Safety Analysis of *Leishmania* Vaccine Used in a Randomized Canine Vaccine/Immunotherapy Trial

Angela Toepp,^{1,2†} Mandy Larson,^{1,2†} Tara Grinnage-Pulley,^{1,2} Carolynne Bennett,^{1,2} Michael Anderson,¹ Molly Parrish,^{1,2} Hailie Fowler,¹ Geneva Wilson,^{1,2} Katherine Gibson-Corely,³ Radhika Gharpure,⁴ Caitlin Cotter,⁴ and Christine Petersen^{1,2*}

¹Department of Epidemiology, College of Public Health, University of Iowa, Iowa City, Iowa; ²Center for Emerging Infectious Diseases, University of Iowa Research Park, Coralville, Iowa; ³Department of Pathology, Carver College of Medicine, University of Iowa, Iowa City, Iowa; ⁴Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland

Abstract. In *Leishmania infantum*–endemic countries, controlling infection within dogs, the domestic reservoir, is critical to public health. There is a need for safe vaccines that prevent canine progression with disease and transmission to others. Protective vaccination against *Leishmania* requires mounting a strong, inflammatory, Type 1 response. Three commercially available canine vaccines on the global veterinary market use saponin or inflammatory antigen components (Letifend) as a strong pro-inflammatory adjuvant. There is very little information detailing safety of saponin as an adjuvant in field trials. Safety analyses for the use of vaccine as an immunotherapeutic in asymptotically infected animals are completely lacking. *Leishmania infantum*, the causative agent of canine leishmaniasis, is enzootic within U.S. hunting hounds. We assessed the safety of LeishTec[®] after use in dogs from two different clinical states: 1) without clinical signs and tested negative on polymerase chain reaction and serology or 2) without clinical signs and positive for at least one *Leishmania* diagnostic test. Vaccine safety was assessed after all three vaccinations to quantify the number and severity of adverse events. Vaccinated animals had an adverse event rate of 3.09%, whereas placebo animals had 0.68%. Receiving vaccine was correlated with the occurrence of mild, site-specific, reactions. Occurrence of severe adverse events was not associated with having received vaccine. Infected, asymptomatic animals did not have a higher rate of adverse events. Use of vaccination is, therefore, likely to be safe in infected, asymptomatic animals.

INTRODUCTION

Leishmaniasis is a protozoan parasitic disease endemic in 98 countries and territories.¹ Visceral leishmaniasis (VL) is endemic in South America and throughout the Mediterranean basin² with both human and canine hosts.^{3,4} Statistical and mathematical models of *Leishmania* zoonotic transmission demonstrated that an effective canine-targeted vaccine would also control human disease.^{5–7} Veterinarians in Brazil, the country with the greatest burden of zoonotic canine leishmaniasis (CanL), have been empirically using vaccination as immunotherapy to treat dogs as they were only recently awarded with the first drug available in Brazil to treat CanL, Miltefosine (Milteforan), licensed in 2017. Before that it had been illegal for veterinarians to treat dogs seropositive for CanL because of the risk of these animals failing treatment and promoting *Leishmania* transmission to people. This report uses a unique cohort of *Leishmania*-infected hunting dogs within the United States to evaluate the safety profile of an anti-*Leishmania* vaccine (LeishTec) in both naïve and infected asymptomatic dogs, for example, as an immunotherapeutic to prevent the progression of infection to disease.

Leishmania infantum, the causative agent of CanL, is endemic within a subpopulation of U.S. hunting hounds.^{8–11} Naturally infected U.S. hounds were shown to be infectious to sand flies; thus, if these dogs were bitten by competent vector(s), they could serve as domestic reservoirs to emergent human infection.^{12–14} Dogs born to infected dams are infected, but remain asymptomatic, through the first 2 or 3 years of life and often test negative by serological screening.

Vertical transmission of *L. infantum* and *Leishmania donovani* has been reported to occur in both dogs and people around the globe.¹⁵ The impact of vertical versus vector-borne transmission is unknown as to date there is not a test that can differentiate the means of transmission.

Currently, all three licensed canine vaccines available globally advise against vaccinating serologically positive animals because of the risk of an adverse event and lack of knowledge as to how vaccination might change the course of disease.^{16–18} Because of vaccination practices in endemic countries, in both Europe and South America, asymptomatic, seronegative, infected dogs are frequently vaccinated. Based on our previous studies,^{8,12,19,20} asymptotically infected dogs may have altered vaccine responsiveness, leading to increased inflammation after vaccination. Assessing the safety of vaccine use within asymptomatic dogs is necessary to assure that vaccination is safe within a population including infected animals.

There is limited evidence regarding anti-*Leishmania* vaccine safety from previous field trials with cohorts with sufficient power to detect less common adverse events. Previous anti-*Leishmania* vaccine studies provided minimal discussion on vaccine safety, with no information regarding risk factors to experiencing an adverse event (e.g., breed and gender).^{21–27} One study compared two commercially available vaccines and concluded that there was no correlation between adverse events and breed, gender, or age.²⁸ Studies of rabies or distemper virus vaccine use in dogs concluded that younger adult, small-breed, neutered dogs were at a higher risk of experiencing an adverse, vaccine-associated event.^{29,30}

This LeishTec vaccine/immunotherapy trial enrolled 557 hunting hounds from seven states within four regions of the United States. Exclusion criteria were established to provide the hounds' immune response the best opportunity to respond to the vaccine effectively and reduce the probability of

* Address correspondence to Christine Petersen, Department of Epidemiology, College of Public Health, University of Iowa, S429 CPHB, 145 N. Riverside Dr., Iowa City, IA 52241. E-mail: christine-petersen@uiowa.edu

† These authors contributed equally to this work.

adverse events. Dogs with clinical CanL were not included because of the likelihood of immune exhaustion against *Leishmania* antigens.⁸ This evaluation found that although local swelling at the site of vaccine injection did occur, 3% of animals had any type of adverse event. Occurrence of severe adverse events was not associated with having received vaccine versus placebo, and asymptomatic animals receiving vaccine did not have an increased rate of adverse events. Providing LeishTec was therefore safe in all dogs, including asymptotically infected animals.

MATERIALS AND METHODS

Animals. Whole blood samples were collected from hunting hounds in four U.S. geographic regions from seven states (Alabama, Colorado, Georgia, Illinois, Maryland, Missouri, and Virginia; $N = 557$). Physical examination was performed at the time of blood collection, and hounds were categorized as 1) uninfected; 2) asymptomatic, with quantitative polymerase chain reaction (qPCR) or serologic evidence of infection; or 3) symptomatic, based on having two or more of the following clinical signs of CanL: alopecia, dermatitis, conjunctivitis, epistaxis, cachexia, hepato- and/or splenomegaly, and lymphadenopathy, as determined in previous studies.^{31,32}

Ethics. Animals were enrolled with signed informed consent of the caretakers and followed protocol 5121575 as approved in December 2016 by the University of Iowa Institutional Animal Care and Use Committee. The University of Iowa is an Association for Advancement and Accreditation of Laboratory Animal Care-accredited institution, and this study meets the guidelines as set out by the Guide for Care and Use of Laboratory Animals, most recently updated in 2011.

LeishTec vaccine and placebo injections. LeishTec, made by Hertape, Brazil, is an A2-based recombinant protein with saponin adjuvant. Vaccine lot 042/15 was used for the trial, made with the manufacturer's instructions in English per United States Department of Agriculture requirements. Placebo was sterile water for 250 random samples in the first series as provided by Hertape, Brazil. Saline was used for all other placebo injections. Vaccine lots were provided by Hertape free of cost and imported through a United States veterinary biological product permit for research and evaluation provided to Dr. Petersen. Ten percent of the vaccine and sterile water lots were tested by Animal and Plant Health Inspection Service to assure that this product was free of contamination by foot-and-mouth disease virus, endemic within Brazil. Permission for experimental vaccination was granted by the state veterinarians of each of the seven states where the vaccine was provided to dogs as a part of this trial.

Sample management and database. All blood and serum samples were stored with unique barcode identifiers. All hound names and matching bar code-identifier numbers were securely stored in an excel database only accessible to a designated researcher. All veterinarians performing physical examinations and researchers performing diagnostics and statistical analyses were blinded and restricted from viewing any identifiers of the hounds to maintain good clinical practices and reduce bias.

Parasite DNA isolation and diagnostic qPCR. QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA) was used for DNA isolation per manufacturer's specifications for 200 μ L blood. The quality and quantity of isolated DNA from whole blood

samples were assessed using NanoDrop 2000 (Thermo Scientific, Waltham, MA). Isolated DNA (neat and 10-fold dilution) was analyzed in duplicate via qPCR in a 96-well plate via Super Mastermix (Rox) (Quanta Biosciences, Gaithersburg, MD). Each qPCR plate contained negative control nuclease-free water and samples of whole blood-extracted DNA from negative dogs. Positive control samples of 10^6 *Leishmania* parasites spiked into canine blood and DNA extracted were tested at full strength, 1:10, and 1:20 dilutions. Ribosomal primer sequences used are as follows: F 5'-AAGTGCTTCCCATC GCAACT, R 5'-CGCACTAAACCCCTCCAA (Invitrogen, Life Technologies, Grand Island, NY), and probe: 5' 6FAM-CGGTTCGGTGTGTGGCGCC-MGBNFQ (Applied Biosystems, Life Technologies, Grand Island, NY). Primers and probe were used at a concentration of 10 nM. The assay was run on an ABI 7000 system machine. Cycling protocol was as follows: 95°C for 2 minutes, 95°C for 1 minute, and 50 cycles of 95°C for 15 seconds, 60°C for 1 minute. Results were analyzed using ABI 7000 System SDS Software (Applied Biosystems, Life Technologies).

Dual path platform[®] canine visceral leishmaniasis (DPP CVL) assay. The DPP CVL test detects *Leishmania*-specific antibodies using the recombinant diagnostic antigen rK28 and colloidal gold particles coupled to protein A.³³ After addition of the last buffer, a timer was started, and the cassette test window was monitored for the appearance of a positive test line next to the control line.³⁴ Test result was visually read and time to positive recorded. A single positive control line was confirmed at 15 minutes for negative test samples.

Vaccination dosing interval and enrollment criteria. A vaccine trial commenced in January 2016 to evaluate a recombinant CanL vaccine, LeishTec, in a population of U.S. hunting hounds. Hounds were excluded based on the following criteria: 1) were pregnant, 2) were < 6 months of age at enrollment, 3) were symptomatic for leishmaniasis, ehrlichiosis, anaplasmosis, Lyme disease, or heartworm disease at enrollment, 4) were not currently on routine vaccinations (i.e., distemper, hepatitis, parainfluenza, and parvovirus combined vaccination and rabies), or 5) had not had a recent deworming medication administered. The vaccination schedule began in February 2016, and consisted of three subcutaneous injections of LeishTec applied in a ventral direction on the left flank just caudal to the scapula. Vaccine was provided via a 3-mL syringe with a 1"-23-gauge needle. Cold chain for the vaccine was maintained via soft-sided coolers throughout the trial and stored at 4°C. The participating population consisted of 557 hounds vaccinated at Day 0 (see Supplemental Figure 1 for schema of vaccination).

Adverse event reporting. Following vaccination at Day 0, Day 14, and Day 28, vaccine trial team members remained at each kennel for 60 minutes to monitor hounds for possible acute reactions. Hound owners were asked to report any adverse events noted within 48 hours after the team member(s) left the premises. Reported adverse events were categorized as 1) mild: soreness or swelling noted at the injection site and/or hound experienced lethargy and/or depression for < 24 hours; 2) moderate: soreness or swelling noted at injection site and/or hound experienced lethargy and/or depression that lasted > 24 hours or required veterinary care; 3) severe: vomiting, diarrhea, central nervous signs (blindness and ataxia), seizures, organ failure, or death within the 48-hour window. Reported adverse events were recorded and

analyzed after all three time points were completed. In the event of a severe event within the 48-hour window that resulted in death or euthanasia of the dog, the animals were stored (frozen) and necropsy performed at the first possible time to evaluate the cause of death and association with vaccination.

Statistical analyses. Fisher's exact test, χ^2 analysis, Pearson's correlation, and Kaplan–Meier survival curves were completed using SAS version 9.4 (SAS Institute Inc., Cary, NC), R version 3.2.1 (R Foundation for Statistical Computing, Vienna, Austria), and GraphPad Prism 6.0 for Windows (GraphPad Software, La Jolla, CA). $P < 0.05$ was defined as significant.

RESULTS

Cohort demographics. Three *Leishmania* vaccines are on the market for use in dogs. Despite this, very little information is available regarding risk factors that may promote mild or severe adverse events following vaccination against *Leishmania*. Because of current laws in Brazil requiring culling of any *Leishmania*-seropositive dog, there is no information available regarding the safety of LeishTec in infected animals. To evaluate the safety of LeishTec vaccine in the U. S. hunting hounds, an initial 650 hounds were assessed for enrollment in the study. Of the 650 hounds, 557 hounds were enrolled in the study and randomized to receive either placebo or vaccine. Five hundred and forty-six hounds were vaccinated and monitored after each vaccination time point (Supplemental Figure 1). Gender was distributed equally across vaccine and placebo groups with 49% male in vaccine group and 47% male in placebo group (Table 1). The mean age was 4 years for both groups. Regional distributions were the same between the placebo and vaccinated groups. Further analyses were performed to establish the rate of adverse events following vaccination with LeishTec and to investigate whether these events correlated with a particular vaccination within the three shot series.

TABLE 1
LeishTec vaccine/immunotherapy trial cohort demographics

Variable	Vaccine	Placebo
Day 0	274	272
Day 14	268	268
Day 28	266	267
Age, mean $\pm$ SD	4.100 $\pm$ 2.291	4.252 $\pm$ 2.611
Gender, % male	48.89	47.46
Region, %		
Midwest	21.11	18.48
South	17.04	15.21
East	50.37	53.63
West	11.48	12.68
Mild reactions (no.)		
Day 0	5	1
Day 14	9	1
Day 28	8	0
Total	22	2
Severe reactions (no.)		
Day 0	1	1
Day 14	0	2
Day 28	2	0
Total	3	3

SD = standard deviation. Bold indicates statistically significant increase in mild reactions in vaccinated group, $P < 0.05$.

Mild adverse events were significantly dependent on having received vaccine. No immediate, anaphylactic-like events occurred in any hound at any time point. All other adverse events were reported to the trial clinical team by phone or e-mail within 72–96 hours of the vaccination visit. In the case of severe events, a necropsy was performed if possible (2/3 animals that received vaccine and had a severe event). Analyses were performed to determine the overall rate of injection-associated adverse events in both groups. Regardless of treatment group, the incidence of any adverse event in the trial was 1.86% (30/1,615) across all three time points. The incidence of adverse events reported across all three time points in hounds that received the vaccine was 3.09% (25/808).

The occurrence of an adverse event was analyzed for an association between receiving an injection of vaccine versus placebo. In addition, we evaluated factors contributing to mild versus severe adverse events; no moderate adverse events were reported. Ninety-eight percent of events were without any change, the vast majority of hounds in the trial were vaccinated without experiencing an adverse event. However, the chance of having an adverse event was significantly dependent on receiving vaccine as compared with placebo (Figure 1A, risk ratio: 4.99, 95% confidence interval [CI]: 1.99–12.57, P value = 0.0002).

To evaluate whether this association also occurred when stratifying for severity of event, events were sorted into mild versus severe adverse event groups based on the definitions provided. Only mild adverse events were significantly dependent on receiving vaccination (RR: 10.99, 95% CI: 2.88–42.05, P value = 0.0001). The risk of having a severe adverse event after injection was not associated with having received vaccine, (RR: 1.02, 95% CI: 0.24–4.42, P value = 0.9766), (Figure 1B, Table 2). Of approximately 300 hounds that received LeishTec vaccine as a part of this study, in total three hounds experienced a severe adverse event across all vaccination time points, as did three dogs in the placebo group.

Although rare adverse effects occurred, benefit of vaccination greatly outweighs risks in hunting dogs. To establish the safety versus benefit impact of the vaccine, the rate of deaths attributed to leishmaniasis in the placebo group across the 12 months of follow-up was compared with the deaths categorized as severe adverse events directly or indirectly attributable to vaccination (Figure 2). There was a statistically significant higher rate of deaths due to leishmaniasis in the placebo group compared with vaccine-related deaths in the vaccinated group. Although the vaccine group did have a necropsy-confirmed severe adverse event that led to euthanasia of the dog, significantly more deaths will be averted by vaccine intervention than associated as a severe adverse event with vaccination.

Experiencing an adverse event was not dependent on time in the vaccination course. To establish whether developing a cellular/adaptive immune response to the vaccine was significantly correlated with adverse events, analyses were performed to establish if there was an association between vaccination time point and the number of mild adverse events. The hypothesis was that if vaccination with saponin mounted a sufficiently robust cell-mediated immune response, by 28 days after the first vaccination, there should be a delayed-type hypersensitivity response within the dermis at the site of vaccination detected as a site reaction. There were

FIGURE 1. Adverse events were significantly associated with receiving anti-*Leishmania* vaccine. Five hundred and forty-six hounds were vaccinated against *Leishmania infantum* over three vaccination time points, each 2 weeks apart. Adverse events were reported by owners to the research team and analyzed. (A) Risk of any adverse event (mild, moderate, or severe) in vaccinated (black) and placebo (white) groups. $P < 0.05$ (B) Risk of developing a severe adverse reaction for vaccine (black) and placebo (white) groups. All statistical analyses were performed using Fisher's exact test.

no significant differences in the occurrence of adverse events across all three vaccination time points (Figure 3). This suggests that these events were unlikely to be due to development of an adaptive immune response and more likely inflammation related.

***Leishmania infantum* status did not affect the rate of adverse events.** To establish whether there was a relationship between the development of an adverse event and *L. infantum* status, a separate analysis was performed comparing *Leishmania* diagnostic status to the rate of vaccine reactions within that diagnostic group. We hypothesized that asymptotically infected dogs may experience an adverse event due to the vaccine boosting the responsiveness of an already activated immune system. Surprisingly, *L. infantum* status was not significantly related to having an adverse event (P value 0.3554), suggesting adverse events were not due to the (re) activation of immunity in *L. infantum*-positive dogs (Figure 4).

Shorter breed experienced more adverse events than taller breeds. Previous studies of vaccine-associated adverse events in dogs found a significant association between smaller dogs and risk of an adverse event. In this trial, there were two hound breeds enrolled. Based on the American Kennel Club breed standards, one breed was not to be taller than 15" at the point of the shoulder, and the other, despite sexual dimorphism, was not to be shorter than 21" in smaller, female hounds. To evaluate the association between the size of hound and the risk of experiencing an adverse event, the data were stratified by breed and statistical analysis performed to compare these breeds. The shorter hound breed was significantly more likely to experience an adverse event compared with the likelihood of an adverse event in taller hounds, (RR: 46.94, 95% CI: 23.06–94.4, P value < 0.0001).

Adverse events within these shorter hounds predominated the total adverse events reported (Figure 5).

DISCUSSION

Current therapeutic measures to decrease infection in dogs and the transmission to humans are often cost-prohibitive and/or not efficacious.^{6,35–41} Treatment of CanL is difficult or even prohibited in some countries.^{37,40–43} This is due to the fear of the development of drug-resistant strains of *Leishmania* based on veterinary use as the same drugs are used to treat disease in dogs and humans.^{37,40–43} The most common treatment of choice for VL in humans worldwide is amphotericin B (liposomal or conventional) with or without the combination of pentavalent antimony.⁴⁴ However, these therapies are often not well tolerated, are cost-prohibitive, and have become complicated by antimonial-resistant *Leishmania* strains in India and Nepal.^{44–47} Other therapeutic options include miltefosine and paromomycin, which either have not

FIGURE 2. Rate of death from leishmaniasis was significantly greater than the rate of death associated with vaccination during 48 hours following vaccination. Rate of deaths due to leishmaniasis in animals that did not receive vaccine and severe adverse events in the vaccinated group depicted via Kaplan-Meier curve. Significance of differences were analyzed using χ^2 analysis, $P = 0.03$.

TABLE 2

Risk ratios for all, mild, and severe adverse events

Adverse event	Fisher's exact P value	RR	CI
All	0.0002	4.99	1.99–12.57
Severe	0.9766	1.02	0.24–4.42
Mild	< 0.0001	10.99	2.88–42.05

CI = confidence interval. Dogs were vaccinated against *Leishmania infantum* over three vaccination time points, each 2 weeks apart. Adverse events were reported by owners and analyzed. Fisher's exact test with significance set at $P < 0.05$, $N = 546$, designated in bold.

FIGURE 3. No association with time of vaccination across vaccination period. Adverse events collected at each time point were compared between the three different vaccination time points using χ^2 analysis, $P = 0.5158$.

been tested for efficacy against visceralizing forms of infection or have a lower efficacy than aforementioned treatments.^{44,48} Using a vaccine as an immunotherapeutic, alone or in conjunction with reduced doses of chemotherapy, would provide a highly desirable alternative to current expensive, toxic, and ineffective options in both people and dogs.

In this canine study population, LeishTec was safe with only a 3.09% incidence of adverse events in vaccinated dogs. A mild adverse event, which includes soreness or swelling at the injection site or lethargy for < 24 hours, is not an undesirable response to a vaccine as it indicates that an appropriate inflammatory response is being mounted at the site of vaccination. An equal amount of severe adverse events were reported in the placebo group as the vaccine group, potentially indicating the causes of these events were other disease processes unrelated to the vaccine trial. Hounds within this study have many high-risk exposures to their overall health within their daily lives due to the intensity and environment in which they are working. This includes long-distance hunts

FIGURE 4. Risk of an adverse event not associated with positive *Leishmania* status. Comparison between *Leishmania* diagnostic status and number of adverse events per number of animals in *Leishmania*-negative and *Leishmania*-positive (asymptomatic) groups made using a one-way Fisher's exact test with Baptista-Pike method. $P = 0.0795$.

FIGURE 5. Shorter hound breed is more likely to experience an adverse event. Hound breeds in the study were stratified by breed height and compared using a χ^2 analysis. RR: 46.94, 95% confidence interval: 23.06–94.40.6, $P < 0.0001$.

through high brush and grass during both hot and cold weather, which can lead to pleurisy, heat stress, cuts, tears, and bite wounds. Based on the even distribution of severe events between placebo and vaccinated groups, severe events are likely to have had to do more with life experiences rather than vaccination.

The shorter hound breed had a higher risk of having a mild adverse event as compared with the other hound breeds in this study. Although the hounds were not weighed individually in this trial, other studies have shown that smaller breed dogs are more likely to experience a vaccine-related adverse event.^{29,30} This may indicate that smaller breeds are more likely to experience a mild adverse event to LeishTec due to a dose-response mechanism. However, this may have been confounded by husbandry differences between breeds used in this study; smaller hounds were individually handled more frequently. This could increase the potential that a slight change postvaccination was more likely to be detected by the owner of the smaller hounds compared with the larger hounds.

Time within the vaccine course and *L. infantum* status were not significantly related to having an adverse event, suggesting that adverse events were not associated with immune response or *L. infantum* positivity. Instead, this response was more likely due to inflammation related to saponin or other vaccine components. Exclusion criteria chosen for this vaccine trial seemed to be efficacious in preventing vaccination of hounds that would have a severe adverse event. Participating hounds were examined every 3 months until the 12-month time point to evaluate the long-term safety, with no additional adverse event findings in this trial cohort.

Safe and efficacious vaccines for CanL are important tools to reduce the transmission and development of disease. There is very limited information available in the literature regarding safety of *Leishmania* vaccination and no evidence of the safety of vaccinating infected, nonclinical animals. This vaccine safety analysis provides a description of LeishTec safety profile and provides evidence that asymptomatic dogs can be vaccinated safely. However, as vaccination usage increases, it will be important to continue monitoring for the possibility of additional moderate, severe, or otherwise rare adverse events. This study provides animal model evidence for safety in development of human immunotherapies/vaccines to decrease

clinical progression in *Leishmania*-infected asymptomatic populations.

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Authors' addresses: Angela Toepp, Mandy Larson, Molly Parrish, and Geneva Wilson, College of Public Health, University of Iowa, Iowa City, IA, E-mails: angela-schneider@uiowa.edu, maklarson@gmail.com, molly-parrish@uiowa.edu, and geneva-wilson@uiowa.edu. Tara Grinnage-Pulley, National Cancer Institute, Frederick, MD, E-mail: tara.grinnage-pulley@nih.gov. Carolyne Bennett and Caitlin Cotter, Centers for Disease Control and Prevention, Decatur, GA, E-mails: nuw8@cdc.gov and caitlincotter@gmail.com. Michael Anderson, Center for State and Territorial Epidemiologists, Atlanta, GA, E-mail: manderson@cste.org. Hailie Fowler, Integrated DNA Technologies, Coralville, IA, E-mail: hflowler@idtdna.com. Radhika Gharpure, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, E-mail: radhika.gharpure@gmail.com. Christine Petersen, Department of Epidemiology, College of Public Health, University of Iowa, Iowa City, IA, and Center for Emerging Infectious Diseases, University of Iowa Research Park, Coralville, IA, E-mail: christine-petersen@uiowa.edu.

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