectant in the absence of *M. bovis*. The purpose of this control was
to determine whether or not the normal bacterial flora, measured in the negative milk controls, was killed by the routine test concentrations of each disinfectant.

Each *M. bovis* strain was reisolated on Herrold’s medium from turbid growth in two separate tubes of DTA; one had survived phenol treatment and the other had survived Environ treatment. Isolated colonies on Herrold’s medium were typed by routine biochemical methods (7).

Smears were prepared from approximately 20% of the final dilutions which produced 0.05 O.D. or greater. They were stained by the Ziehl-Neelsen method (7) to determine if acid-fast test cultures or non-acid-fast milk flora had produced the turbid growth.

**Statistical analysis**

The sign test (5) was used to determine the statistical significance of exposure temperature, exposure time, and disinfectant concentration on the tuberculocidal activity of Environ and phenol. An analysis was made of the number of times each variable caused an increase, a decrease, or no change in the tuberculocidal activity of each disinfectant.

**RESULTS AND DISCUSSION**

The tuberculocidal activity of both disinfectants increased with increased temperature, time, or disinfectant concentration (Tables 1, 2). Four comparisons were excluded due to contamination. In 63 of 116 trials (41 Environ, 22 phenol), the tuberculocidal activity was greater at 23 than at 4 °C. In 53 trials (19 Environ, 34 phenol) there was no difference in activity at the two temperatures. In 56 of 116 trials (38 Environ, 18 phenol), the tuberculocidal activity of the disinfectants was increased when the exposure time was extended from 1 to 6 h. There was no increased activity in 60 of the 116 trials (22 Environ, 38 phenol). Increased disinfectant concentrations caused increased tuberculocidal activity in 96 of 156 trials (63 Environ, 33 phenol). In 60 trials (17 Environ, 43 phenol), there was no difference in disinfectant activity between consecutive two-fold dilutions.

In the statistical analysis, the computed chi-square values all exceeded 15.0, indicating a highly significant effect of exposure temperature, exposure time, and disinfectant concentration on tuberculocidal activity (P = 0.005). These data are summarized in Table 2.

The highest dilution of phenol which was capable of killing the five *M. bovis* strains was 1:32 when exposure was made at 23 C for 1 or 6 h. When exposure was at 4 C for 6 h, the highest effective dilution was 1:16. Growth occurred in all dilutions of phenol when exposure was at 4 C for 1 h. The highest dilution of Environ which killed the five *M. bovis* cultures was 1:8 when exposure was made at 23 C for 6 h; 1:4 at 23 C for 1 h. Growth occurred in all dilutions of Environ when exposure was made at 4 C.

Although no precise comparisons of strain-related sensitivity to disinfectants were made, similarities between strains were evident in their rate of growth. In positive dilution control tests (no disinfectant), strain 878 first appeared at 11 days while the other four strains first appeared at 8 days. The earliest appearance time for strains treated with disinfectants was 8 days. Strain 878 did not appear until 29 days. The latest appearance time was 36 days. The variations in appearance time are shown in Table 1.

The opacity of the untreated milk caused a relatively high initial O.D. (average 1.2) in the 1:100 dilution. The turbidity increased rapidly to an average maximum O.D. of 1.7 in 24 h. The initial O.D. was 0 for all 1:10,000

<p>| TABLE 1. The effect of temperature, time and disinfectant concentration on appearance time of growth. Average of 5 M. bovis isolates. |</p>
<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Expa</th>
<th>Environ</th>
<th>Phenol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:4b</td>
<td>1:8</td>
<td>1:16</td>
</tr>
<tr>
<td>23</td>
<td>1</td>
<td>36*</td>
<td>24.46*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>36*</td>
<td>(11-36*)</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>14.8</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>34.6</td>
<td>(22-36*)</td>
</tr>
</tbody>
</table>

aExposure time in hours.
bDisinfectant dilution.
cFirst appearance of growth. Upper number = mean days. Numbers in parentheses = range of days.

<p>| TABLE 2. Summary of effects of temperature, time and disinfectant concentration on tuberculocidal activity of Environ and phenol. |</p>
<table>
<thead>
<tr>
<th>Tuberculocidal activity</th>
<th>Exposure temperature (4 to 22 °C)</th>
<th>Exposure time (1 h to 6 h)</th>
<th>Disinfectant concentration (two-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E</td>
<td>P</td>
<td>E</td>
</tr>
<tr>
<td>Increased</td>
<td>41</td>
<td>22</td>
<td>38</td>
</tr>
<tr>
<td>No change</td>
<td>19</td>
<td>34</td>
<td>22</td>
</tr>
<tr>
<td>Decreased</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chi-square</td>
<td>39.0</td>
<td>20.0</td>
<td>36.0</td>
</tr>
</tbody>
</table>

aNumber of trials representing Environ.
bNumber of trials representing phenol; four trials excluded due to contamination.
cComputed chi-square, comparing number of increases vs. number of decreases in tuberculocidal activity.
dilutions of untreated milk and then increased rapidly to an average maximum O.D. of 0.65 in 4 to 8 days of incubation. No growth occurred in any of the chemically treated milk controls indicating a complete kill of the normal milk bacterial flora.

Each M. bovis strain was reisolated from DTA following separate exposure to phenol and Environ. Biochemical typing was typical for M. bovis (7).

All smears were positive for acid-fast bacilli except those made from untreated milk and from DTA which developed turbid growth in 1 to 4 days.

The reproducibility of tests was measured in terms of agreement of results in duplicate tests. Of 120 comparisons, 111 (94.9%) were in agreement (similar appearance times), six (5.1%) were in disagreement (one test with growth, one without), and three were eliminated because of contamination.

The data collected in this study revealed the individual influence of four test factors used in evaluating the tuberculocidal properties of two disinfectants. Exposure temperature, exposure time, and disinfectant concentration were important but the effect of M. bovis strain on sensitivity to disinfectants was apparently unimportant. Decontamination was more effective at 23 than at 4 C, thus eliminating the expense of mechanically cooling the unmarketable milk before treatment with disinfectants.

Because the effectiveness of disinfectants increased with greater exposure time, further increases in tuberculocidal activity might be expected with exposures beyond 6 h. However, in actual practice additional exposure time could be impractical. The 1- and 6-h exposure times were selected because the milking schedules of many dairy farms allow 6 h for decontamination plus 2 h for preparing equipment for the next milking.

In preliminary tests, the incubation period for test cultures was 11 days; a period which was considered adequate for detecting growth in untreated subcultures of M. bovis. However, in subsequent tests it became apparent that growth of many treated M. bovis cultures was only delayed and a false kill would have been recorded in tests using an 11-day incubation period. An adequate incubation period was determined to be 36 days, after conducting incubation periods up to 63 days. It should be noted that in three instances the first appearance time of M. bovis in DTA was 36 days; however, all three were in the 4 C temperature category which is not recommended for actual use. The intervals between O.D. readings were unequal and could cause a misconception of the actual time involved. For example, if the actual appearance time of a culture was 16 days, it would have been recorded as 22 days, the next reading date.

It has been reported that small amounts of milk, serum, feces, or similar matter in the test environment can effect reductions of up to 90% of the bacteriocidal activity of phenol (6). In this study all of the milk was obtained under ideal conditions from one cow. It is not known whether or not milk from other sources would have altered the results.

Foaming and gelation were observed when Environ was mixed with milk, but phenol caused neither foaming nor gelation. Although no erratic results were specially noted when gelation was conspicuous, the consistency of the clots suggested that contact between disinfectant and M. bovis within the clots was probably restricted.

The data presented here indicate that Environ or phenol could be used in the chemical decontamination of milk from tuberculous cows.

ACKNOWLEDGMENT

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REFERENCES