Clinical and Vaccine Immunology

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Diagnostic Value of Animal-Side Antibody Assays for Rapid Detection of *Mycobacterium bovis* or *Mycobacterium microti* Infection in South American Camelids[▽]

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Tuberculosis (TB) in South American camelids (SAC) is caused by *Mycobacterium bovis* or *Mycobacterium microti*. Two serological methods, rapid testing (RT) and the dual-path platform (DPP) assay, were evaluated using naturally infected SAC. The study population included 156 alpacas and 175 llamas in Great Britain, Switzerland, and the United States. TB due to *M. bovis* (*n* = 44) or *M. microti* (*n* = 8) in 35 alpacas and 17 llamas was diagnosed by gross pathology examination and culture. Control animals were from herds with no TB history. The RT and the DPP assay showed sensitivities of 71% and 74%, respectively, for alpacas, while the sensitivity for llamas was 77% for both assays. The specificity of the DPP assay (98%) was higher than that of RT (94%) for llamas; the specificities of the two assays were identical (98%) for alpacas. When the two antibody tests were combined, the parallel-testing interpretation (applied when either assay produced a positive result) enhanced the sensitivities of antibody detection to 89% for alpacas and 88% for llamas but at the cost of lower specificities (97% and 93%, respectively), whereas the serial-testing interpretation (applied when both assays produced a positive result) maximized the specificity to 100% for both SAC species, although the sensitivities were 57% for alpacas and 65% for llamas. Over 95% of the animals with evidence of TB failed to produce skin test reactions, thus confirming concerns about the validity of this method for testing SAC. The findings suggest that serological assays may offer a more accurate and practical alternative for antemortem detection of camelid TB.

Tuberculosis (TB) remains one of the most challenging and widespread zoonotic diseases in many countries (5, 6, 34). *Mycobacterium tuberculosis* and *Mycobacterium bovis* are the primary etiologic agents for human and bovine TB, respectively (8, 30, 39). *M. bovis* in particular is also known to cause TB in a broad range of domestic and wild mammal species (24, 35). Infections with *M. bovis* have been shown to affect both New and Old World species of camelids (14, 36, 44, 45). Tuberculosis in South American camelids (SAC), particularly in alpacas and llamas, has recently gained particular attention in Europe and in the United States, where these animals are being increasingly traded and kept in growing numbers as an alternative livestock industry (1). In recent years, *M. bovis* infections have been documented in alpaca and llama herds in parts of Europe, such as Great Britain (GB) (7, 37, 38), Ireland (32), and Spain (11), where this infection is endemic in cattle and wildlife. Also, SAC are known to be susceptible to *M. microti*, another member of the *M. tuberculosis* complex that can cause generalized disease with a fatal outcome in SAC (26, 29, 46). These infections are usually difficult to recognize in live animals because of the lack of reliable antemortem diagnostic tests and the nonspecific nature of the clinical signs of TB (4, 12). The intradermal tuberculin test is used in many countries,

but that method is cumbersome and has poor accuracy in SAC testing (10, 33, 36). Serological assays may constitute a promising alternative approach (7, 22), but additional studies using sufficient numbers of well-characterized individuals from various SAC populations of known TB status are needed to validate the diagnostic performance of such diagnostic methods.

Previous work demonstrated the potential for the multiantigen print immunoassay (MAPIA) and lateral-flow-based rapid testing (RT) to detect serum antibodies in multiple host species with TB (13, 21, 23, 40, 41). Recently, a new-generation animal-side TB assay using innovative dual-path platform (DPP) technology was described (14). The goal of the present study was to evaluate two serological methods, RT and the DPP assay, in SAC naturally infected with *M. bovis* or *M. microti*.

MATERIALS AND METHODS

Animals. The study was performed using 156 alpacas (*Vicugna pacos*) and 175 llamas (*Lama glama*) from several herds in GB, Switzerland, and the United States (Table 1). Each SAC species was represented by three groups: (i) a confirmed-TB group (animals naturally infected with *M. bovis* or *M. microti*); (ii) a group presumed to be free of TB (SAC from herds with no history of TB); and (iii) a TB-exposed group (culture-negative animals with no gross lesions from the infected herds). Group 1 consisted of 34 alpacas and 10 llamas with positive *M. bovis* culture results and/or typical gross TB lesions at postmortem examination that were slaughtered in GB during 2006 to 2010. Group 1 also included 1 alpaca and 7 llamas diagnosed with *M. microti* infection during 2004 to 2007 in Switzerland. Generalized disease due to *M. microti* was diagnosed in these animals by positive culture (1 alpaca and 4 llamas) or was strongly suspected based on the presence of gross lesions consistent with mycobacteriosis and/or suggestive symp-

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TABLE 1. Study population

Group	Category	Country	Etiologic agent	No. of alpacas	No. of llamas
1	Confirmed TB	GB	*M. bovis*	34	10
		Switzerland	*M. microti*[a]	1	7
2	Presumed TB-free	Switzerland	ND[b]	14	22
		United States	ND	82	100
3	Culture-negative (from exposed herds)	GB	None	9	36
		Switzerland	None	16	0

[a] Generalized disease due to *M. microti* was diagnosed by positive culture (1 alpaca and 4 llamas) or was strongly suspected (3 llamas from the known *M. microti*-infected herd) based on gross lesions consistent with mycobacteriosis and/or suggestive symptoms.
[b] ND, not done.

toms (3 llamas from the infected herd), as previously described (22). Except for 2 *M. bovis*-infected llamas, all SAC in group 1 were skin test nonreactors. Group 2 (negative controls) included 96 alpacas and 122 llamas from herds known to be TB free in Switzerland (a country officially free of bovine TB) and the United States (a country largely free of bovine TB). Group 3 (exposed animals) included 25 asymptomatic culture-negative alpacas from *M. microti*-infected and *M. bovis*-infected herds in Switzerland and in GB, respectively, and 36 asymptomatic culture-negative llamas that were removed as part of depopulation procedures carried out with *M. bovis*-infected herds in GB. All serum samples were obtained from live animals and stored frozen at −70°C until use in antibody assays.

Clinical and postmortem examination. In Switzerland, all SAC were examined clinically according to a standardized procedure (9). Animals suspected of harboring an *M. microti* infection were humanely euthanized, and necropsy was performed as described previously (26). Lung, liver, kidney, and lymph node specimens were submitted for culture (Mycobacterial Growth Indicator Tube; Becton Dickinson, Sparks, MD) and for molecular testing by the amplified *Mycobacterium tuberculosis* Direct Test (GenProbe, San Diego, CA). *M. microti* was identified in culture-positive cases by spoligotyping (26). In GB, animals from SAC herds with established *M. bovis* infection were tested antemortem (by clinical evaluation, the single comparative intradermal tuberculin test, and serology) and examined postmortem (by inspection for gross lesions, histopathology, and culture) as described earlier (7, 37, 38). All testing procedures and the protocols for euthanasia and postmortem examination complied with legal requirements and were approved by the national committees for animal care and protection.

Rapid test (RT). The immunochromatographic VetTB Stat-Pak kit (Chembio Diagnostic Systems, Inc., Medford, NY) uses selected *M. tuberculosis* antigens (ESAT-6, CFP10, and MPB83) and a blue latex signal detection system for rapid detection of antibodies, as previously described (22, 23). To perform the test, 30 µl of serum and 3 drops of sample buffer (included in the kit) were added to the device and results were read visually after 20 min. Any visible band other than the control line in the test area was considered to represent an antibody-positive result, whereas the absence of a test band was considered to represent a negative result.

Dual-path platform (DPP) assay. The DPP format uses two nitrocellulose strips that are connected in a "T"-shaped configuration inside the device to allow independent delivery of the test sample and the antibody-detecting reagent (14). A mixture of two *M. bovis* recombinant proteins, MPB70 and MPB83, printed on a membrane at a concentration of 0.25 mg/ml and a recombinant hybrid of staphylococcal protein A and streptococcal protein G (protein A/G) conjugated to colloidal gold microparticles were used to detect camelid antibody responses in the DPP assay. The test required 5 µl of serum and 2 drops of buffer to be added to the sample well, followed by placement of 4 drops of buffer in the

conjugate well. Results were read at 15 min using the DPP optical reader device, which measures reflectance in relative light units (RLU). Cutoff values were established by receiver operating characteristic (ROC) analysis as described below.

Data analysis. The diagnostic performance of the serologic assays was evaluated using SAC sera from groups 1 (confirmed TB) and 2 (TB-free) for assessing test sensitivity and specificity, respectively. Cutoff values for the DPP assay were determined using ROC analysis of the reader-generated data and Prism 5 software (GraphPad, San Diego, CA). DPP cutoff values for each host species were set at 98% specificity. Area under the curve (AUC) and *P* values were calculated. Results from group 3 (exposed animals) were not used in the evaluation of the test performance characteristics, because the true infection status of those animals could not be reliably determined. Test sensitivity (proportion of positive results found among infected animals), specificity (proportion of negative results found among noninfected animals), and accuracy (proportion of correct results among all tested animals) were separately calculated using available software (20) for each assay and host species; data are presented with 95% confidence intervals (CI).

RESULTS

Antibody detection in alpacas. The RT was read visually, whereas DPP seroreactive results for alpacas were determined based on a cutoff of 8.25 RLU (AUC, 0.9087; 95% CI, 0.8353 to 0.9821; $P < 0.0001$). In group 1, of the 35 culture-positive alpacas (34 infected with *M. bovis* and 1 infected with *M. microti*), 25 (71%) were reactive in RT, while 26 (74%) reacted in the DPP assay (Table 2). One of the *M. microti*-infected alpacas included in the study was seropositive by both tests. Figure 1A shows the antibody responses obtained with the DPP assay in the three different groups of alpacas. The RT and DPP kits showed similar and relatively high individual specificities of 98% each in evaluations of 96 negative-control sera from animals in group 2. When the two methods were combined for parallel test interpretations (either assay may be reactive for a positive result), 89% of the animals with confirmed TB were seropositive, but the specificity marginally decreased to 97% (Table 2). In contrast, when RT and the

TABLE 2. Diagnostic performance of antibody tests for TB in alpacas

| Test	No. of true positive test results/no. of infected animals tested (% sensitivity [95% CI])			No. of true negative test results/no. of uninfected animals tested (% specificity [95% CI])	No. of true TB detection test results/total no. of animals tested (% accuracy [95% CI])
	M. bovis	*M. microti*	TB total		
RT	24/34	1/1	25/35 (71.4 [53.7–85.4])	93/95 (97.8 [92.6–99.7])	117/130 (90.0 [83.5–94.6])
DPP	25/34	1/1	26/35 (74.3 [56.7–87.5])	94/96 (97.6 [92.7–99.8])	120/131 (91.6 [85.5–95.7])
RT and/or DPP	30/34	1/1	31/35 (88.6 [73.3–96.8])	92/95 (96.8 [91.1–99.3])	123/130 (94.6 [89.2–97.8])
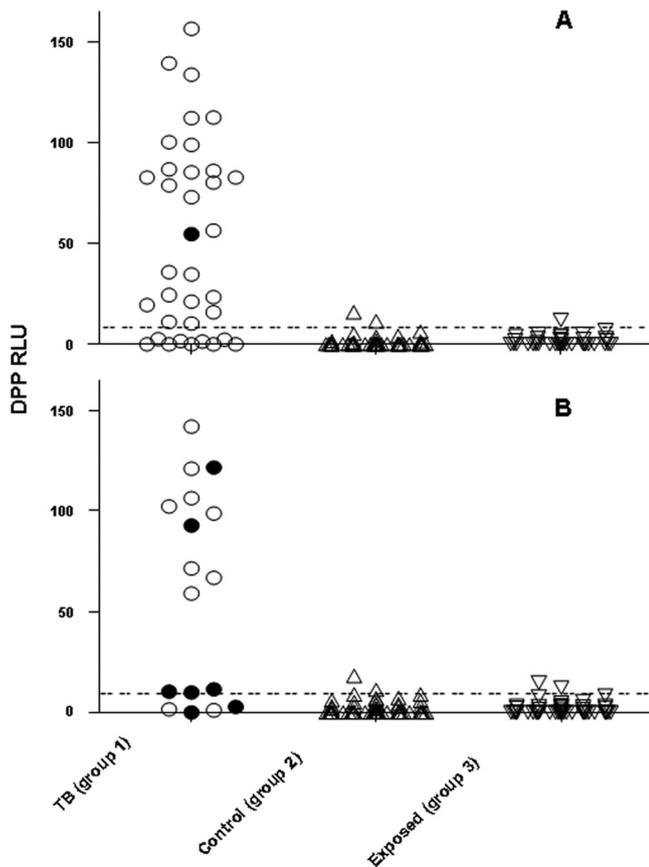
FIG. 1. Quantitative DPP detection of serum IgG antibodies in SAC diagnosed with TB. Reflectance values quantified in relative light units (RLU) generated by the DPP reader are shown for sera from 156 alpacas (A) and 175 llamas (B). Group 1, confirmed TB (open circles, *M. bovis*; filled circles, *M. microti*); group 2, negative-control animals (triangles); group 3, culture-negative animals from TB-exposed herds (inverted triangles). Cutoff values of 8.25 RLU for alpacas and of 9.55 RLU for llamas (dotted lines) were established by receiver operating characteristic (ROC) analysis at a test specificity of 98% using sera from groups 1 and 2 as described in Materials and Methods.

DPP assay were used in series (both assays must be reactive for a positive result), the specificity was 100%, although only 57% of the alpacas with confirmed TB produced positive results (data not shown). Overall, the combined application of these antibody assays with the parallel interpretations yielded the highest (95%) diagnostic accuracy in correctly identifying infected alpacas compared to that seen with either test used alone (90% to 92%) (Table 2).

Antibody detection in llamas. Fig. 1B shows the antibody responses obtained for all groups of llamas by the use of a DPP

TABLE 4. Rates of seroreactive results for culture-negative SAC from TB-exposed herds

Test	% antibody-positive results (no. of positive results/total no. of animals tested) for indicated species	
	Alpacas	Llamas
RT	8.0 (2/25)	8.3 (3/36)
DPP	4.0 (1/25)	5.5 (2/36)
RT and/or DPP	12.0 (3/25)	14.3 (5/36)

cutoff of 9.55 RLU (AUC, 0.9216; 95% CI, 0.8497 to 1.0; $P < 0.0001$). Group 1 consisted of 10 *M. bovis*-positive llamas (GB) and 7 *M. microti*-infected llamas (Switzerland). Of those 17 animals, 13 (77%) were seroreactive by each of the two immunoassays. The rate of antibody detection in the *M. microti* subgroup appeared to be lower than that seen with *M. bovis*-infected llamas, especially for RT (Table 3). The diagnostic specificity for 122 negative-control sera of llamas from presumably TB-free herds was similar to that seen with the alpacas in the DPP assay (98%), but the specificity was noticeably lower for RT (94%). As with the alpacas, combining the two tests in parallel for the llamas resulted in higher sensitivity (88%) and lower specificity (93%), whereas using them in series provided 100% specificity and 65% sensitivity (data not shown). In contrast to the results seen with the alpacas, the combined application of the antibody tests for the llamas did not yield improved diagnostic accuracy (Table 3), which appeared to be highest with the DPP assay alone (96%) for this species.

Serological reactivity in exposed SAC. Group 3 included 61 alpacas and llamas with likely TB exposure for which the true infection status could not be reliably determined. Although those animals were clinically healthy, had no gross lesions, and gave culture-negative results at the postmortem examination, they had all been in herds where TB cases caused by *M. bovis* (GB) or *M. microti* (Switzerland) had been diagnosed. Since the presence of infection among the exposed SAC could not be ruled out completely, it was reasonable to hypothesize that some of them could produce antibody responses. Test results obtained from group 3 revealed that moderately elevated rates of seroreactivity were found with both assays for each host species (Table 4, Fig. 1). When RT and DPP data were analyzed in combination as parallel tests, 12% alpacas and 14% llamas were seropositive.

DISCUSSION

Camelid species are known to be susceptible to pathogenic mycobacteria. TB due to *M. bovis* has been most commonly

TABLE 3. Diagnostic performance of antibody tests for TB in llamas

Test	No. of true positive test results/no. of infected animals tested (% sensitivity [95% CI])			No. of true negative test results/no. of uninfected animals tested (% specificity [95% CI])	No. of true TB detection test results/total no. of animals tested (% accuracy [95% CI])
	M. bovis	*M. microti*	TB total		
RT	9/10	4/7	13/17 (76.5 [50.1–93.2])	115/122 (94.3 [88.5–97.7])	128/139 (92.1 [86.3–96.0])
DPP	8/10	5/7	13/17 (76.5 [50.1–93.2])	120/122 (98.0 [93.0–99.8])	133/139 (95.7 [60.8–98.4])
RT and/or DPP	9/10	6/7	15/17 (88.2 [63.6–98.5])	113/122 (92.6 [86.5–96.6])	128/139 (92.1 [8233–96.0])

found (3, 17, 36, 44), but infections with *M. caprae*, *M. kansasii*, or *M. paratuberculosis* have also been documented (2, 16, 28). In the last decade, cases of generalized TB in alpacas and llamas caused by *M. microti*, a member of the *M. tuberculosis* complex, have been increasingly reported (26, 46). Small rodents are believed to be the natural hosts for this pathogen (19, 43), but recent findings have revealed that *M. microti* may cause clinical disease in other animal species (18, 31) and in humans (25, 27). The zoonotic potential of camelid TB must not be overlooked, because SAC are often kept as family pets (i.e., in close contact with children), as companion animals in nursing homes (in contact with elderly and weak persons) or, in the case of llamas, as pack animals for trekking.

Antemortem diagnosis of TB in nonbovine animals, including camelids, is challenging due to serious limitations of the existing methods (4, 12). Clinical signs (wasting, inappetence, respiratory distress, etc.) are not specific to the disease and may not be present in all infected animals (37, 38, 46). Until recently, the intradermal tuberculin test was the only test available for camelid species and it is still considered the official TB screening and pre-export certification test for SAC in many countries (37, 44). However, the skin tests are difficult to standardize and can reportedly produce unacceptably high rates of false-positive and/or false-negative results (10, 33, 36, 38, 45). In two Swiss herds of SAC where 10 animals were diagnosed with generalized *M. microti* infection, the skin test detected no reactors (22). Of 14 animals infected with *M. bovis* that were members of a large British llama herd, only 2 yielded positive results in the comparative tuberculin test, even when a severe interpretation mode was used (7). Most recently, in another *M. bovis* outbreak in llamas, 4 skin test-negative results were documented for 5 culture-confirmed TB cases (38). Finding so few (*M. bovis* infection) or no (*M. microti* infection) tuberculin reactors in the reported studies suggests a lack of sensitivity of this test for SAC.

The present report describes an alternative and potentially useful approach for laboratory-free diagnosis of TB in live SAC on the basis of serum antibody detection by animal-side rapid *in vitro* techniques. The RT and DPP immunoassays evaluated in this study were able to correctly identify *M. bovis* or *M. microti* infections with high accuracy (90% to 92% for alpacas and 92% to 96% for llamas). Importantly, the use of these tests in combination could offer either significantly improved diagnostic sensitivity (88% to 89%, using parallel testing interpretations) or maximized specificity (100%, using serial testing interpretations). This observation suggests that the differential algorithms may be considered for determination of the most efficient antemortem testing strategies in various settings, such as investigation of known infected herds with high disease prevalence (for maximized sensitivity) versus routine surveillances in presumably TB-free populations (for highest specificity). It should be noted that all alpacas ($n = 35$) and most llamas (15/17) with evidence of TB in the present study presented as skin test-negative animals; therefore, the infections would have probably escaped traditional antemortem diagnosis.

The DPP assay detected antibody responses in SAC animals infected with *M. bovis* and with *M. microti* in identical proportions (75% [33/44 and 6/8, respectively]). Earlier work demonstrated that most of alpacas and llamas with TB produced IgG

antibodies against the MPB83 protein, irrespective of whether the etiologic agent was *M. bovis* or *M. microti* (7, 22). This antigen is known to be a major serological target for the immune responses to infections caused by organisms of the *M. tuberculosis* complex in various host species (13, 15, 23, 40, 41). Both RT and the DPP assay include the MPB83 protein in their test antigen composition, which could partly explain the reasonable agreement of the tests with respect to identification of infected animals.

The findings presented here indicate that the serologic assays for SAC TB infections may have diagnostic performance superior to that of the tuberculin skin test. Similar observations were recently made in a study of an outbreak of *M. bovis* infection in a mixed herd of elk and fallow deer in Nebraska (42) in which the tuberculin skin test failed to detect most of the infected animals with gross lesions and positive culture found at postmortem examination, whereas the antibody assays showed encouraging diagnostic potential.

The study had certain limitations with respect to assessing the immunoassay performance. Some llamas in *M. bovis*-infected herds were skin tested a few weeks before blood specimens were collected for serological assays. Therefore, the antibody responses measured in those animals might have been boosted by the recent tuberculin injections, as previously suggested (7). Further, most of the animals from populations presumed to be free of TB that were included in group 2 could not be euthanized to confirm true negative status by the gold standard methods at postmortem examination.

In conclusion, this study demonstrated the diagnostic potential of the two animal-side antibody detection tests, RT and the DPP assay, for rapid identification of alpacas and llamas infected with *M. bovis* or *M. microti*. The serologic techniques appear to be more sensitive than the intradermal tuberculin tests for SAC. The diagnostic accuracy of antemortem TB detection in llamas and alpacas can be maximized by combining available immunoassays.

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