

REVIEW

Diagnosis of Tuberculosis in Camelids: Old Problems, Current Solutions and Future Challenges

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Summary

In spite of great efforts for its control and eradication, tuberculosis remains one of the most important zoonosis worldwide. Its causative agents, the members of the *Mycobacterium tuberculosis* complex, have a wide host range that complicates the epidemiology of this disease. Among susceptible species to these pathogens, camelids from the New World (llama, alpaca and vicuña) and Old World (Bactrian camel and dromedary) are acquiring an increasing importance in several European countries because of its growing number and could act as reservoirs of the disease for livestock and humans in their natural habitat. In addition, tuberculosis caused by a number of *M. tuberculosis* complex members is a life-threatening disease in these animal species. Although tuberculosis has been known to affect camelids for a long time, ante-mortem diagnosis is still challenging because of the lack of standardized diagnostic techniques and the limited sensitivity and specificity of the most widely applied tests. However, in recent years, several techniques that can at least partially overcome these limitations have been developed. This paper reviews the results and advances achieved in tuberculosis diagnosis in camelids in the last decade as well as the progresses on ongoing investigations, with special attention to the remaining challenges that still have to be faced to assure the availability of reliable tools for the detection of tuberculosis-infected animals and herds.

Introduction

Animal tuberculosis is a zoonotic disease caused by members of the *Mycobacterium tuberculosis* complex; among these organisms, the species most frequently involved in animal infections are *M. bovis* and, to a lesser extent, *M. caprae*. Both of them can infect a wide number of animal species, especially *M. bovis*, which have been shown to have an extraordinary wide host range (Cousins and Florisson, 2005). This host range includes both domestic and wild animals and can complicate the fight against animal tuberculosis in those species subjected to eradication/control programmes (mainly cattle), as several animal species can act as reservoirs of infection and therefore as potential sources of tuberculosis for livestock and humans.

Tuberculosis in New World camelids – NWC – (or South American camelids, SAC) have gained greater importance in Europe in the last decades, because of the growing number of animals (mainly llamas – *Lama glama* – and alpacas – *Vicugna pacos*) being imported into several European countries to serve as pets, pack animals or for productive purposes (Barlow et al., 1999; D'Alterio et al., 2006). In certain countries, reports of tuberculosis on SAC have increased: for instance, 42 outbreaks of tuberculosis were diagnosed in alpaca or llama herds between 2003 and 2009 in Great Britain, with a growing incidence in the last years (Twomey et al., 2010a). Moreover, Old World camelids (Bactrian camel – *Camelus bactrianus* – and dromedary – *Camelus dromedaries*) can be also infected by *M. tuberculosis* complex members (Kinne et al., 2006; Pate et al., 2006; Moser et al., 2008).

Tuberculosis has been reported to occur more often when animals are managed under intensive conditions and/or close to cattle herds (Barlow et al., 1999; Kinne et al., 2006), with little incidence of infection in NWC in their natural habitat. This could indicate a lower susceptibility to mycobacterial infections under natural conditions (Oevermann et al., 2004). In contrast, a high susceptibility of llamas to *M. bovis* infection has been also suggested (Stevens et al., 1998), thus indicating that the low number of reports of tuberculosis in certain countries may be because of non-exposure or under-reporting (Connolly et al., 2008). Within-herd transmission between alpacas (Twomey et al., 2009) and llamas (Oevermann et al., 2004) has been also suspected in European herds.

Infection by *M. bovis* in llamas and alpacas seems to occur mainly by the respiratory route (Twomey et al., 2009), as in most domestic (McNair et al., 2007) and wild ruminants (Martin-Hernando et al., 2010), and thus lesions are mainly located in lung, pleura and associated lymph nodes, usually in the form of small multifocal white-yellowish caseous nodules, although larger abscesses have been also described (Barlow et al., 1999; Twomey et al., 2007, 2010a). However, generalized disease is not uncommon, and additional locations of macroscopical pathology include the pericardial sac, liver and bronchial and mediastinal lymph nodes (Barlow et al., 1999; Twomey et al., 2007). Presence of tuberculous lesions in the gastrointestinal tract and mesenteric lymph nodes could be owing to oral infection, secondary spread or swallowing of infected sputum (Twomey et al., 2010a). Discharging skin lesions associated with tubercular lymphadenitis in the draining lymph nodes have been also reported (Twomey et al., 2010a). Likewise, tuberculosis-like lesions have been also described in the lung, liver (Ryan et al., 2008), kidney (Twomey et al., 2009) and hepatic, bronchial, mediastinal and mesenteric lymph nodes in *M. bovis*-infected alpacas (Ryan et al., 2008; Garcia-Bocanegra et al., 2010). Because of the uncertainty regarding time of infection in lesioned animals, the time necessary to develop well-formed tuberculosis lesions cannot be determined. However, in an experimental infection study using heavy *M. bovis* challenge doses (2–5 µg wet weight) injected through the skin into the lower trachea, all challenged llamas ($n = 6$) developed multifocal granulomas in lung and other thoracic and abdominal locations between 68 and 158 days post-infection (Stevens et al., 1998). *M. microti*, another member of the *M. tuberculosis* complex that has been traditionally considered as an unimportant animal pathogen (except in its primary hosts, rodents) (Smith et al., 2009), is able to cause similar lesions as those observed in *M. bovis* infections in SAC (Oevermann

et al., 2004; Smith et al., 2009; Zanolari et al., 2009), suggesting higher susceptibility of these animals to the bacteria (Xavier Emmanuel et al., 2007).

Fewer reports describing tuberculosis lesions in camels and dromedaries exist. Areas of mineralization, solid abscesses and granulomatous lesions have been observed in lungs, respiratory lymph nodes and other internal organs because of *M. bovis* infection in both animal species (Paling et al., 1988; Kinne et al., 2006; Pate et al., 2006; Wernery et al., 2007). Moreover, mineralization in lung and caseous foci in lung lymph nodes have been also described in a camel infected with *M. pinnipedii*, another member of the *M. tuberculosis* complex that is closely related to *M. microti* (Huard et al., 2006) and usually causing tuberculosis in pinnipeds (Moser et al., 2008).

The tuberculosis lesion patterns usually observed in camelids, often involving generalized dissemination of the pathogen, highlight the potential role of these animal species as potential sources of tuberculosis for livestock but also for humans (Twomey et al., 2010b). It also emphasizes the detrimental effect this disease has on the New and Old World camelids themselves. Therefore, the diagnosis of infected animals and their early removal is critical for the management of infected herds. However, currently, there are several limitations to the *in vivo* diagnosis of tuberculosis in camelids that remain unsolved. This review describes the advances achieved on the diagnosis of tuberculosis in camelids over the last 10 years, focusing on the application of both established diagnostic tests and novel techniques recently developed. Development of reliable diagnostic tests for tuberculosis in camelids is critical to control the spread of the disease in these animal species, not only because of its clinical importance but also because of the potential role of camelids as reservoirs of the disease for cattle, target of eradication programmes implemented in several developed countries that have not achieved the officially tuberculosis-free status yet (Starnes and Wood, 2007).

Diagnosis of Tuberculosis in Camelids

Detection of tuberculosis in camelid-infected herds often occurs through the detection of clinical symptoms in one or more animals, followed with the observation of compatible lesions during the necropsy of some of these animals (that either die because of the disease or are euthanized because of poor body condition). After the initial detection of infected animals in a herd, *in vivo* diagnostic tests are used in the remaining animals, usually based on the detection of the specific immune response of infected animals.

Clinical Diagnosis

Tuberculosis usually causes severe disease in affected camelids, but symptoms are unspecific. The most often described clinical presentations in *M. bovis*- or *M. microti*-infected llamas and alpacas involve respiratory distress (that can be acute or occur in a chronic form) with occasional coughing (Barlow et al., 1999; Twomey et al., 2007, 2009; Zanolari et al., 2009; Garcia-Bocanegra et al., 2010). However, more unspecific signs such as lethargy, anorexia and/or appetite loss leading to cachexia with none or mild respiratory problems can be patent (Barlow et al., 1999; Oevermann et al., 2004; Twomey et al., 2009; Zanolari et al., 2009). Usually, animals do not respond to antibiotic treatments and, if not euthanized, die of clinical tuberculosis. Sudden death of apparently healthy animals (whose necropsy reveals extensive lesions) has been also described (Barlow et al., 1999; Zanolari et al., 2009; Twomey et al., 2010a). The presentation of clinical symptoms can be of variable duration, and in chronic cases, symptoms can be observed for months (Oevermann et al., 2004; Ryan et al., 2008; Garcia-Bocanegra et al., 2010). However, because of the lack of knowledge of the time of infection in most cases, the latent incubation period of the disease before symptoms are evident cannot be estimated. In the only *M. bovis* experimental infection performed in llamas, respiratory symptoms and loss of body condition were observed as early as 60–70 days after a challenge with high infectious doses (Stevens et al., 1998).

Tuberculosis-infected Old World camelids also show non-specific symptoms similar to those observed in SAC, with gradual weight loss and inappetence (Kinne et al., 2006) or respiratory symptoms (Moser et al., 2008).

Diagnosis Based on the Detection of the Immune Response

Indirect diagnosis of tuberculosis in these species is challenging, as common diagnostic tests have proved to lack both sensitivity and specificity (Wernery and Kaaden, 1995). Evaluation of the reliability of available techniques is seriously impaired by the lack of systematic studies specifically aimed at determining their usefulness in animals of known infectious status. A number of reports describing the application of tests based on the detection of the cellular (tuberculin test) and humoral (serological tests) immune responses on infected hosts have been published in the last decade. However, the lack of knowledge of the true status of the majority of tested animals allows very limited interpretation of the available data. Tables 1 and 2 show the performance of cellular and humoral immune response-based tests when used on animals with a known infection status. Results have been included regardless of

sample size, although 95% confidence intervals for sensitivity and specificity estimates have been included in an attempt to reflect the uncertainty associated with small numbers of studied animals. For specificity estimation purposes, only results from animals with no known exposure to *M. tuberculosis* complex members have been included (thus, all animals from herds where infected individuals were detected have been excluded from the analysis).

Tests targeting the cellular immune response

Ante-mortem diagnostic techniques aimed at the detection of cellular immune response of infected animals have been the basis of tuberculosis control and eradication programmes in domestic ruminants and thus have been the most widely investigated tests in camelids. Data on the use of both single intradermal tuberculin (SIT) and single comparative intradermal tuberculin (SCIT) tests are shown in Table 1; in an attempt to minimize the false-positive reactions because of sensitization to environmental mycobacteria, the SCIT test has been used more frequently. To enhance the sensitivity, several injection sites have been used (mainly neck and axillary sites, but also under the tail), and because of the thick and resilient nature of the skin of the neck, the axillary site is usually preferred (Simmons, 1989; Lyashchenko et al., 2007; Ryan et al., 2008). Different reading times have been also evaluated to measure the skin fold increase, with maximum values obtained 72–96 h post-inoculation of the antigens in NWC (Stevens et al., 1998), although there is limited information on this subject. SCIT test performed in infected Old World camelids (Wernery et al., 2007) showed that larger reactions were obtained 5 days post-inoculation, although the test was only performed on two animals.

Recently, an interferon-gamma (IFN- γ) assay recognizing SAC IFN- γ in an ELISA format based on a set of cross-reactive monoclonal antibodies has been developed at the Veterinary Laboratories Agency (M. Vordermeier and S. G. Rhodes, unpublished data). This assay, when used in conjunction with the highly sensitive Meso Scale Discovery (MSD) platform using an electrochemiluminescence readout, detected 26/29 (93%) of alpacas with confirmed bovine tuberculosis that were available for analysis (M. Vordermeier and S. G. Rhodes, unpublished data). However, this test's specificity is as yet undefined. A validation study to determine this assay's sensitivity and specificity is now underway in GB. Another approach to the diagnosis of tuberculosis by IFN- γ detection was proposed by Harrington and collaborators using a real-time reverse transcription PCR for quantification of IFN- γ mRNA after stimulation with specific antigens (bovine purified protein

Table 1. Sensitivity and specificity of skin tests performed on camelids with known infection status

Test	Animal species tested (n)	True status (bacterial species)	Sensitivity (95% CI)	Specificity (95% CI)	Comments	Reference	
Single intradermal tuberculin (SIT) test	Alpaca (16)	Experimentally infected (<i>M. bovis</i>)	100 (79.4–100)	ND	No data on site of inoculation and interpretation criteria	R. de la Rua-Domenech, personal communication (cited in Cousins and Florisson, 2005)	
	Llama (5)	Experimentally infected (<i>M. bovis</i>)	80 (28.4–99.5)	ND	Axillary site, day 80 post-challenge, readings at 96 h post-inoculation	Stevens et al., 1998	
	Llama (3)	Experimentally infected (<i>M. bovis</i>)	100 (29.2–100)	ND	Axillary site, day 143 post-challenge, readings at 96 h post-inoculation	Stevens et al., 1998	
	Dromedary (2)	Naturally infected (<i>M. bovis</i>)	100 (15.8–100)	ND	Axillary site, reading 5 days post-inoculation, standard interpretation	Wernery et al., 2007	
	Alpaca (12)	Non-infected	ND	100 (73.5–100)	No data on site of inoculation and interpretation criteria	R. de la Rua-Domenech, personal communication (cited in Cousins and Florisson, 2005)	
	Llama (2)	Non-infected	ND	100 (15.8–100)	Axillary site, performed twice in both animals	Stevens et al., 1998	
	Single comparative intradermal tuberculin (SCIT) test	Alpaca (2)	Naturally infected (<i>M. bovis</i>)	0 (0–84.2)	ND	Test performed in the month before the onset of clinical signs.	Garcia-Bocanegra et al., 2010
		Alpaca (21)	Experimentally infected (<i>M. bovis</i>)	76.2 (52.8–91.8)	ND	No data on site of inoculation and interpretation criteria	R. de la Rua-Domenech, personal communication (cited in Cousins and Florisson, 2005)
		Llama (14)	Naturally infected (<i>M. bovis</i>)	14.3 (1.8–42.8)	ND	Axillary site, no data on interpretation criteria (standard?)	Dean et al., 2009
		Llama (7)	Naturally infected (<i>M. microti</i>)	0 (0–41)	ND	Axillary site, standard interpretation	Lyashchenko et al., 2007
		Llama (24)	Experimentally infected (<i>M. bovis</i>)	87.5 (67.6–97.3)	ND	No data on site of inoculation and interpretation criteria	F. Stuart, personal communication (cited in Cousins and Florisson, 2005)
		Alpaca (5)	Naturally infected (<i>M. bovis</i>)	0 (0–52.18)	ND	Axillary and cervical sites assayed. No data on site of inoculation	Ryan et al., 2008
Single intradermal tuberculin (SIT) test	Dromedary (2)	Naturally infected (<i>M. bovis</i>)	100 (15.8–100)	ND	Axillary site, day 143 post-challenge, readings at 96 h post-inoculation	Wernery et al., 2007	
	Alpaca (12)	Non-infected	ND	100 (73.5–100)	No data on site of inoculation and interpretation criteria	R. de la Rua-Domenech, personal communication (cited in Cousins and Florisson, 2005)	
	Llama (12)	Non-infected	ND	100 (73.5–100)	No data on site of inoculation and interpretation criteria	F. Stuart, personal communication (cited in Cousins and Florisson, 2005)	

ND, Not determined.

Table 2. Sensitivity and specificity of serological tests performed in camelids with known infectious status

Test	Animal species tested (n)	True status (bacterial species)	Sensitivity (95% CI)	Specificity (95% CI)	Comments	Reference
MAPIA	Llama (14)	Naturally infected (<i>M. bovis</i>)	100 (76.8–100)	ND	MPB83 was the serodominant antigen	Dean et al., 2009
	Llama (7) and Alpaca (1)	Naturally infected (<i>M. microti</i>)	87.5 (47.3–99.7)	ND	MPB83 was the serodominant antigen	Lyashchenko et al., 2007
	Alpaca (13)	Non-infected	ND	100 (75.3–100)		Lyashchenko et al., 2007
	Llama (33)	Non-infected	ND	100 (89.4–100)		Lyashchenko et al., 2007
	Dromedary (3)	Naturally infected (<i>M. bovis</i>)	100 (29.2–100)	ND		Wernery et al., 2007
Rapid test	Llama (14)	Naturally infected (<i>M. bovis</i>)	64.3 (35.1–87.2)	ND		Dean et al., 2009
	Llama (6)	Naturally infected (<i>M. bovis</i>)	100 (54.1–100)	ND	Anamnestic test (three weeks after SCIT test)	Dean et al., 2009
	Llama (7) and Alpaca (1)	Naturally infected (<i>M. microti</i>)	62.5 (24.5–91.5)	ND		Lyashchenko et al., 2007
	Alpaca (13)	Non-infected	ND	92.3 (64–99.8)		Lyashchenko et al., 2007
	Llama (33)	Non-infected	ND	87.9 (71.8–96.6)	Animals from two herds; all false-positive reactors came from the same herd	Lyashchenko et al., 2007
	Dromedary (3)	Naturally infected (<i>M. bovis</i>)	100 (29.2–100)	ND		Wernery et al., 2007

ND, Not determined.

derivative, PPD) (Harrington et al., 2007); however, although this approach proved successful for other animal species, no amplification could be obtained when working with llama samples.

Tests targeting the humoral immune response

Although the humoral immune response is known to occur primarily in advanced stages of infection (Pollock and Neill, 2002; Welsh et al., 2005) (and therefore its detection would have less usefulness for early detection purposes), a number of in-house and commercial assays have been applied to the tuberculosis diagnosis in camelids (Table 2). As reported in some studies, serological tests have been able to detect infected animals before the onset of clinical disease (Lyashchenko et al., 2007). Among them, in-house ELISAs for specific detection of antibodies against crude extracts of mycobacteria or recombinant antigens have been used with variable success (Stevens et al., 1998). Other approaches, such as the multiantigen print immunoassay (MAPIA; Chembio, New York, NY, USA), capable of detecting antibodies against a cocktail of selected antigens applied to nitrocellulose membranes (Lyashchenko et al., 2000) and a lateral-flow-based rapid test (RT) detecting antibodies against a set of recombinant tuberculosis antigens and giving a result in

15–20 min (Lyashchenko et al., 2006; Waters et al., 2006), were useful alone or in combination with skin testing. However, the lack of light chains in camelid antibodies (Hamers-Casterman et al., 1993; Muyldermans, 2001) can cause a limited flexibility of the immune complex formed by sample antibody and antigen-coated latex particles; this could limit the sensitivity of lateral flow assays in certain cases (Lyashchenko et al., 2007). The use of the fluorescence polarization assay for the detection of specific anti-MPB70 antibodies in *M. bovis*-infected llamas was also evaluated, achieving a similar accuracy to that of the ELISA, although only three infected and six non-infected sera were tested (Lin et al., 1996).

Review of Studies Reporting *In Vivo* and *In Vitro* Ante-Mortem Diagnosis of Tuberculosis in Camelids

Studies performed in New World Camelids

Stevens et al., (1998) challenged six llamas with different doses of *M. bovis* to measure their immune responses using the SIT test (performed in the axillary site) and an ELISA aimed at the detection of specific antibodies against *M. bovis*, *M. avium* and *M. avium* subsp. *paratuberculosis* antigens at different times post-infection. Skin tests were performed 80 days after infection on five

animals (4/5 reactors with increase in skin thickness above 2 mm) and in only three animals 143 days post-infection (3/3 positive animals with reactions above 2 mm) because of premature death of some of the animals as a consequence of tuberculosis infection. Reactions were maximal 96 h post-bovine PPD inoculation. Two non-infected control animals showed no reaction. Results of serological analysis using *M. bovis* antigens were consistently positive in most animals from day 83 post-infection onwards, although one animal reacted as soon as 34 days after challenge. However, extrapolation of test performances to what could be expected under natural conditions is difficult because of the heavy infection induced in all challenged animals (with three of six infected animals dying before the completion of the experiment) and the antibiotic treatment given to all animals but one.

In another study, the SIT test detected all 16 experimentally infected alpacas 100 days after inoculation with *M. bovis* and correctly classified 12 non-infected animals. In an additional trial, the SCIT test detected 16 of 21 alpacas challenged with *M. bovis* and also showed a perfect specificity when performed on 12 non-infected alpacas (R. de la Rúa-Domenech, personal communication; cited in Cousins and Florisson, 2005). A similar experiment was performed on 24 llamas experimentally infected with *M. bovis* and 12 uninfected controls: the SCIT test detected 21 of the challenged animals, and all controls tested negative. An ELISA detecting antibodies against bovine and avian PPDs detected all 24 infected animals, but no results on specificity of the assay were provided (F. Stuart, personal communication, cited in Cousins and Florisson, 2005).

These relatively high sensitivity and specificity values of the tuberculin skin tests contrast with the performance of tests in naturally infected animals. As an example of lack of sensitivity of the SCIT test in an infected herd under field conditions, Twomey et al. (2007) reported the results obtained in a llama herd in Great Britain with culture-confirmed tuberculosis infection: after finding the first positive animal (euthanized because of poor body condition), all the herds ($n = 84$) were tested every 90 days using the SCIT test. After two rounds with all animals testing negative, a new clinically ill animal was detected, from which *M. bovis* was isolated. Three months later, a new SCIT test was performed, with three animals showing positive reactions, although no data on the infection status of these animals were provided by the authors. Animals leaving the herd in the 12 months before the onset of the outbreak were traced back and skin tested, but all yielded negative results. In this herd, the serological RT was performed after the second SCIT test, and 19 adults and two crias were culled based on serological results or

epidemiological suspicions, from which tuberculosis infection was confirmed in four adults. A more detailed report on the performance of diagnostic techniques (SCIT test and RT and MAPIA serological test) on a subset of llamas from this herd was published by Dean et al. (2009). Skin test was only able to detect two of 14 animals with macroscopical lesions typical of tuberculosis (and culture positive to *M. bovis* in 12 cases), while MAPIA detected all 14 individuals. RT performed before the tuberculin test detected nine of the 14 infected animals; the RT was repeated in six animals (four positive and two negative in the previous round) 3 weeks after the skin test and all tested positive, suggesting a potential anamnestic increase in the sensitivity. Nevertheless, up to 54 animals from this herd yielded positive results at different herd group rapid tests; all of them were culled, but tuberculosis could only be detected post-mortem in 10 of them (Twomey et al., 2010a). Twenty of these animals, whose infection could not be confirmed, were negative at their first RT but positive when the test was repeated 3 weeks after the skin test. The absence of lesions and positive culture in these llamas cannot exclude a possible infection; however, considering these animals could be non-infected, and a potential decrease in the specificity of the RT when performed after the skin test cannot be ruled out.

Over the last years, 830 alpacas from herds with confirmed tuberculosis were tested in GB with the RT, with 9.8% (81/830) testing positive. Of the 76 RT-positive animals that underwent post-mortem examination, 76.3% (58/76) were confirmed to have bovine tuberculosis by culture and visible pathology (M. Vordermeier, unpublished data). Interestingly, only 3.5% (2/57) of animals from disease-free herds tested RT positive, which suggested a high degree of test specificity (96.5%, 95% CI: 87.9, 99.6) (M. Vordermeier, unpublished data). Other serological assays based on novel technology developed for cattle (e.g. DPP (Buddle et al., 2010), a modified lateral flow device, or laboratory-based serology based on the Enferplex TB assay (Whelan et al., 2010) have been evaluated at the Veterinary Laboratories Agency in South American Camelids (M. Vordermeier, unpublished data), and these assays are now being validated in further studies in GB.

The risk of introduction of tuberculosis because of *M. bovis* associated with animal movement was demonstrated in three alpaca herds (Twomey et al., 2009). Once more in this case, tuberculosis-infected animals were detected because of clinical symptoms, but once diseased animals were removed from the herd, the SCIT test was performed in all three herds and no reactors were found. Again, lack of information of the true status of negative SCIT test individuals makes it difficult to ascertain whether all were 'true-negative' animals.

A lack of sensitivity of the SCIT test in alpacas infected with *M. bovis* was also reported by Garcia-Bocanegra et al., (2010), as two infected herds (of 32 and four animals) were analysed (1 month before the clinical diagnosis of one infected animal in one case, and 1 week after observing clinical symptoms in another animal in the second herd) and all animals were negative. The usefulness of the IFN- γ detection assay designed for livestock (Bovigam, Prionics AG, Switzerland) was evaluated, but no positive results were obtained.

In another outbreak extensively studied in an alpaca herd in Ireland, the SCIT test performed in the cervical and the axillary skin could not detect any of the five tuberculosis-infected animals that were present in the herd (Connolly et al., 2008; Ryan et al., 2008). A state of anergy (deduced from the severe lesions detected in all infected alpacas) was deemed as a possible cause of this lack of sensitivity (Ryan et al., 2008). No serological tests, which could be useful for the detection of such advanced cases of tuberculosis, were applied in the herd.

Other members of *M. tuberculosis* complex such as *M. microti* (Oevermann et al., 2004) can also cause tuberculosis in NWC, thus provoking the same diagnostic problem. In this scenario, the SCIT test was performed on two llama herds from Switzerland after one *M. microti*-infected animal was detected in each one; negative results obtained in two rounds at 6-week intervals in both herds were interpreted as a sign that no more infected animals remained in both herds (Oevermann et al., 2004). Interestingly, one of the infected animals came from South America in a group where six of 83 llamas showed inconclusive reactions in the tuberculin skin test, maybe indicating an early exposure to the tuberculosis causative agent. The second infected animal was an offspring of a female from this same group, thus revealing a potential epidemiological link.

Serological diagnostic assays have also been tested in alpacas and llamas infected or exposed to *M. microti*, showing promising results (Lyashchenko et al., 2007). RT detected five of eight confirmed or strongly suspected *M. microti*-infected animals (seven llamas and one alpaca) while all animals reacted in the MAPIA test. Estimated specificity of both tests was also high (89.9 and 97.5% for RT and MAPIA test, respectively), although some exposed animals were included among the true-negative individuals because of the lack of symptoms (if these animals are excluded, specificity values would be 89.9 and 100%). Interestingly, false-positive response rates to the RT in non-exposed animals varied depending on the herd of origin (from 0 to 20% of non-specific responses detected), thus indicating potential differential exposure to cross-reacting antigens. No significant differences were

observed between the responses obtained from alpaca or llama samples.

In another report, SCIT, RT and MAPIA tests were used in herds from which *M. microti* animals had been detected (Zanolari et al., 2009). No reactors at the SCIT test were found in six herds of llamas and alpacas, even though two of them were monitored annually for 4 years, thus leading to the conclusion that no more cases remained. In these two herds, two animals showed inconclusive results in the MAPIA test, the RT or both. However, in spite of regular clinical examinations and more serological analysis, tuberculosis could not be confirmed in neither of the animals, and therefore, inconclusive results were interpreted as false-positive results.

Studies performed on Old World camelids

There are few detailed reports on the effectiveness of ante-mortem diagnostic tests in Old World camelids, and especially cases of tuberculosis in Bactrian camels appear to be rare events. High levels (10–20%) of non-specific reactions were reported (Schillinger, 1987) after inoculation of avian and bovine antigens in non-infected Australian dromedaries (as demonstrated by the absence of compatible lesions). Another study using the SCIT test in the neck of dromedaries in Kenya (Paling et al., 1988) reported comparable percentages of reactors to bovine PPD (22%) and avian PPD (34%) from 41 analysed animals; only two individuals had a larger response to bovine tuberculin than to avian tuberculin. One animal was slaughtered, and lesions compatible with tuberculosis (fibrosis and mineralization) were observed, although no isolation of *Mycobacteria* spp. was achieved. Therefore, the true infection status of animals could not be fully determined.

An infection caused by *M. caprae* in one dromedary was reported in the context of a larger outbreak affecting also bison in a zoological garden in Slovenia (Pate et al., 2006). This infected animal was not skin tested, but another dromedary kept in the same pen reacted in the SCIT test and was slaughtered: no lesions were observed, and bacteriology was negative, thus indicating a possible false-positive reaction in the tuberculin test or a lack of sensitivity of post-mortem analysis.

M. pinnipedii was determined as the causative agent of another outbreak involving a range of mammalian species kept in two zoological gardens (four Bactrian camels among them) (Moser et al., 2008). *M. pinnipedii* was only isolated from one Bactrian camel, which unfortunately could not be subjected to ante-mortem testing. The remaining three animals were analysed using an in-house ELISA detecting antibodies against three antigens: extracts prepared from *M. avium* and *M. bovis* and recombinant

MPB70 antigen. Only one animal showed significant titres of antibodies against both *M. avium* and *M. bovis* antigens but not against MPB70. Although this animal suffered from a severe respiratory disease, lesions found in the post-mortem analysis were not considered pathognomonic of tuberculosis and *M. pinnipedii* could not be cultured nor was its DNA detected by PCR, therefore obscuring the significance of high titres against mycobacterial antigens. The other two Bactrian camels remained healthy and were not subjected to further analysis (Moser et al., 2008).

Wernery et al., (2007) reported the use of both single and comparative skin tests and two serological assays (RT and MAPIA) on a dromedary racing herd ($n = 57$) in which one animal had been diagnosed of having tuberculosis. Parallel inoculation of avian and bovine PPDs in the middle of the neck, the axillary site and under the tail (only bovine PPD) demonstrated that higher responses were obtained in two infected animals when the axillary site was used; in the same way, differences in skin thickness in these animals were maximal if the tests were read 5 days after the tuberculin inoculation. Overall results from the 57 animals yielded a total of four positive reactors and four inconclusive reactors to the SIT test. When both avian and bovine PPDs were considered in the SCIT test, only two animals were classified as positives and one more as an inconclusive reactor. A high proportion of reactors to avian PPD was recorded in this herd (27% of the herd had increased in skin thickness over 2 mm), which was considered as a signal of exposure to environmental mycobacteria. The whole herd was tested thrice using RT and MAPIA serological test, and only two animals (in addition to the index case) tested positive in both tests (those that were positive at the SCIT test). These two animals were euthanized and subjected to post-mortem examination although they did not show any clinical symptoms; both were infected with *M. bovis* as demonstrated by culture. No tuberculin reactivity was reported after retesting the herd 6 months later. The authors concluded that serological tests were able to detect all confirmed infected animals, while more non-specific reactions were found in the SCIT and especially SIT tests. However, as no attempt was made to determine the infection status of seronegative animals, one cannot exclude the presence of additional infected animals that escaped detection by RT and MAPIA test.

Conclusions

The review of ante-mortem strategies to diagnose tuberculosis in camelids revealed that, in spite of substantial progresses over the last decade, serious limitations on the capacity of available diagnostic tests still exist:

- 1 There is a lack of data on the performance of diagnostic tests in naturally infected and non-infected camelids (with known infection status). When data from numbers of known infected animals are analysed and are available, sample sizes are usually very limited, and thus, performance estimates suffer from large confidence intervals and a high degree of uncertainty.
- 2 Most of the studies reported negative test results from exposed and potentially infected animals without further follow-up or post-mortem examination. Therefore, it is not possible to exclude the possibility that some of them could be truly infected.
- 3 Tests performed on experimentally infected animals show better sensitivity than those performed on naturally infected animals. This suggests that challenge of camelids might not mimic realistically natural infection. Thus, care should be taken when transferring data from experimental infection systems to natural infection conditions.
- 4 Usually, skin tests in camelids are performed following protocols in use for cattle, but further studies to define the best dosage of PPDs and time interval chosen to read the test should be carried out to optimize and standardize these techniques in these animal species.
- 5 Generally, only a small proportion of animals react in the skin tests after infection is detected in a herd. This can suggest a lack of sensitivity of the tests but also a low transmission rate of tuberculosis between camelids (several studies report only one or two clinically infected animals in herds where no more reactors are detected in consecutive tests). However, generalized lesions observed in infected animals suggest a high risk of large bacterial excretion. Further studies characterizing tuberculosis pathogenesis in camelids are needed to shed light on this aspect.
- 6 Recent data on the performance of diagnostic tests in Old World camelids are scarce, even for tuberculin testing. The available evidence suggests that tuberculin test should be performed on the axillary site and read 5 days after inoculation, but studies on larger number of animals are required to validate these initial observations.
- 7 *M. microti* appears to be highly pathogenic in New World Camelids, as confirmed by the reports of generalized disease. The usefulness of skin tests using bovine and avian PPD should be assessed before using this technique as the reference diagnostic test, as no specific intradermal tuberculin tests using *M. microti* antigens have been developed yet.
- 8 Serological and IFN- γ detecting tests offer promising results for the ante-mortem tuberculosis diagnosis in camelids. Although initial studies suggest that these

tests will display higher sensitivities than tuberculin skin tests, their performances need to be fully determined, with the main emphasis placed on studying disease-free animals.

Challenges for the Future

Because of the lack of knowledge on many aspects of tuberculosis diagnosis in camelids and the need to improve available diagnostic tests to help the control and eradication of the disease in these animal species (and to prevent potential transmission to domestic livestock), research aiming to accurately determine the sensitivity and specificity of available diagnostic tests (including skin tests and serological techniques) should be performed, ideally under two different field conditions:

- 1 For estimation of tests' sensitivity, animals from infected herds should be tested and both positive and negative animals slaughtered and subjected to detailed post-mortem analysis including *M. bovis* culture and histopathological evaluation.
- 2 For specificity analysis, animals from herds known to be free of tuberculosis should be tested. If positive responses are observed, reactors need to be subjected to post-mortem analysis and the involvement of other cross-reacting bacteria (such as environmental mycobacteria) should be established to define their infectious status.

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