Contribution of environmental mycobacteria to false-positive serum ELISA results for paratuberculosis

Jason B. Osterstock, DVM; Geoffrey T. Fosgate, DVM, PhD, DACVP; Bo Norby, CMV, PhD, MPVM; Elizabeth J. B. Manning, MPH, MBA, DVM; Michael T. Collins, DVM, PhD, DACVP; Allen J. Roussel, DVM, MS, DACVIM

Objective—To evaluate the effect of exposure to environmental mycobacteria on results of 2 commercial ELISAs for paratuberculosis in cattle.

Design—Experimental trial.

Animals—19 weaned crossbred beef calves.

Procedures—Calves were inoculated SC with 1 of 5 mycobacterial isolates (3 calves/isolate) derived from herds with high proportions of false-positive serologic reactions for paratuberculosis in calves, Mycobacterium avium subsp. paratuberculosis (MAP; positive control inoculum; 2 calves), or mineral oil (negative control inoculum; 2 calves). Sera were assessed at intervals by use of 2 ELISAs (A and B) for paratuberculosis in cattle, and all calves underwent tuberculin testing at the end of the study.

Results—Neither mineral oil–inoculated calf had positive results with either ELISA during the study. Both MAP-inoculated calves were identified as seropositive via ELISA-A, and 1 calf was identified as seropositive via ELISA-B. By use of ELISA-A, ≥ 1 false-positive reaction over time was detected in 2, 3, 3, and 1 of the 3 calves injected with Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium scrofulaceum, or Mycobacterium terrae, respectively. By use of ELISA-B, only M. scrofulaceum induced false-positive reactions (2/3 calves). Calves that had at least 1 positive ELISA-A result were more likely to be classified as suspect reactors via the caudal fold tuberculin test.

Conclusions and Clinical Relevance—False-positive serologic reactions may occur during use of commercially available ELISAs for paratuberculosis in calves experimentally exposed to environmental mycobacteria; naturally occurring exposures with these mycobacteria may represent a cause for high proportions of false-positive serologic reactions for paratuberculosis in some cattle herds. (J Am Vet Med Assoc 2007;230:896–901)

Paratuberculosis is a chronic, debilitating intestinal infection of ruminants caused by MAP. The disease is associated with notable economic losses in the beef and dairy industries because of increased culling, decreased value of culled cattle, diagnostic and treatment costs, and decreases in production.1–3 Serologic tests are an important component of paratuberculosis management programs when applied as herd-screening tools. Specificity estimates for commercially available paratuberculosis ELISAs are varied; reported estimates range from 95.3% to 99.8% in uninfected dairy cattle.8–11 Results from herd screening performed by use of a commercially available ELISA in beef cattle in Texas have indicated that the proportions of false-positive results were greater than that expected on the basis of reported assay specificities.12 A potential cause for these false-positive test results in beef cattle is their exposure to Mycobacterium spp that may have antigenic similarity to MAP and hence induce production of serum antibodies that cross-react with antigens in conventional serologic tests. These Mycobacterium spp are often referred to as environmental or atypical mycobacteria.13

In analyses of isolates obtained from bacterial cultures of feces from cattle and camels, Cousins et al14 identified sufficient genetic homology among MAP and environmental mycobacteria to cause false-positive reactions on PCR tests for paratuberculosis.

Exposure to environmental mycobacteria has been associated with cross-reactions during intradermal tuberculin testing in both cattle and humans, and delayed-type hypersensitivity reactions have been used to characterize the geographic distribution of environmental mycobacteria exposure among people in the United States.15–18 The US Navy has measured respons-

ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP</td>
<td>Mycobacterium avium subsp. paratuberculosis</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
</tr>
<tr>
<td>S:P ratio</td>
<td>Test sample to positive control sample ratio</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
</tbody>
</table>

From the Departments of Large Animal Clinical Sciences (Osterstock, Fosgate, Roussel) and Veterinary Integrative Biosciences (Fosgate, Norby), College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843; and the Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706 (Manning, Collins). Funded by USDA-APHIS-VS Award No. 2004-9100-0792-GR. Presented in part at the 8th International Colloquium on Paratuberculosis, Copenhagen, August 2005, and the 86th Conference of Research Workers in Animal Diseases (CRWAD), St Louis, December 2005. Address correspondence to Dr. Osterstock.
es to both PPD tuberculin and antigen from an environmental mycobacterial isolate (PPD-B tuberculin). The results of that screening program have been used to examine the incidence of tuberculosis, the extent of exposure to environmental mycobacteria, the potential for interference with tuberculosis test results by these environmental mycobacteria, and the geographic distribution of the environmental mycobacteria exposures. The screening program revealed that the prevalence of exposure to environmental mycobacteria is greatest in the southern and southeastern United States, including parts of Texas from which herds with high proportions of false-positive results for paratuberculosis (determined via ELISA) have been identified.

In a previous prevalence survey in Texas, several herds were identified for which the results via ELISA and microbial culture of feces were discordant, suggesting a high proportion of false-positive results. A subsequent study was performed in which environmental mycobacteria were isolated from bacterial cultures of feces collected in these herds with greater frequency than they were from bacterial cultures of feces collected in herds with low seroprevalence for paratuberculosis. The purpose of the study reported here was to further evaluate the effect of exposure to environmental mycobacteria on results of 2 commercial ELISAs for paratuberculosis in cattle.

Materials and Methods

Nineteen weaned Angus crossbred heifers (mean weight, 162.3 kg [357.1 lb]) were obtained from a single commercial cow-calf operation in central Texas. All calves were free from paratuberculosis, confirmed by results of microbial culture of feces and assessment of serum via ELISA-A and ELISA-B. Calves were housed in concrete pens in groups of 9 or 10 with no direct contact with other cattle or exposure to soil. They were fed coastal hay ad libitum, received a 14% crude protein commercial creep supplement (approx 4.5 kg/head/d [10 lb/head/d]), and had free access to salt and mineral blocks. All animal research procedures were reviewed and approved by the Texas A&M University Laboratory Animal Care Committee.

Mycobacterial isolates were obtained from microbial cultures of feces collected from cattle in herds with high proportions of apparent false-positive results determined on the basis of high seroprevalence for paratuberculosis but low prevalence of MAP isolated via bacterial culture of feces and no history of MAP-associated clinical disease. Isolates were obtained by use of radiometric culture methods in liquid medium, as previously described. Briefly, the medium was supplemented with mycobactin J, egg yolk suspension, and antimicrobials. Fecal samples were decontaminated with 1.0% hexadecylpyridinium chloride and concentrated via filtration. The resulting filter membrane was placed into radiometric culture medium and evaluated weekly for growth by use of an ionization detector.

For acid-fast organisms, a PCR assay for the IS900 gene insertion element was used to identify MAP. Mycobacterial isolates that were negative for IS900 were termed environmental mycobacteria and identified via high-performance liquid chromatography of extracted mycolic acids. From among the mycobacteria species isolated, Mycobacterium terrae, Mycobacterium celatum, Mycobacterium scrofulaceum, Mycobacterium intracellulare, and Mycobacterium avium subsp avium were selected for inoculation on the basis of their prevalence in the herds that had discordant diagnostic findings and on previous clinical experience.

One milliliter of each mycobacterial isolate and 1 mL of an MAP isolate (obtained in the same manner as the other isolates) were each inoculated into 10 mL of mycobacterial growth broth. Each culture was incubated until it became turbid, at which time it was evaluated for the presence of acid-fast bacteria. Ten milliliters of each pure culture was then inoculated into 100 mL of the growth broth. When the culture was turbid, it was again examined for the presence of acid-fast organisms. All resultant pure cultures were homogenized and the organisms killed by repeated passage through a 26-gauge needle and addition of formalin to a final concentration of 50% by volume. The cultures were shaken for 24 hours and each washed 3 times with PBS solution. One milliliter of each killed mycobacterial isolate was inoculated into a separate bottle of liquid culture medium to confirm that no viable organisms were present. Approximate organism concentrations for each isolate were determined visually; the number of organisms present in 3 separate 0.2-mm² areas was counted by each of 2 observers, and the mean count (cells/mL) was calculated. Final concentration was estimated by multiplying the mean number of organisms per milliliter by the total volume of the killed product (Appendix). Cultures were centrifuged and decanted, and killed organisms were stored as pellets at room temperature (approx 21°C [70°F]) pending inoculation.

Calves were randomly assigned to receive 1 of 7 treatments: inoculation SC with 1 of the 5 environmental mycobacterial isolates (3 calves/isolate), MAP (positive control inoculum; 2 calves), or mineral oil (negative control inoculum; 2 calves). At the time of inoculation, an isolate was suspended in 1.5 mL of sterile water and mixed with 2.0 mL of mineral oil. The mixture was emulsified through a double Luer-lock needle to ensure mixing and facilitate injection. Each calf assigned to receive inoculation with MAP or 1 of the 5 environmental mycobacterial isolates was administered 1.2 mL of killed mycobacterial suspension in mineral oil. For all treatments, calves were sedated by use of xylazine hydrochloride (0.3 mg/kg [0.14 mg/lb], IM) and then inoculated SC in the brisket region. Blood samples were collected via coccygeal or jugular venipuncture immediately prior to inoculation (week 0) and every 14 days thereafter for 10 weeks (at weeks 2, 4, 6, 8, and 10). Serum was separated from each blood sample and submitted for serologic evaluation by use of ELISA-A and ELISA-B. Laboratory personnel were unaware of calf identity (ie, treatment received) when processing samples. Results of the serologic tests were interpreted by use of the manufacturer’s recommended cutoff values provided in the diagnostic kit instructions. Results from ELISA-A were classified as positive if the S:P ratio was ≥ 0.25. The S:P ratio was calculated as the ratio of the negative control OD subtracted from the sample OD to the negative control OD subtracted from the positive control OD. Results from ELISA-B were classified as positive if subtraction of the
cutoff value (determined by adding 0.1 to the mean OD of the duplicate negative controls) from the sample OD and multiplication by 100 yielded a value that was > 0. The S:P ratios were calculated for ELISA-B for purposes of comparison of ELISAs and statistical analyses, not for results classification. Bacterial culture of feces was performed at the conclusion of the study by use of liquid culture media as previously described. At the conclusion of the study, all calves were evaluated for tuberculosis according to the Uniform Methods and Rules for Bovine Tuberculosis Eradication. All calves were injected with 0.1 mL of bovine PPD tuberculin in the caudal fold at the base of the tail; the area was palpated by 2 experienced veterinarians (AJR and JBO) 72 hours later to evaluate the reaction. Calves that had a suspect reaction on the basis of findings during palpation of the injection site underwent a comparative cervical test administered by regulatory officials from the Texas Animal Health Commission.

Differences in serologic response over time and among isolates were evaluated by use of a mixed-effects model with time modeled as a random effect. Pairwise comparisons were used to detect significant differences in serologic responses between treatment groups with Bonferroni adjustment for multiple comparisons. A first-degree autoregressive covariance structure was used to adjust for correlation of serologic responses among time points. By use of a Fisher exact test, proportions of calves with suspect reactor classification on the basis of caudal fold tuberculosis test results that had positive serologic results at any time during the study period and those that remained seronegative were compared. For all statistical tests, a value of \( P \leq 0.05 \) was considered significant. Statistical analyses were performed by use of commercially available statistical software.

**Results**

The ELISA results for the study calves varied among the different challenge isolates and between the 2 ELISA kits (Figures 1 and 2). Calves injected with mineral oil remained seronegative (as determined by use of both ELISAs) for the duration of the experiment. Of the 2 calves inoculated with killed MAP, both had positive results via ELISA-A at 2, 4, 6, 8, and 10 weeks after inoculation; 1 had positive results via ELISA-B at 6, 8, and 10 weeks after inoculation. Of the 3 calves inoculated with \( M. \) scrofulaceum, 2 (calves 1 and 3) had positive results via ELISA-A at week 8, and all 3 had positive results at week 10. When sera from these 3 calves were evaluated by use of ELISA-B, 1 (calf 2) had a positive result on week 6, and 1 (calf 1) had positive results at weeks 6, 8, and 10. Among the 3 calves inoculated with \( M. \) intracellulare, 1 had positive results via ELISA-A at week 2, and all 3 had positive results at weeks 4, 6, 8, and 10. None of the calves inoculated with \( M. \) intracellulare had positive results via ELISA-B during the study period. One of 3 calves inoculated with \( M. \) terrae had a positive result via ELISA-A at week 10. None of the serum samples from calves inoculated with \( M. \) terrae yielded positive results via ELISA-B during the study period. Of the 3 calves inoculated with \( M. \) avium, 1 had positive results via ELISA-A at weeks 2, 4, 6, 8, and 10, and 1 calf had positive results via ELISA-A at weeks 4 and 6. The third calf in this group did not have positive results via ELISA-A during the study period. None of the serum samples from these calves yielded positive results via ELISA-B during the study period. None of the serum samples from calves inoculated with \( M. \) celatum yielded positive results via either ELISA-A or ELISA-B during the study period.

For both ELISA-A and ELISA-B, S:P control ratios varied significantly (\( P < 0.001 \)) among isolates over time adjusted for the correlation associated with repeated observations on the same individual (Tables 1 and 2). Mean S:P ratios for calves inoculated with MAP were significantly greater than those for the mineral oil–treated (negative control) calves (\( P < 0.001 \)), \( M. \) scrofulaceum–treated calves (\( P = 0.025 \)), \( M. \) celatum–treated calves (\( P < 0.001 \)), \( M. \) terrae–treated calves (\( P < 0.001 \)), and \( M. \) avium–treated calves (\( P < 0.001 \)) by use of ELISA-A. Similarly, mean S:P ratios for calves inoculated with \( M. \) intracellulare were significantly greater than those for the mineral oil–treated (negative control) calves (\( P < 0.001 \)), \( M. \) scrofulaceum–treated calves (\( P < 0.001 \)), \( M. \) celatum–treated calves (\( P < 0.001 \)), \( M. \) terrae–treated calves (\( P < 0.001 \)), and \( M. \) avium–treated calves (\( P < 0.001 \)).
A at least once, compared with those that had negative
0.001) among calves that had positive results via ELISA-
test. The proportion of suspect reactors was greater (P
or oil
vical testing. None of the calves inoculated with mineral
these calves yielded negative results via comparative cer-
tracellulare
calves inoculated with
M scrofulaceum
B. Similarly, mean S:P ratios for calves inoculated with
M terrae
for the mineral oil–treated (negative control) calves
by use of ELISA-A. Mean S:P ratios for calves inocu-
0.001) by use of ELISA-A. Mean S:P ratios for calves
inoculated with M scrofulaceum were significantly (P
0.049) greater than those for M celatum–treated calves
by use of ELISA-A. Mean S:P ratios for calves inocu-
ated with MAP were significantly greater than those
for the mineral oil–treated (negative control) calves
(P = 0.008), M celatum–treated calves (P = 0.008), and
M terrae–treated calves (P = 0.046) by use of ELISA-
B. Similarly, mean S:P ratios for calves inoculated with
M scrofulaceum were significantly greater than those for
the mineral oil–treated (negative control) calves (P
0.041), M celatum–treated calves (P = 0.008), and M ter-
rae–treated calves (P = 0.046) by use of ELISA-B. For all
calves, results of microbial cultures of feces performed
at the end of the study were negative for mycobacteria.

Caudal fold tests for tuberculosis resulted in identi-
fication of 10 calves as suspect reactors. This subgroup
included 1 of 3 calves inoculated with M terrae, 2 of 3
calves inoculated with M avium, 2 of 3 calves inoculated
with M scrofulaceum, all 3 calves inoculated with M in-
tracellulare, and both calves inoculated with MAP. All of
these calves yielded negative results via comparative cen-
vical testing. None of the calves inoculated with mineral
oil or M celatum had suspect reactions to the caudal fold
test. The proportion of suspect reactors was greater (P
0.001) among calves that had positive results via ELISA-
A at least once, compared with those that had negative
results via ELISA-A for all samples. The proportion of
suspect reactors was not significantly different among
those classified as seropositive via ELISA-B at least once
during the study period and those that were seronegative
via ELISA-B for all samples (P = 0.620).

Discussion

The results of the present study have indicated that
cattle may develop an immune response after experimen-
tal inoculation with environmental mycobacteria, which
may generate false-positive results when the calves
are tested by use of an ELISA for paratuberculosis.
This is consistent with the findings of Jørgensen,
who evaluated the effects of oral exposure to various en-
vironmental mycobacteria, including M avium and M in-
tracellulare, on serologic tests for paratuberculosis used
approximately 25 years ago. Jørgensen also reported
that this exposure resulted in suspect reactions to ID
caudal fold injection of bovine PPD, which is similar to
the results of our study and other investigations
that assessed the effects of exposure to environmental
mycobacteria on tuberculin sensitivity in cattle. The
false-positive ELISA reactions attributable to environ-
mental mycobacteria exposure appear to markedly al-
ter the reliability of conventional interpretation of such
test results in affected beef cattle herds. Additionally,
the frequency of false-positive results differed between
the 2 ELISAs. Overall, at least 1 false-positive reaction
was detected in 9 of 15 calves during the study by use of
ELISA-A, compared with 2 of 15 calves by use of ELISA-B.
However, 1 of the calves inoculated with MAP remained
seronegative throughout the study, according to results of
ELISA-B. These results suggest that current specificity esti-
mates within herds with potential exposure to environ-
mental mycobacteria may be too high.

The source of exposures to these mycobacteria is
unclear, but beef cattle are most likely exposed to sappho-
thetic environmental mycobacteria while grazing. Alterna-
tively, cattle may be exposed via aerosolization of mycobacteria associated with water sources. This
type of exposure has been reported to be the most
common among humans with nontuberculous myco-
bacterial disease. It should be noted that the route of
exposure in the experiments of this report was unnatu-
ral and that the organisms were formalin-killed; either
factor might account for differences in immunologic
responses, compared with those after natural exposure,
although the responses in the present study were con-
sistent with findings in the field. The level of exposure
is likely affected by environmental factors and can be
predicted geographically. Falkinham et al determined
that the frequency of isolation of mycobacteria includ-
ing M avium, M intracellulare, and M scrofulaceum from
water sources was greater in the southeastern United
States than in northeastern states. Furthermore, it is
likely that there are differences in susceptibility and se-
rologic responses among breeds and individuals after
exposure to environmental mycobacteria. Exposure to
mycobacteria could also alter the host response to sub-
sequent exposure to pathogenic mycobacteria includ-
ing MAP.

Our findings are important because they suggest that
false-positive ELISA results may overestimate the

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Mean S:P ratio</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral oil*</td>
<td>0.000</td>
<td>NA</td>
</tr>
<tr>
<td>MAP</td>
<td>0.500</td>
<td>0.244–0.756</td>
</tr>
<tr>
<td>Mycobacterium avium</td>
<td>0.188</td>
<td>0.111–0.266</td>
</tr>
<tr>
<td>Mycobacterium scrofulaceum</td>
<td>0.238</td>
<td>0.098–0.350</td>
</tr>
<tr>
<td>Mycobacterium celatum*</td>
<td>0.000</td>
<td>NA</td>
</tr>
<tr>
<td>Mycobacterium terrae†</td>
<td>0.064</td>
<td>0.000–0.152</td>
</tr>
<tr>
<td>Mycobacterium intracellulare</td>
<td>0.558</td>
<td>0.368–0.749</td>
</tr>
</tbody>
</table>

*SP values are constant; no CI reported. †Lower limit of 95% confidence interval is bound by zero.
A,B,C Values with different superscripts are significantly (P < 0.05) different.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Mean S:P ratio</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral oil*</td>
<td>0.000</td>
<td>NA</td>
</tr>
<tr>
<td>MAP</td>
<td>0.500</td>
<td>0.244–0.756</td>
</tr>
<tr>
<td>M avium</td>
<td>0.188</td>
<td>0.111–0.266</td>
</tr>
<tr>
<td>M scrofulaceum</td>
<td>0.238</td>
<td>0.098–0.350</td>
</tr>
<tr>
<td>M celatum*</td>
<td>0.000</td>
<td>NA</td>
</tr>
<tr>
<td>M terrae†</td>
<td>0.064</td>
<td>0.000–0.152</td>
</tr>
<tr>
<td>M intracellulare</td>
<td>0.558</td>
<td>0.368–0.749</td>
</tr>
</tbody>
</table>

See Table 1 for key.
prevalence of paratuberculosis in beef cattle in some regions. To date, paratuberculosis prevalence estimates in beef cattle have largely been based on results of testing via ELISA and range from 0.4% to 8.6%.

Many of these estimates are based on results from samples evaluated by use of ELISA-A. A substantial number of these estimates are from cattle in the southern United States, which appears to be associated with increased probability of exposure to environmental mycobacteria. Interestingly, in a national survey to determine the prevalence of serum antibodies against MAP in cattle, Texas had the lowest proportion of seropositive herds among those sampled.

Although several significant differences in serologic reactions to different mycobacterial isolates were detected in the present study, it is important to note that these results were not derived from a randomly selected population and the serum S:P ratios were not normally distributed. Analysis of residuals did not identify severe departures from normality, but the limited sample size and relatively short duration of observation in our study likely limit the stability of the model used to test these hypotheses. Therefore, it would be inappropriate to use the results of the present study to predict how serologic responses to environmental mycobacteria exposure might differ at additional time points or with alternative means of exposure. The data indicated changes in S:P ratios over a fixed period following experimental exposure and highlight cumulative differences in S:P ratios and relative differences in rate of S:P ratio increase following exposure to different mycobacteria.

The results of the present study indicated that exposure of cattle to environmental mycobacteria, specifically M. avium, M. intracellulare, and M. scrofulaceum, may cause them to have false-positive results via 2 ELISAs that are widely used for diagnosis of paratuberculosis. Furthermore, there may be differences in the frequency of such false-positive reactions between these 2 ELISAs. In our study, the association of the isolates with false-positive reactions did not result from random mycobacterial isolate selection, but instead purposeful selection of isolates collected from herds with high seropositivity in the absence of supporting clinical history or culture results. Results of the present study and previous work suggest that the potential for natural exposure of cattle to Mycobacterium spp is high and appears to be associated with geographic differences in risk of exposure. On the basis of our findings, we suggest that veterinarians consider their geographic location and previous experiences when deciding which screening test for paratuberculosis to use. Producers should be forewarned that false-positive results may occur and that final management decisions should be based on findings of confirmatory tests if initial ELISA results are not consistent with herd clinical history.

References
8. Collins MT, Sockeet DC, Ridge S, et al. Evaluation of a commer-
13. Hornick DB, Schlesinger LS. Mycobacterioses other than tuber-
18. Edwards LB, Acquaviva FA, Lively VE, et al. An atlas of sensi-
20. Collins MT, Kenefick KB, Sockeet DC, et al. Enhanced radi-

Appendix

Results of manual counts of mycobacteria isolates to establish concentration of organisms prior to final centrifugation and inoculum preparation.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Cells/mL*</th>
<th>Volume (mL)</th>
<th>Estimated total No. of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP†</td>
<td>ND</td>
<td>42</td>
<td>ND</td>
</tr>
<tr>
<td>Mycobacterium intracellulare</td>
<td>$4.90 \times 10^8$</td>
<td>45</td>
<td>$2.21 \times 10^{10}$</td>
</tr>
<tr>
<td>Mycobacterium celatum†</td>
<td>$3.53 \times 10^6$</td>
<td>40</td>
<td>$1.41 \times 10^8$</td>
</tr>
<tr>
<td>Mycobacterium avium</td>
<td>$1.77 \times 10^8$</td>
<td>43</td>
<td>$7.61 \times 10^9$</td>
</tr>
<tr>
<td>Mycobacterium terrae†</td>
<td>ND</td>
<td>40</td>
<td>ND</td>
</tr>
<tr>
<td>Mycobacterium scrofulaceum</td>
<td>$1.79 \times 10^4$</td>
<td>45</td>
<td>$8.06 \times 10^4$</td>
</tr>
</tbody>
</table>

*Cells were counted in 3 separate 0.2-mm² areas by each of 2 observers and averaged to determine mean cells per milliliter. †Cells were too clumped for an accurate count. ‡Some large clumps and chains of cells were not counted.

ND = No data.