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## Diagnosis of tuberculosis in camelids

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Due to the increasing importance of tuberculosis in camelids and the trading of these species in Europe, the European Commission requested to the EU-RL for Bovine Tuberculosis the determination of the sensitivity (Se) and specificity (Sp) of available diagnostic tuberculosis (TB) tests in camelids.

A recent review of the published studies (Alvarez et al., 2011) reported some aspects regarding diagnosis of tuberculosis in New World camelids (NWC): (1) A lack of data on performance of diagnostic tests in naturally infected and non-infected camelids (with known infection status) and using a sufficient number of animals, (2) a better sensitivity when tests were applied on experimentally infected animals, (3) few reactions in the skin tests after the infection is detected in a herd, suggesting a lack of sensitivity of the tests but also a low transmission rate of tuberculosis, (4) absence of data about performing of skin test using different inoculation sites and times of reading, and (5) promising results of the serological tests although its usefulness for early diagnosis and the lack of false positive reactions in animals exposed/infected with other mycobacteria remains to be fully determined

Taking into account these limitations, the EU-RL carried out different studies to evaluate Se and Sp of intradermal test using different inoculation sites and times of reading in *M. bovis* naturally-infected animals and in TB-free herds respectively. Moreover, sera samples were assayed using a novel serological assay (Ingenasa, Spain) and results were compared with those obtained using other assays and reported in previous studies (Dean et al., 2009; Lyashchenko et al., 2011; Rhodes et al., 2012). Several samples from infected animals were also tested using the experimental assay developed by researchers of the AHVLA (UK) to compare the results with those previously published.

### 1. Sensitivity study results.

The aim of this study was to evaluate (1) the intradermal tuberculin test using different inoculation sites (axillary, prescapular and cervical) and times of reading (72 and 120 h) and (2) a novel serological assay based on MPB83 antigen in a *M. bovis* naturally infected alpaca herd in order to provide reliable data about the performance of these assays for the diagnosis of TB in this species. Results reported in this document correspond to three herd testings carried out in the infected herd during the study.

1.1. Tuberculin tests.

Se of single intradermal tuberculin (SIT) test at 72 husing standard and severe interpretation criteria was 37.5% (95% CI, 15.2-64.6) and 56.2% (95% CI, 29.9-80.2) respectively in the first herd testing. Reading at 120 h the Se was 31.2% and 50% respectively (Table 1). Regarding the comparative (SCIT) test, severe interpretation achieved a Se of 37.5% (95% CI, 15.2-64.6) of Se. Reading at 120 h after PPD injection and using severe interpretation criteria, the Se of SCIT test was 43.7% (95% CI, 19.8-70.1) (Table 1).

Results of SIT/SCIT tests in the second herd testing are indicated in Table 1. The highest Se using SIT test in the axillary site was obtained using the severe interpretation criteria and reading at 72 h (95% CI, 53.8%, 40.8-74.5). Using SCIT test in the axillary site, Se was identical to SIT test (53.8%) using severe interpretation but decreased to 5.1% (95% CI, 0.6-17.3) using standard interpretation. Although prescapular site has not been widely used for skin testing in previous studies in camelids the Se obtained did not differ markedly from that obtained inoculating in the axillary site. In fact, using the severe interpretation of the SIT test and

reading at 72 h, the Se was higher than that obtained in the axillary site (58.3% *vs.* 53.8%. Table 1).

A cervical inoculation was also assayed in the third herd testing. The highest number of positive reactors, reading at 72 h, was detected using the prescapular site (eight against six using axillary or cervical sites). However, Se in the different inoculation sites was similar (Table 1). Reading at 120 h, Se of SIT was similar to that obtained reading at 72 h regardless the inoculation site (Table 1). SCIT test was again less sensitive than the single test, specially using standard interpretation and time of reading at 120 h. Under the best conditions (severe interpretation and time of reading at 72 h) the highest number of positive reactors among the infected animals were detected using the prescapular inoculation site (Se=60%, 95% CI, 26.2-37.8, Table 1).

In overall terms, the skin fold thickness in axillary and cervical sites after bovine PPD injection site were slightly higher reading at 120 h (data not shown), although the differences were not significant (*Mann-Whitney* test, $p>0.05$). On the other hand, significant differences were obtained reading at 72 h in the prescapular site (*Mann-Whitney* test, $p=0.007$).

1.2. Serology.

In the first herd testing, Se of the serological assay was 18.7% (95% CI, 4-45.6) (Table 2). The Se increased to 88.9% (95% CI, 51.8-99.7) using samples collected 15 days after PPD injection and it detected five animals that were negative to the most sensitive skin test (SIT test using severe interpretation and reading at 72 h). In fact, applying serology (15 d) and severe interpretation of the SIT test in the axillary site and reading at 72 h in parallel the Se increased to 100% (95% CI, 66.4-100; Table 3).

In the second herd testing, the Se using samples taken at 0 h was 43.6% (95% CI, 27.8-60.4). Thirty days after PPD inoculation, the Se of the serological assay increased to 76.9% (95% CI, 60.7-88.9; Table 2). Furthermore, interpretation of this serological result in parallel with the most sensitive skin test in this herd testing (severe interpretation of the SIT test in the prescapular site and reading at 72 h) increased the Se to 94.4% (95% CI, 81.3.-99.3; Table 3).

Finally, in the third herd testing, Se of the serological assay was 80% (95% CI, 44.4-97.5). Using samples taken 15 days after PPD injection Se increased to 100% (95% CI, 69.2-100; Table 2). Under these conditions, two animals that were not positive reactors with the most sensitive skin test (severe interpretation of the SIT test in the prescapular site and reading at 72 h) were classified as infected using the serological assay.

1.3. IFN-ɣ assay.

Thirty-two *M. bovis* infected alpacas were tested using the experimental IFN-ɣ developed by the AHVLA (Rhodes et al., 2012). Using the threshold described in the manuscript (positive if: bovPPD-avPPD > 0.1; ESAT-6/CFP-10-nil > 0.1), the best Se was achieved considering as positive all the animals reacting against PPD and/or ESAT-6/CFP-10 (EC) [40.6% (95% CI, 23.7-59.4), Table 4]. If only both PPD+ and EC+ alpacas were considered as positive reactors, the Se achieved was only 6.2% (95% CI, 0.8-20.8)(Table 4). The absence of adequate Se was caused by the presence of higher responses against the av PPD in most of the animals. Applying other interpretation criteria used in Europe (e.g in Spain: positive if bovPPD > avPPD and bovPPD-nil > 0.05) and reducing also the threshold for EC, the Se increased to 56.2% (95% CI, 37.7-73.6) under the best conditions (positive if PPD+ and/or EC+)(Table 4).

1.4. Conclusions and recommendations.

Axillary site has been traditionally preferred by veterinarians to perform the skin testing due to the limited presence of hair and the nature of the skin in that region (Lyashchenko et al., 2007; Ryan et al., 2008) but results from this study showed that the Se of SIT using axillary or cervical site was similar and even better using the prescapular site and reading at 72 h (severe interpretation) after PPD inoculation. SCIT test showed the highest Se injecting the PPDs at the prescapular site, reading at 72 h and using severe interpretation although similar values were observed reading at 120 h in axillary site. Using axillary site and standard interpretation, the highest Se of SCIT test was obtained reading at 120 h, obtaining Se results similar to those reported in previous studies in naturally infected llamas and alpacas (Dean et al., 2009; Garcia-Bocanegra et al., 2010).

Regarding serology, in the present study, Se increased in every consecutive herd testing likely due to the booster effect caused by previous skin tests. Results obtained using samples taken 15-30 d after skin testing were promising and showed that this serological assay could be a useful TB diagnostic tool in NWC. As it has been previously reported, diagnosis based on detection of specific antibodies showed better Se than the skin testing. However, these studies were performed using animals that showed gross lesions (Rhodes et al., 2012; Twomey et al., 2012).

This study has demonstrated that the use of ante-mortem skin test and a serological assay in parallel increase the Se and, therefore, is suitable to enhance the detection of infected animals since some positive reactors to one assay are not reactors to the other. Rhodes and collaborators (2012) recommended the use of more than one serological assay or their combination with the IFN-γ assay to maximize the detection of infected animals although skin test was not evaluated. Due to the low Se achieved by IFN-γ assay, the difficulties to performed it using whole blood samples from alpacas and its high logistical and economical demands, the combination of the skin test and a serological assay could be a more realistic approach, obtaining similar Se results.

In summary, this study demonstrate that the intradermal test shows, in general, a low Se when performed in alpacas from *M. bovis* naturally infected herd, although the use of severe interpretation of the SIT test, the prescapular site for PPD inoculation and the time of reading of 72 h after PPD inoculation can improve the detection of infected animals. Moreover, the novel serological assay tested showed promising results using samples collected 15-30 days after PPD inoculation and its implementation in parallel with the most sensitive skin testing could maximize the detection of infected animals.

## 2. Specificity study results.

The aim of this study was to evaluate the Sp of the intradermal tuberculin test for diagnosis of TB under field conditions in different TB-free herds of llamas and alpacas located in different regions in Peru.

2.1. Tuberculin tests.

One hundred and twenty alpacas and 40 llamas were subjected to SIT and SCIT tests in herds A and B respectively. Different inoculation sites (cervical, axillary and prescapular) and two different times of reading (72 h and 120 h) were used in herd A. Llamas were inoculated in the prescapular site using bovine and avian PPDs (2500 IU) reading 72 and 120 hours post-PPD inoculation in herd B.

In Table 5 the number of reactors and the Sp of SIT and SCIT tests (standard interpretation) is shown. In herd A, only one reactor was detected using the SIT test at the cervical site and reading 72 h after PPD inoculation. No reactors were detected using SIT test at the prescapular or axillary site, showing a high Sp. Using the SCIT test, no reactors were detected using either the inoculation sites and times of reading and yielding a comparable Sp to the SIT test at least when the test was carried out at the cervical site (Table 5). Regarding response against bovine PPD, no significant differences were observed depending on the inoculation site and time of reading as all animals (excluding one alpaca) were non-reactors (Table 5).

In herd B, maximum Sp was achieved since no reactors were detected using either of the intradermal test and times of reading (Table 5).

2.2. Conclusions and recommendations.

Maximum Sp was achieved using the SCIT test, demonstrating its usefulness to determine the TB-free status. Furthermore, these results demonstrate that SIT is a specific test, although no avian reactions were observed in these animals. Anyway, studies to evaluate the Sp in presence of other infections that may interfere in the diagnosis are still needed. Regarding SIT test, no significant differences were observed depending on the inoculation site and time of reading as all animals (excluding one alpaca) were non-reactors. Therefore, the Sp reported was similar in all the cases unlike the Se that differed between inoculation sites and times of reading.

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**Table 1.** Sensitivity and 95% confidence intervals (*Fisher´s*) of the single and comparative intradermal tuberculin (SIT and SCIT) tests using different inoculation sites (axillary, prescapular and cervical) and two different times of reading (72 and 120 hours) in three different herd testing (January, March and June, 2012).

| Herd testing (N alpacas) | Test | Time of reading (h) | Inoculation site | Reactors/ infected | Sensitivity (95% CI) | Reactors/ infected | Sensitivity (95% CI) |
|---|---|---|---|---|---|---|---|
| | | | | Standard interpretation | | Severe interpretation | |
| Jan (107) | SIT | 72 | axillary | 6/16 | 37.5 (15.2-64.6) | 9/16 | 56.2 (29.9-80.2) |
| | | 120 | | 5/16 | 31.2 (11-58.7) | 8/16 | 50 (24.7-75.3) |
| | SCIT | 72 | | 0/16 | 0 (0-20.6) | 6/16 | 37.5 (15.2-64.6) |
| | | 120 | | 1/16 | 6.25 (2-30.2) | 7/16 | 43.7 (19.8-70.1) |
| Mar (64) | SIT | 72 | axillary | 10/39 | 25.6 (13-42.1) | 21/39 | 53.8 (37.2-69.9) |
| | | 120 | | 5/39 | 12.8 (4.3-27.4) | 8/39 | 20.5 (9.3-36.5) |
| | | 72 | prescapular | 10/36 | 27.8 (14.2-45.2) | 21/36 | 58.3 (40.8-74.5) |
| | | 120 | | 3/36 | 8.3 (1.8-22.5) | 6/36 | 16.6 (6.4-32.8) |
| | SCIT | 72 | axillary | 2/39 | 5.1 (0.6-17.3) | 21/39 | 53.8 (37.2-69.9) |
| | | 120 | | 2/39 | 5.1 (0.6-17.3) | 8/39 | 20.5 (9.3-36.5) |
| | | 72 | prescapular | 1/13 | 4.3 (0.2-36) | 6/13 | 46.1 (19.2-74.9) |
| | | 120 | | 0/13 | 0 (0-14.8) | 3/13 | 23.1 (5-53.8) |
| Jun (17) | SIT | 72 | axillary | 5/10 | 50 (18.7-81.3) | 6/10 | 60 (26.2-87.8) |
| | | 120 | | 5/10 | 50 (18.7-81.3) | 6/10 | 60 (26.2-87.8) |
| | | 72 | prescapular | 5/10 | 50 (18.7-81.3) | 8/10 | 80 (44.4-97.5) |
| | | 120 | | 3/10 | 30 (6.7-65.2) | 7/10 | 70 (34.8-93.3) |
| | | 72 | cervical | 5/10 | 50 (18.7-81.3) | 6/10 | 60 (26.2-87.8) |
| | | 120 | | 2/10 | 20 (2.5-55.6) | 5/10 | 50 (18.7-81.3) |
| | SCIT | 72 | axillary | 1/10 | 10 (0.3-44.5) | 5/10 | 50 (18.7-81.3) |
| | | 120 | | 1/10 | 10 (0.3-44.5) | 6/10 | 60 (26.2-87.8) |
| | | 72 | prescapular | 3/10 | 30 (6.7-65.2) | 6/10 | 60 (26.2-87.8) |
| | | 120 | | 3/10 | 30 (6.7-65.2) | 5/10 | 50 (18.7-81.3) |
| | | 72 | cervical | 2/10 | 20 (2.5-55.6) | 4/10 | 40 (12.2-73.8) |
| | | 120 | | 1/10 | 10 (0.3-44.5) | 5/10 | 50 (18.7-81.3) |

**Table 2.** Number of positive reactors detected and sensitivity and 95% confidence intervals (*Fisher´s*) obtained using the serological assay using sera samples taken at different times after skin testing (0 h, 15 d, 30 d and 42 d) in three different herd testing (January, March and June, 2012).

| | **Time of sampling** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **0 h** | | | **15 d** | | | **30 d** | | | **42 d** | | |
| **Herd testing** | **Sero+** | **Se (95% CI)** | | **Sero+** | **Se (95% CI)** | | **Sero+** | **Se (95% CI)** | | **Sero+** | **Se (95% CI)** | |
| **Jan** | 3/18 | (3/16) | 18.7 (4-45.6) | 10/11 | (8/9) | 88.9 (51.8-99.7) | | ND | | 2/7 | (2/7) | 28.6 (3.7-71) |
| **Mar** | 17/39 | (17/39) | 43.6 (27.8-60.4) | | ND | | 30/39 | (30/39) | 76.9 (60.7-88.9) | | ND | |
| **Jun** | 12/18 | (8/10) | 80 (44.4-97.5) | 10/14 | (10/10) | 100 (69.2-100) | | ND | | | ND | |

**Table 3.** Skin test and antibody test combinations and sensitivity and 95% confidence intervals (*Fisher´s*).

| Herd testing | Test combination | | | Reactors/ infected | % Sensitivity (95% CI) |
|---|---|---|---|---|---|
| | Skin test and inoculation site (severe interpretation) | Time of reading (h) | Serology (time of sampling after skin test: 0 h, 15 d, 30 d) | | |
| Jan | SIT axillary | 72 | 0 | 11/16 | 68.7 (41.3-89) |
| | | | 15 | 9/9 | 100 (66.4-100) |
| | | 120 | 0 | 11/16 | 68.7 (41.3-89.9) |
| | | | 15 | 9/9 | 100 (66.4-100) |
| Mar | SIT axillary | 72 | 0 | 29/39 | 74.4 (57.8-86.9) |
| | | | 30 | 36/39 | 92.3 (79.1-98.4) |
| | | 120 | 0 | 22/39 | 56.4 (39.6-72.2) |
| | | | 30 | 32/39 | 82.1 (66.5-92.5) |
| | SIT prescapular | 72 | 0 | 26/36 | 72.2 (54.8-85.8) |
| | | | 30 | 34/36 | 94.4 (81.3-99.3) |
| | | 120 | 0 | 19/36 | 52.8 (35.5-69.6) |
| | | | 30 | 30/36 | 83.3 (67.2-93.6) |
| Jun | SIT axillary | 72 | 0 | 9/10 | 90 (55.5-99.7) |
| | | | 15 | 10/10 | 100 (69.2-100) |
| | | 120 | 0 | 8/10 | 80 (44.4-97.5) |
| | | | 15 | 10/10 | 100 (69.2-100) |
| | SIT prescapular | 72 | 0 | 9/10 | 90 (55.5-99.7) |
| | | | 15 | 10/10 | 100 (69.2-100) |
| | | 120 | 0 | 8/10 | 80 (44.4-97.5) |
| | | | 15 | 10/10 | 100 (69.2-100) |
| | SIT cervical | 72 | 0 | 8/10 | 80 (44.4-97.5) |
| | | | 15 | 10/10 | 100 (69.2-100) |
| | | 120 | 0 | 8/10 | 80 (44.4-97.5) |
| | | | 15 | 10/10 | 100 (69.2-100) |

**Table 4.** IFN-γ test sensitivity and Fisher´s 95% CI using different interpretation criteria and two cut-off points.

| | bovPPD-avPPD > 0.1; ESAT-6/CFP-10-nil > 0.1 | | bovPPD > avPPD and bovPPD-nil > 0.05; ESAT-6/CFP-10-nil > 0.05 | |
|---|---|---|---|---|
| **Test Result** | **Reactors/Infected** | **Se (95% CI)** | **Reactors/Infected** | **Se (95% CI)** |
| PPD+ | 3/32 | 9.4 (1.9-25) | 4/32 | 12.5 (3.5-28.9) |
| EC+ | 12/32 | 37.5 (21.1-56.3) | 16/32 | 50 (31.9-68.1) |
| EC+PPD- | 10/32 | 31.2 (16.1-50) | 14/32 | 43.7 (26.4-62.3) |
| PPD+EC+ | 2/32 | 6.2 (0.77-20.8) | 2/32 | 6.2 (0.77-20.8) |
| PPD+/EC+ | 13/32 | 40.6 (23.7-59.4) | 18/32 | 56.2 (37.7-73.6) |

**Table 5.** Number of reactors detected using SIT and SCIT tests (standard interpretation) reading 72 and 120 hours after PPDs inoculation and specificity (Wilson´s 95% I.C.) obtained at each inoculation site.

| Herd | Site | SIT test | | | | SCIT test | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N72 | Sp | N120 | Sp | N72 | Sp | N120 | Sp |
| A | Cervical | 1/23 | 79-99.2 | 0/20 | 83.9-100 | 0/21 | 84.5-100 | 0/17 | 81.6-100 |
| | Prescapular | 0/60 | 94-100 | 0/60 | 94-100 | 0/58 | 93.8-100 | 0/57 | 93.7-100 |
| | Axillary | 0/60 | 94-100 | 0/60 | 94-100 | 0/59 | 93.9-100 | 0/59 | 93.9-100 |
| B | Prescapular | 0/40 | 91.2-100 | 0/40 | 91.2-100 | 0/39 | 91-100 | 0/38 | 90.8-100 |

N72. Number of reactors 72 hours post- PPD inoculation/total number of animals; N120: Number of reactors 120 hours post-PPD inoculation/total number of animals.

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