Fecal shedding of *Giardia duodenalis*, *Cryptosporidium parvum*, *Salmonella* organisms, and *Escherichia coli* O157:H7 from llamas in California

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Objective—To evaluate fecal shedding of *Giardia duodenalis*, *Cryptosporidium parvum*, *Salmonella* organisms, and *Escherichia coli* O157:H7 from llamas in California with respect to host factors and management practices.

Animals—354 llamas from 33 facilities.

Procedure—Fecal specimens were collected and examined for *G duodenalis* and *C parvum* by means of immunofluorescent microscopy. *Salmonella* organisms were cultured by placing feces into selenite enrichment broth followed by selective media. *Escherichia coli* O157:H7 was cultured by use of modified tryptocase soy broth followed by sorbitol MacConkey agar, with suspect colonies confirmed by means of immunofluorescent microscopy.

Results—12 of 354 fecal specimens (3.4%) had *G duodenalis* cysts. Younger llamas (crias) were more likely to be shedding cysts, compared with older llamas. Farm-level factors that increased the risk of shedding were large numbers of yearlings on the property (> 10), smaller pen sizes, large numbers of crias born during the previous year (> 10), and large pen or pasture populations (> 20). None of the 354 fecal specimens had *C parvum* oocysts. Seventy-six (from 7 facilities) and 192 (from 22 facilities) llamas were tested for *Salmonella* organisms and *E coli* O157:H7, respectively. All fecal specimens had negative results for these bacteria.

Conclusions and Clinical Relevance—Shedding of *G duodenalis* was primarily limited to crias 1 to 4 months old. Llamas from properties with large numbers of crias born in the previous year, resulting in large numbers of yearlings in the current year, were at greater risk of infection. In addition, housing llamas in smaller pens or pastures and managing llamas and crias in large groups also increased the risk of *G duodenalis* shedding. (*Am J Vet Res* 2001;62:637–642)

A growing concern among public health officials and water quality agencies in the United States is the potential public and environmental health risks associated with the storage and disposal of animal feces and urine.[1] Of particular concern are fecal-oral microbial pathogens that can be transmitted from livestock populations to humans, either from ingestion of contaminated water or through the consumption of unprocessed foods such as fresh fruits and vegetables that have been irrigated with water contaminated with infective manure or effluents. Although much of the regulatory attention is now focused on large confined and unconfined livestock operations,[1-3] consideration of smaller facilities such as recreational equine, llama, and commercial packstock operations has increased.[4-6]

Water quality concerns regarding llama operations are similar to packstock (equine and mule) facilities; the public and environmental health risks associated with the storage and disposal of animal waste is focused on the stacked manure pile (if one exists), fecal material in the holding pens and corrals, and the manure deposited along recreational trails if llamas are used as packstock. The first step in assessing the microbial risk to surface water quality attributable to llama operations is to generate a prevalence estimate of shedding of potentially zoonotic microbial pathogens. Although natural infections of llamas with *Giardia duodenalis*,[3,7] *Cryptosporidium* organisms,[8] *Listeria monocytogenes*,[9] and *Salmonella* serotype Typhimurium and *S* serotype Cholerasuis var *kunzendorf*[10] have been reported, to our knowledge, little information is available regarding the prevalence of fecal shedding of these and other potentially zoonotic microbial pathogens in nonhospitalized llama populations and in clinically healthy llamas that are more likely to be used as packstock on important watersheds.

The following cross-sectional survey was undertaken to determine the prevalence of fecal shedding of *Giardia duodenalis*, *Cryptosporidium parvum*, *Salmonella* organisms, and *E coli* O157:H7 in llamas in California. In addition, we evaluated whether various host and management practices were associated with the probability of shedding any of these 4 microorganisms. Our goal was to establish a baseline prevalence of fecal shedding of these microorganisms and to identify host factors and management practices that were associated with a reduced prevalence of fecal shedding from individual llamas. Such factors and practices could serve as potential good management practices for reducing the microbial risk to surface water quality attributable to llama operations.

Materials and Methods

Study population—Members of the California International Llama Association were solicited for voluntary participation to obtain widespread geographic testing. Fresh fecal specimens were collected from llamas ranging in age from 3 weeks to 23 years from private facilities located in 1 of 11 counties in California. Refrigerated fecal specimens were shipped overnight to the Veterinary Medicine Teaching

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and Research Center at the University of California-Davis in Tulare for diagnostic analysis. Specimens were collected between November 1996 and July 1997.

Examination for G *duodenalis* and *C parvum*—Approximately 10 g of feces were collected from each llama. Specimens were placed into plastic containers, refrigerated at 4 C, and diagnostic procedures were initiated within 48 hours of collection. Using 30 ml of Tween water (0.2% wt/vol Tween 80 in deionized water), 5 g of specimen was washed through folded surgical gauze into a 50-ml tube and centrifuged at 1,000 × g for 10 minutes. Supernatant was aspirated, and the pellet was resuspended in 2 to 3 ml of Tween water. Ten microliters were smeared onto a commercially prepared glass slide, air dried overnight, and a direct immunofluorescent assay[a] was performed according to the manufacturer's instructions. The entire smear was examined at 400× magnification for *Giardia* cysts and *Cryptosporidium* oocysts, with specimens containing 1 or more 4- to 6-µm diameter oocysts or 10- × 15-µm diameter cysts recorded as having positive results for *C parvum* or *G duodenalis*, respectively; otherwise, specimens were recorded as having negative results for these organisms.

***Salmonella* culture—**One gram of llama fecal material was inoculated into selenite enrichment broth at a 1:10 ratio (specimen:broth) and incubated overnight (12 to 18 hours) at 37 C. Selenite broth was streaked onto selective Salmonella-Shigella plating media, or xylose-lysine tergitol 4 and incubated for 24 to 48 hours at 37 C. The use of delayed secondary enrichment procedure was used for selected specimens. For delayed secondary enrichment, a cotton swab saturated with the original selenite broth was placed into 10 ml of fresh selenite broth and incubated 12 to 18 hours (overnight) at 37 C. Selenite broth was streaked onto selective Salmonella-Shigella plating media and suspect colonies were inoculated to triple sugar iron and urea media slants and incubated at 37 C for 24 hours. Colonies that produced an alkaline slant with an acidic butt, hydrogen sulfide induced pigment on triple sugar iron, and had negative results for urea hydrolysis were serotyped with commercial *Salmonella* serotyping reagents.[b]

***E coli* O157:H7 detection—**One gram of llama fecal material was inoculated into modified tryptocase soy broth containing desoxycholate (1 g/L), sodium citrate (1 g/L), tellurite (2.5 mg/L), and vancomycin (40 mg/L) in a 1:5 ratio and incubated 12 to 18 hours (overnight). The modified tryptocase soy broth was streaked onto sorbitol MacConkey agar containing tellurite (2.5 mg/L) and vancomycin (40 mg/L) and incubated at 30 C for 12 to 18 hours (overnight). Suspect colonies[3-8] were subcultured onto a MacConkey agar and a sorbitol MacConkey agar plate. Lactose positive and sorbitol negative suspect coliforms were stained with an affinity-purified fluorescein-conjugated goat antibody targeted against O157:H7.[c]

Statistical analysis—Fixed effects logistic regression[11] was used to test and quantify the association between host factors (eg, age, sex), structural features of housing (eg, pen size), management practices (eg, manure disposal, density of llamas in the corral), month of fecal collection, and the odds of shedding *C parvum* oocysts, *G duodenalis* cysts, *Salmonella* organisms, or *E coli* O157:H7. Forward stepping algorithm was used, with *P* value of ≤ 0.10 for inclusion of the term in the model, using the **likelihood ratio test (LRT)**. Goodness-of-fit for the final model was calculated, using the deviance and the Hosmer-Lemeshow test, with a χ^2 test performed on the appropriate *df* to determine *P* values.[11]

In the event that the observed prevalence was zero for *C parvum* or *G duodenalis*, the highest probable prevalence of shedding would be calculated given the current regulatory interest in fecal shedding of these waterborne protozoa from livestock populations. This variable is estimated from the binomial distribution by solving *P* (equation 1),

$$\left\{\frac{n}{x}\right\}(P)^x(1-P)^{n-x} \geq a$$

where n is the number of total specimens (in our study n = 354), x is the number of observed specimens with positive results (in this instance x = 0), P is the calculated maximum apparent prevalence of shedding for these protozoa for the population of llamas, and α is the probability of observing no specimens with positive results among n specimens given our point estimate of P.[6] Setting n = 354, x = 0, and $\alpha \geq 0.05$, the upper value for P is calculated by solving the following equation (equation 2):

$$P \geq 1 - 0.05^{1/354}$$

Such a calculation assumes sensitivity and specificity of the diagnostic test are 100%. To adjust the maximum apparent prevalence of shedding to account for the sensitivity and specificity of the diagnostic test (thereby calculating the maximum true prevalence of shedding), the maximum true prevalence can be calculated as (equation 3),[12]

$$\text{Maximum true prevalence} = \frac{(\text{Maximum apparent prevalence} + Sp - 1)}{(Se + Sp - 1)}$$

where the maximum apparent prevalence, P, is derived from equation 2, and specificity (Sp) and sensitivity (Se) are the diagnostic attributes of the immunofluorescent assay when applied to the study population of llamas.

Results

A single fecal specimen was collected from 354 llamas from 1 of 33 different private llama facilities locat-

Table 1—Host and management factors associated with fecal shedding of *Giardia duodenalis* in llamas from California

Variables	Presence of *Giardia* cysts
Age of llamas (m)	
0.1–2.0	6/24 (25.0%)
2.1–4.0	4/32 (12.5%)
4.1–12	1/19 (5.3%
13–24	1/31 (3.2%)
25–48	0/93 (0.0%)
49–72	0/60 (0.0%)
73–96	0/50 (0.0%)
> 96	0/45 (0.0%)
No. of crias born on the property during the previous year	
0	0/20 (0.0%)
1–5	0/177 (0.0%)
6–10	0/34 (0.0%)
11–15	8/85 (9.4%)
> 15	4/38 (10.5%)
No. of yearlings on the property	
0	0/40 (0.0%)
1–5	1/172 (0.6%)
6–10	0/36 (0.0%)
11–15	11/110 (10.0%)
No. of llamas and crias in the pen or pasture	
1–10	2/202 (1.0%)
11–20	1/67 (1.5%)
21–30	5/47 (10.6%)
> 30	4/38 (10.5%)
Size of pen or pasture (acres)	
0.1–0.5	1/62 (1.6%)
0.6–1.0	3/86 (3.5%)
1.1–5.0	8/159 (5.0%)
> 5.0	0/47 (0.0%)

ed in California (mean of 11 llamas tested/facility; range of 1 to 38 llamas tested/facility). Twelve (3.4%) of the 354 fecal specimens had 1 or more *G duodenalis* cysts (**Table 1**). Median age of llamas was 48 months; median number of crias born on the property during the previous year was 4; median number of animals per pen was 9; median number of acres was 2; median number of yearlings on the property was 4.

None of the 354 fecal specimens had detectable concentrations of *C parvum* oocysts. From the 354 llamas from 1 of 33 different llama facilities, a subpopulation of 76 (7 facilities) and 192 (22 facilities) llamas were tested for *Salmonella* organisms and *E coli* O157:H7, respectively. All fecal specimens had negative results for these bacteria.

Herd-level prevalence for fecal shedding of *G duodenalis* ranged from 0 to 11%, with 29 herds having all llamas with negative results and 4 herds having 1 or more llamas with positive results. Odds of shedding *G duodenalis* cysts were not associated with sex of llama nor month of fecal collection (data not shown). Using logistic regression to test the association between various host, structural, and management factors and the odds of shedding *G duodenalis* cysts, 2 alternative multivariate statistical models were developed for the association between various risk factors and the odds of shedding *G duodenalis* among llama populations. Each model provided an equally good fit and was biologically plausible.

For the first model (cria-acreage), we found that younger llamas were significantly more likely to be shedding cysts, compared with older llamas, with active shedding primarily limited to crias 4 months of age or younger (**Table 2, Fig 1**; LRT for age = 30 on 1 *df*, $P < 0.001$). The number of crias born during the previous year was associated with the probability of llamas shedding *G duodenalis* cysts this year (LRT for No. of crias = 13 on 1 *df*, $P < 0.001$). Effect modification existed between this factor and the size of the pen or pasture containing the tested llama, indicating that the relationship between the number of crias born during the previous year and the odds of shedding *G duodenalis* was influenced by the size of the pen or pasture containing the tested llama (LRT for interaction term = 3.7 on 1 *df*, $P = 0.056$). For facilities that keep their llamas and crias in pens or pastures smaller than 6 acres, the odds of a llama shedding *G duodenalis* increased with an increase in the number of crias born during the previous year; however, this association between the number of crias born during the previous year and the odds of shedding *G duodenalis* became negligible once the pen or pasture size was greater than 6 acres. The overall model did not significantly differ from the raw data. Goodness-of-fit for the cria-acreage model was deviance = 44 on 267 *df*, $P = 1.00$; Hosmer-Lemeshow statistic = 0.5 on 8 *df*, $P = 0.99$.

Regarding the second model (yearling-population), age of the llamas was associated with odds of shedding *G duodenalis*, just as in the cria-acreage model (**Table 3, Fig 2**; LRT = 28 on 1 *df*, $P < 0.001$). The number of yearlings on the property (but not nec-

Table 2—Alternative fixed effects logistic regression cria-acreage model for risk factors associated with fecal shedding of *Giardia duodenalis* in llamas from California

Factor	Median (range)	Odds ratio (95% CI)	Likelihood ratio test *P* value*
Age of llama (m)	48.0 (0.1–276)	0.90 (0.83, 0.98)	< 0.001
CRIA	4.0 (0–25)	1.46 (1.13, 1.89)	< 0.001
ACRE	2.0 (0.1–100)	1.32 (0.60, 2.90)	0.52
CRIA × ACRE	NA	0.94 (0.88, 1.00)	0.06

*Tests the null hypothesis that the logistic regression coefficient is zero (odds ratios = 1) for the specified term, using the likelihood ratio test.
CI = Confidence interval. CRIA = Number of crias born during the previous year. ACRE = Size of pen or pasture. NA = Not applicable.

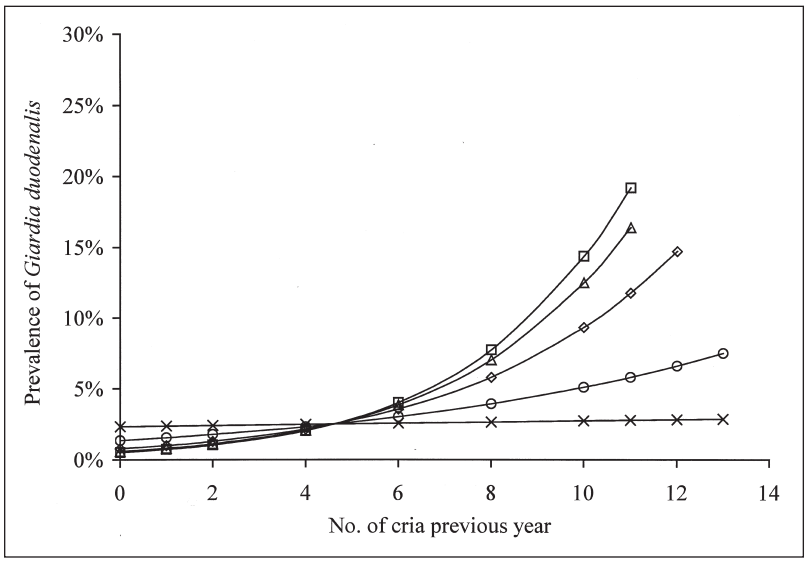

Figure 1—Association between total number of crias born on the property during the previous year and the prevalence of shedding *Giardia duodenalis* cysts, stratified across different corral or pasture sizes to indicate effect modification between number of crias and corral or pasture size (corral or pasture size in acres: □ = 0.5; △ = 1.0; ◇ = 2.0; ○ = 4.0, X = 6.0). Prevalence of shedding modeled for 4-month-old crias.

essarily sharing the same pen or pasture with the tested llama) was associated with shedding *G duodenalis* cysts (LRT for No. of yearlings = 14 on 1 *df*, $P < 0.001$). Effect modification existed between this factor and the total number of llamas sharing the same pen or pasture with the tested llama, indicating that the relationship between the number of yearlings on the property and the odds of shedding *G duodenalis* was influenced by the total number of llamas sharing the same pen or pasture with the tested llama (LRT for interaction term = 4.1 on 1 *df*, $P = 0.04$). In other words, the odds of shedding *G duodenalis* increased for llamas on properties with a high number of yearlings, but this association between the number of yearlings on the property and the odds of shedding *G duodenalis* became less pronounced once the pen population exceeded 20 llamas. The overall model did not significantly differ from the raw data. Goodness-of-fit for the yearling-population model was deviance = 40 on 267 *df*, $P = 1.00$; Hosmer-Lemeshow statistic = 0.2 on 8 *df*, $P = 1.00$.

None of the 354 llamas had detectable concentrations of *C parvum* oocysts. The maximum apparent prevalence of shedding *C parvum*, setting the confidence to be ≥ 5 % (ie, eliminating scenarios with < 5% probability of occurrence), was ≤ 0.84%. To calculate the maximum true prevalence for our study population, estimates for the sensitivity and specificity of the diagnostic assay[a] in this population of llamas were needed. Given that the observed number of positive test results was zero, there was no opportunity for false-positives. Hence, specificity should have approximated 100% in our cohort of llamas. Diagnostic attributes for the performance of the assay in llama populations are currently not available, but the specificity of the detection kit was recently estimated at 100%, using flow cytometry as the criterion standard for a population of 95 horses.[13] In addition, 3 independent evaluations of the detection kit on human fecal specimens found 100% specificity.[14-16] The sensitivity of the assay, defined as the probability of detecting 1 or more oocysts/fecal smear for fecal specimens with positive results,[17] was estimated at 43%, using flow cytometry as the criterion standard for a population of 95 horses.[13] In addition, 4 independent determinations of the sensitivity of the assay for human fecal specimens ranged from 83 to 100%.[14-16,18] Using the more conservative of these point estimates (43%) as an approximation of the sensitivity of the assay in llamas, the estimated maximum true prevalence of shedding of *C parvum* for llamas in central California would be ≤ 2.0% of the population.

Table 3—Alternative fixed effects logistic regression yearling-population model for risk factors associated with fecal shedding of *Giardia duodenalis* in llamas from California

Factor	Median (range)	Odds ratio (95% CI)	Likelihood ratio test *P* value*
Age of llama (m)	48.0 (0.1–276)	0.89 (0.81, 0.99)	< 0.001
YRL	4.0 (0–14)	2.86 (0.83, 9.88)	< 0.001
POP	9.0 (1–66)	1.46 (0.79, 2.70)	0.10
YRL × POP	NA	0.97 (0.92, 1.01)	0.04

YRL = Number of yearlings on the property. POP = Number of llamas or crias in the pen or pasture.
See Table 2 for key.

Discussion

In our study, we found no llamas shedding *C parvum*, *E coli* O157:H7, or *Salmonella* organisms at the time of testing, and fecal shedding of *G duodenalis* limited primarily to crias < 4 months of age. Furthermore, using conservative scenarios (worst-case) for estimating the maximum true prevalence of shedding of *C parvum*, it would appear that < 2.0% of llamas in central California are likely to be shedding *C parvum* at any given time. The observed 0% (0/248) prevalence of *G duodenalis* shedding for llamas 2 years of age or older was significantly lower than the 4.6% prevalence of *G*

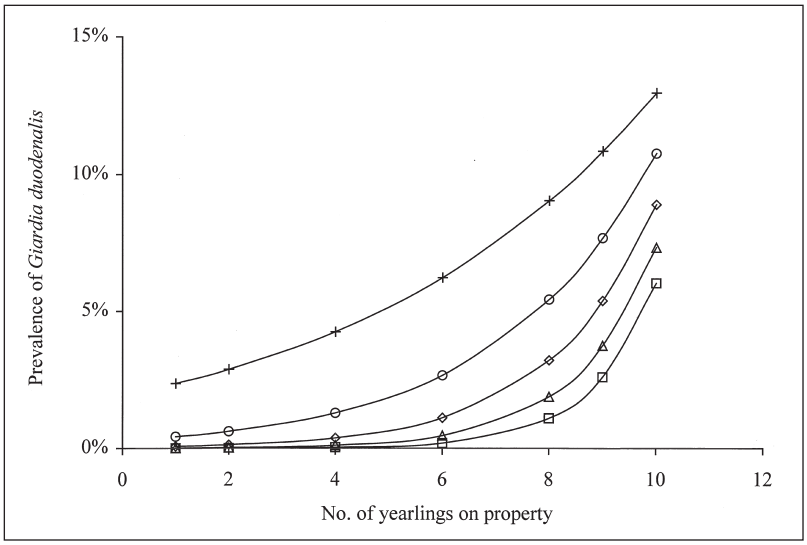

Figure 2—Association between total number of yearlings currently on the property and the prevalence of shedding *Giardia duodenalis* cysts, stratified across different population sizes (total No. of llamas regardless of age in the pen or pasture with the tested llama) to indicate effect modification between number of yearlings and population size (population sizes: □ = 5; △ = 10; ◇ = 15; ○ = 20, X = 25). Prevalence of shedding modeled for 4-month-old crias.

duodenalis shedding for the horses and mules at commercial facilities in the Sierra Nevada Range (2-sided $P < 0.001$, on the basis of results of a Fisher exact test),[6] but not significantly different from the 0.7% (2/300) prevalence of *G duodenalis* shedding among recreational trail horses tested in Colorado (2-sided $P = 0.50$, on the basis of results of a Fisher exact test).[4] The limited specimen sizes for *E coli* O157:H7 and *Salmonella* organism detection precludes us from making strong inferences regarding the true prevalence of fecal shedding for these important bacterial zoonoses. It is also important to be aware that little, if any, incontrovertible evidence exists of documented transmission of any of these 4 microorganisms from llamas to humans. Furthermore, for *G duodenalis*, 2 reviews of the scientific literature concluded that evidence for its zoonotic potential is incomplete.[19,20] In contrast, *Giardia* organisms obtained from a Gambian giant pouched rat (*Critetomys gambianus*) was infectious for a human volunteer.[21] In addition, results of 2 recent molecular epidemiologic studies, using enzyme electrophoresis and restriction fragment length polymorphism of polymerase chain reaction amplicons, indicated that isolates from humans and several domestic and wild animal hosts were genetically similar.[22,23] Although this evidence is compelling, its relevancy is unclear with respect to the risk that llama *G duodenalis* poses to humans in rural areas under standard sanitary and culinary conditions (eg, filtering or boiling water, adequate personal hygiene, cooking food).

Young crias from properties that had larger numbers of crias born in the previous year, leading to larger numbers of yearlings in the current year, were at greater risk of infection. This tentatively suggests that endemic infection on a llama facility is maintained by the previous year's infected crias, leading to a subset of infected yearlings this year, which then serves to transmit the infection to this year's cria population. Absence of fecal shedding among llamas 2 years of age or older may indicate that a 2-year cessation of newborns on the property may be sufficient to break the cycle of endemic giardiasis in closed populations. Alternatively, minimizing contact between crias and yearlings may serve to reduce the number of infections in crias. What is not clear at this time is whether minimizing *G duodenalis* infection among crias will result in increasing the prevalence of infection later in life once the llamas become exposed to *G duodenalis* through whatever means. This would have the unintentional effect of moving the median age of shedding to a later age and thereby would increase the likelihood that infected llamas be used in recreational settings, rather than the current scenario of *G duodenalis* infection being primarily limited to llamas < 12 months of age.

Managing llamas in smaller pens or pastures and maintaining llamas in larger groups were associated with a higher risk of *G duodenalis* shedding. This is consistent with the notion that the likelihood of fecal-oral transmission between infected and susceptible hosts is higher when interanimal or fecal-oral contact rates are increased as the result of spatial confinement. Large group sizes can serve to prolong the circulation of *G duodenalis* between infected and susceptible hosts and increase the likelihood that at least 1 llama in the group is actively infected. We observed increased *G duodenalis* shedding in horses and mules kept at higher corral densities for corrals of 6,000 square feet or less, but this association between the number of animals and the likelihood of shedding *G duodenalis* became negligible once the corral exceeded 6,000 square feet.[6] Managing llamas on large pastures or, if space is limited, partitioning the large group into a set of smaller independent groups through the use of multiple pens or pastures may function to reduce the prevalence of infection among crias.

In conclusion, given the generally low prevalence of fecal shedding for these 4 pathogens among adult llamas, recreational use of this age of llama should pose only a negligible risk of waterborne transmission to humans. Furthermore, if fecal material from crias is managed to reduce the viability of protozoal and bacterial microorganisms of zoonotic concern (eg, composting, spreading, and drying) and if recreational llama use on backcountry trails is designed to exclude crias and is managed to minimize fecal deposition in proximity to surface water, llamas and crias are unlikely to be an important waterborne source of these microbes for humans.

[a]MERIFLUOR *Cryptosporidium/Giardia* direct immunofluorescence detection kit, Meridian Diagnostics Inc, Cincinnati, Ohio.

[b]Salmonella O Antiserum Group series, Difco Laboratories, Detroit, Mich.

[c]*Fluorescein*-labeled Affinity Purified Antibody to E coli O157:H7, Kirkegaard & Perry, Gaithersburg, Md.

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Correction: Bacteriologic and histologic features in mice after intranasal inoculation of *Brucella melitensis*

In the article "Bacteriologic and histologic features in mice after intranasal inoculation of *Brucella melitensis*" (*AJVR*, Mar 2001), the figure legend for figure 8 on page 402 is incorrect. The correct information is that the photomicrographs are sections of liver obtained 28 days (top) and 1 day (bottom) after mice received intranasal inoculation of *B melitensis* organisms.