DOI: 10.7589/2013-07-159 *Journal of Wildlife Diseases*, 50(1), 2014, pp. 140–142

Utility of a Fecal Real-time PCR Protocol for Detection of *Mycobacterium bovis* Infection in African Buffalo (*Syncerus caffer*)

Annette Roug,[1,7] Claire Geoghegan,[2] Elizabeth Wellington,[3] Woutrina A. Miller,[1] Emma Travis,[3] David Porter,[3] David Cooper,[4] Deana L. Clifford,[1,5] Jonna A. K. Mazet,[1] and Sven Parsons[6] [1]Wildlife Health Center & One Health Institute, School of Veterinary Medicine, 1089 Veterinary Medicine Drive, University of California, Davis, California 95616, USA; [2]Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, Private bag X20 Hatfield, Pretoria 0028, South Africa; [3]School of Life Sciences, University of Warwick, Coventry, CV4 7AL, UK; [4]Ezemvelo KZN Wildlife Game Capture Unit, PO Box 13053 Cascades, Pietermaritzburg 3202, South Africa; [5]California Department of Fish and Wildlife, 1701 Nimbus Road, Suite D, Rancho Cordova, California 95670, USA; [6]Faculty of Medicine and Health Sciences, Stellenbosch University, PO Box 19063, Tygerberg, 7505, South Africa; [7]Corresponding author (email: aroug@ucdavis.edu)

ABSTRACT: A real-time PCR protocol for detecting *Mycobacterium bovis* in feces was evaluated in bovine tuberculosis–infected African buffalo (*Syncerus caffer*). Fecal samples spiked with 1.42×10^3 cells of *M. bovis* culture/g and Bacille Calmette–Guérin standards with 1.58×10^1 genome copies/well were positive by real-time PCR but all field samples were negative.

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis*, is a concern in many wildlife species (Michel et al. 2006), with African buffalo (*Syncerus caffer*) as important maintenance hosts (De Vos et al. 2001). Current diagnostic tests for bTB require animal capture, and noninvasive techniques would greatly expand screening options. A sensitive real-time PCR assay has been utilized for detection of *M. bovis* in badger feces (Sweeney et al. 2007). The assay had 100% specificity and 97% sensitivity in spiked fecal samples with $\geq10^5$ *M. bovis* cells/g (Travis et al. 2011) and detected *M. bovis* in 12 of 12 infected badger latrines (Sweeney et al. 2007).

We evaluated the utility of the DNA extraction and real-time PCR assay protocol as a diagnostic herd screening tool for *M. bovis* infection in African buffalo. Fecal samples were collected from 229 buffaloes from three herds in Hluhluwe-iMfolozi Park, South Africa. Buffaloes were corralled into a temporary capture facility, immobilized with etorphine hydrochloride (Novartis Animal Health, Isando, South Africa) and azaperone (Janssen Pharmaceutica, Woodmead, South Africa), branded, and screened for bTB using single intradermal tuberculin skin tests. Bovine-purified protein derivative (0.1 mL) was injected, and skin thickness was measured at 0 and 72 hr. Reactors with an increase in skin thickness of ≥4 mm (World Organization for Animal Health 2013) were slaughtered for postmortem examination.

Fecal samples were collected rectally on the first test day. Age class and sex were recorded, and body condition was scored from 1 (emaciated) to 5 (obese; Ezenwa et al. 2009). Fecal samples were subdivided into 2-mL microcentrifuge tubes and frozen at −20 C. DNA was extracted at the Faculty of Health Sciences, University of Stellenbosch, South Africa, with the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, Ohio, USA) with the following modifications: 0.1 g of feces was used and ribolyzed 2 times for 40 sec at 6 m/sec. DNA was stored at −20 C until processing using real-time PCR. Eight samples from skin test–negative buffaloes were spiked with heat-killed *M. bovis* over a dilution range of 1.42×10^7 to 1.42×10^1 cells/g feces (Sweeney et al. 2007). A reference standard curve was generated using purified *M. bovis* Bacille Calmette–Guérin (BCG) DNA in a dilution series of between 1.58×10^5 and 1.58×10^{-2} genome copies per reaction. Real-time PCR targeting *M. bovis* sequences flanking a region of difference (RD4) deletion, and utilizing a fluorescent probe hybridizing with the 5′ and 3′ RD4 deletion-flanking sequences that only are adjacent to each other in *M. bovis* (Brosch et al.

2002), was performed on collected samples, spiked samples, standards, and negative controls in triplicate from each extraction (Sweeney et al. 2007). The threshold, 0.30 delta Rn with an automatic baseline, was set at the midexponential phase of the amplification curve. A sample was positive if each of the triplicates gave cycle threshold (Ct) values above the threshold and negative if all were below the threshold. If two thirds or one third of the triplicate was positive, the sample was considered inconclusive and rerun. Inhibition by contaminants was assessed using an inhibition control assay (Pontiroli et al. 2011). A delta Ct value >1.5 was considered significant inhibition.

Thirty of 229 buffaloes (13%) were skin test reactors; 17 were adult females, 10 adult males, two subadult males, and one subadult female. Body condition scores (BCSs) ranged from 3 to 4 (good to excellent) in 27 of 30 animals, whereas 2 of 30 and 1 of 30 had BCSs of 2 (thin) and 1 (emaciated), respectively. Fecal samples were available from 29 of 30 skin test reactors (missing one adult male), and postmortem examination results were available from 18 of 30 reactors (missing from seven adult females and four adult males).

At postmortem examination, 5 of 18 buffaloes (27.8%) had visible tuberculous lesions in the mediastinal, bronchial, and parotid lymph nodes, as well as in tonsils and lungs; 9 of 18 (50%) had limited lesions in one or more tonsils, parotid lymph nodes, lungs, or bronchi and mediastinal lymph nodes; and 4 of 18 (22.2%) had no lesions.

DNA standards with at least 1.58×10^1 genome copies per PCR and fecal samples spiked with at least $\sim1.42\times10^3$ cells/g feces were positive. For spiked samples, the extraction protocol included a final dilution step into 100 μL of water. Ten μL of this diluent was added to the real-time PCR well, and the lowest detectable concentration was 1.42×10^1 genome copies, which coincided with the lower limit of BCG standards. All samples from skin test reactors were negative, and one sample had mild inhibition (delta Ct = 1.8).

The real-time PCR assay had high sensitivity to detect *M. bovis* DNA in spiked samples but failed to detect any in feces of naturally infected animals, including buffaloes with extensive thoracic lesions. Two factors determine the utility of a fecal diagnostic test: rate of shedding and ability to detect pathogens. Infection in cattle and buffaloes is predominantly pulmonary (Neill et al. 1994; Laisse et al. 2011), with intermittent shedding of *M. bovis* in feces and nasal secretions documented in livestock (Kao et al. 2007, Srivastava et al. 2008). Little information exists on fecal shedding in buffalo, but one study of Asian buffaloes cultured *M. bovis* in feces from 3 of 36 skin test reactors (Jha et al. 2007). Conventional culture is known to have low sensitivity in feces, and our utilization of PCR techniques may improve fecal screening, such as recently when fecal shedding was detected in pastoralist cattle by the authors using this protocol (unpubl.). In our limited sample size, infected buffalo might not have shed *M. bovis* in feces because, in contrast to badgers, *M. bovis* discharged from the lungs was destroyed in the complex gastrointestinal tract, or the real-time PCR assay was not sensitive enough to detect *M. bovis* diluted in the large fecal volume. Combining real-time PCR with immunomagnetic capture (Sweeney et al. 2006) to allow DNA extraction from more feces or increasing the cycle number may improve sensitivity. Although we cannot recommend this fecal real-time PCR protocol as a herd screening tool for detection of *M. bovis* infection in African buffalo, the test may have utility as a noninvasive diagnostic tool in other species that have shorter digestive tracts or acquire more systemic infection.

We thank Ezemvelo KZN Wildlife, Hluhluwe-iMfolozi Park Game Capture, Management, and Hluhluwe Research

Centre; and Dr. Jenny du Preiss, Warren and Alicia McCall, and Jacoline Winterbach from the South African Department of Agriculture, Forestry and Fisheries, Directorate of Food and Veterinary Services (Hluhluwe). This project was supported by the Wildlife Health Center, University of California, Davis, USA (UCD); approved by the Institutional Animal Care and Use Committee at UCD (Protocol 15919); the Ezemvelo KZN Wildlife Scientific Research Committee (Permit registration E/5077/05), and the Department of Agriculture, Forest and Fisheries (Reference 12/11/1/5, Section 20 of the Animal Diseases Act No. 35, 1984).

LITERATURE CITED

Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, Garnier T, Gutierrez C, Hewinson G, Kremer K, et al. 2002. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proc Natl Acad Sci USA* 99:3684–3689.

De Vos V, Bengis RG, Kriek NP, Michel A, Keet DF, Raath JP, Huchzermeyer HF. 2001. The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa. *Onderstepoort J Vet Res* 68:119–130.

Ezenwa VO, Jolles AE, O'Brien PO. 2009. A reliable body condition scoring technique for estimating condition in African buffalo. *Afr J Ecol* 47:476–481.

Jha VC, Morita Y, Dhakal M, Besnet B, Sato T, Nagai A, Kato M, Kozawa K, Yamamoto S, Kimura H. 2007. Isolation of *Mycobacterium* spp. from milking buffaloes and cattle in Nepal. *J Vet Med Sci* 69:819–825.

Kao RR, Gravenor MB, Charleston B, Hope JC, Martin M, Howard CJ. 2007. *Mycobacterium bovis* shedding patterns from experimentally infected calves and the effect of concurrent infection with bovine viral diarrhoea virus. *J R Soc Interface* 4:545–551.

Laisse CJM, Gavier-Widen D, Ramis G, Bila CG, Machado A, Quereda JJ, Agren EO, Van Helden PD. 2011. Characterization of tuberculous lesions in naturally infected African buffalo (*Syncerus caffer*). *J Vet Diagn Invest* 25:1022–1027.

Michel AL, Bengis RG, Keet DF, Hofmeyr M, De Klerk LM, Cross PC, Jolles AE, Cooper D, Whyte U, Buss P, et al. 2006. Wildlife tuberculosis in South African conservation areas: Implications and challenges. *Vet Microbiol* 112:91–100.

Neill DF, Pollock JM, Bryson DB, Hanna DJ. 1994. Pathogenesis of *Mycobacterium bovis* infection in cattle. *Vet Microbiol* 40:41–52.

World Organization for Animal Health. 2013. Bovine tuberculosis. In: *Manual of diagnostic tests and vaccines for terrestrial animals 2013*. Chapter 2.4.7, http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/. Accessed September 2013.

Pontiroli A, Travis ER, Sweeney FP, Porter D, Gaze WH, Mason S, Hibberd V, Holden J, Courtenay O, Wellington EM. 2011. Pathogen quantitation in complex matrices: A multi-operator comparison of DNA extraction methods with a novel assessment of PCR inhibition. *PLoS One* 6:e17916.

Srivastava K, Chauhan DS, Gupta P, Singh HB, Sharma VD, Yadav VS, Sreekumaran S, Thakral SS, Dharamdheeran JS, Nigam P, et al. 2008. Isolation of *Mycobacterium bovis* and *M. tuberculosis* from cattle of some farms in north India—Possible relevance in human health. *Indian J Med Res* 128:26–31.

Sweeney FP, Courtenay O, Ul-Hassan A, Hibberd V, Reilly LA, Wellington EM. 2006. Immunomagnetic recovery of *Mycobacterium bovis* from naturally infected environmental samples. *Lett Appl Microbiol* 43:364–369.

Sweeney FP, Courtenay O, Hibberd V, Hewinson RG, Reilly LA, Gaze WH, Wellington EM. 2007. Environmental monitoring of *Mycobacterium bovis* in badger feces and badger sett soil by real-time PCR, as confirmed by immunofluorescence, immunocapture, and cultivation. *Appl Environ Microb* 73:7471–7473.

Travis ER, Gaze WH, Pontiroli A, Sweeney FP, Porter D, Mason S, Keeling MJ, Jones RM, Sawyer J, Aranaz A, et al. 2011. An interlaboratory validation of a real time PCR assay to measure host excretion of bacterial pathogens, particularly of *Mycobacterium bovis*. *PLoS One* 6:e27369.

Submitted for publication 10 July 2013.
Accepted 30 July 2013.