Current development in the diagnosis of tuberculosis

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Specific tasks:

1. Maximise the **interactions between Member States** through active collaboration.

2. **Harmonise the protocols** across countries of the European Union.

3. Contribute to the **scientific, technical, and policy objectives** proposed by the Commission.
Diagnosis of bovine tuberculosis

- Objectives:
  - to detect the highest number of infected animals
  - to confirm the disease

- Bovine tuberculosis eradication program: test-and-slaughter.

1. Clinical signs.
2. Cellular immune response (SITT, IFN-\(\gamma\))
3. Postmortem analysis (lesions, pathology)
4. Bacteriological culture
In vivo diagnosis of bovine tuberculosis

Official diagnostic tests (64/432/ECC)
1. Tuberculin skin test

- *In vivo* cellular immune response.
- **Keystone** of the eradication programmes.
- Good **herd test** although limited individual value.
- Single intradermal tuberculin test (**SITT**) or comparative (**SICTT**): bovine PPD/avian PPD.
- **Interpretations** (standard, severe).
- Variable performance (**Se, Sp**): gold standard and population study (chronic infections, lesions, etc.).
### Single intradermal tuberculin test (SITT)

<table>
<thead>
<tr>
<th>Se</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>63-100% (interval)</td>
</tr>
<tr>
<td></td>
<td>83.9% (most probable)</td>
</tr>
<tr>
<td></td>
<td>95% &gt; 60%</td>
</tr>
<tr>
<td></td>
<td>&lt; in certain circumstances (repetitions)</td>
</tr>
<tr>
<td></td>
<td>&gt; 90% at herd level (IMP!)</td>
</tr>
<tr>
<td></td>
<td>It is not perfect (but TB has been erradicated under certain conditions)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sp</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75-99% (interval)</td>
</tr>
<tr>
<td></td>
<td>96.8% (most probable)</td>
</tr>
<tr>
<td></td>
<td>95% &gt; 75%</td>
</tr>
<tr>
<td></td>
<td>Season effect?</td>
</tr>
<tr>
<td></td>
<td>Field studies → ↑ Sp</td>
</tr>
<tr>
<td></td>
<td>It is not perfect</td>
</tr>
</tbody>
</table>

### Single intradermal comparative tuberculin test (SICTT)

- Sensitization with other mycobacteria (MAC)
- Se: 52-100% (80-93.5% depends on interpretation)
- Sp: 78-100% (99.5%)
- If there is disease → > probability of leaving infected animals
- It is not perfect
Factors that could affect the performance of the skin test:

1. Related with the animal (difficult to control):
   - Pre-allergic stage (too soon)
   - Anergic state (too late)
   - Cross reactions (other mycobacteria)

2. Related with the technique:
   - **PPD quality** (conservation, expiry date, potency)
   - Poor inoculation (site, volume, intradermal)
   - Identification of animals
   - Poor reading (skin thickness, clinical signs)
   - Bad practice
Tuberculins

Biological potency assays

• OIE Manual / European Pharmacopoeia.
• Guinea pigs / naturally infected tuberculous cattle.
## Bovine PPD biological potency

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Type infection</th>
<th>Number animals/group</th>
<th>PPD inoculation</th>
<th>Interpretation results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pigs</td>
<td>Experimental infection (0.5 ml IM, 5 weeks)</td>
<td>9</td>
<td>2 PPDs and IS/group, 3 dilutions (1:200, 1:1000, 1:5000), 4 injections 0.2 ml in each side (8), Intradermal injection, Change position (latin square design)</td>
<td>28 hours, Digital calliper, Erythema area</td>
</tr>
<tr>
<td>Cattle</td>
<td>Naturally infected (reactors)</td>
<td>8</td>
<td>3 PPDs and IS/group, 2 dilutions (1 and 0.2 mg/ml), 4 injections 0.1 ml in each side (8), Intradermal inoculation, Change position (latin square design), Severe interpretation (&gt; 2mm, signs)</td>
<td>72 hours, Calliper, Skin thickness</td>
</tr>
</tbody>
</table>

Bovine PPD: 1: Cranial dorsal R/L; 2: Caudal dorsal R/L; 3: Cranial ventral R/L; 4: Caudal ventral R/L; Avian PPD: 5: Avian PPD R/L
OIE Manual and European Pharmacopoeia

- Statistical analysis (parallel lines) → biological potency (IU/ml).
- Minimum: 20,000 IU/ml (2,000 IU/dose).
- Bovine PPD estimated potency must be not less than 66% and not more than 150% of the potency stated on the label.

Determine the biological potency; test different batches; report to EC.
2. IFN-γ assay

- *In vivo* cellular immune response.
- Early detection of infected animals.
- Optical density value (**cut-off value**) → less subjective interpretation.
- Strategic use of the IFN-γ assay: parallel testing (Annex B.3. 64/432/ECC) to detect the maximum number of infected animals in a herd or region of high prevalence.

*In vitro* blood culture
<table>
<thead>
<tr>
<th>Se</th>
<th>Sp</th>
</tr>
</thead>
<tbody>
<tr>
<td>• 73-100% (interval): cut-off value</td>
<td>• 80-99% (interval): cut-off value</td>
</tr>
<tr>
<td>• 92% (most probable); 95% &gt;80%</td>
<td>• 90% (most probable); 95% &gt;80%</td>
</tr>
<tr>
<td>• Early diagnosis</td>
<td>• Sensible to slight modifications of the protocol</td>
</tr>
<tr>
<td>• IFN &gt; SITT in field conditions</td>
<td>• SITT&gt;&gt;IFN in field conditions → use in infected herds</td>
</tr>
<tr>
<td>• It is not perfect</td>
<td>• It is not perfect</td>
</tr>
</tbody>
</table>
Factors that could affect the performance of the IFN-γ test:

1. Related with the animal (difficult to control):
   - Pre-allergic stage (too soon)
   - Anergic state (too late)
   - Cross reactions (other mycobacteria)

2. Related with the technique:
   - Skin test (> 60 days, age, before skin test)
   - Blood collection (lithium heparin, volume)
   - Identification of each tube – ear tag
   - Transport (room temperature)
   - Technique (PPDs, incubation, plasma, EIA)
   - Time to stimulate (variable)
   - Interpretation of results (cut-off value)
## Cut-off values in the IFN-γ

<table>
<thead>
<tr>
<th>Number</th>
<th>bPPD vs. PBS (cut-off A)</th>
<th>bPPD vs. aPPD (cut-off B)</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; – PBS&lt;sub&gt;OD&lt;/sub&gt; ≥ 0.05</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; &gt; PPDa&lt;sub&gt;OD&lt;/sub&gt;</td>
<td>Spain, Portugal, Poland*</td>
</tr>
<tr>
<td>2</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; – PBS&lt;sub&gt;OD&lt;/sub&gt; ≥ 0.1</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; &gt; PPDa&lt;sub&gt;OD&lt;/sub&gt;</td>
<td>Germany*</td>
</tr>
<tr>
<td>3</td>
<td>--</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; – PPDa&lt;sub&gt;OD&lt;/sub&gt; ≥ 0.1</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>4</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; – PBS&lt;sub&gt;OD&lt;/sub&gt; &gt; 0.1</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; – PPDa&lt;sub&gt;OD&lt;/sub&gt; &gt; 0.1</td>
<td>Austria*</td>
</tr>
<tr>
<td>5</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; – PBS&lt;sub&gt;OD&lt;/sub&gt; ≥ 0.1</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; – PPDa&lt;sub&gt;OD&lt;/sub&gt; &gt; 0.1</td>
<td>Hungary</td>
</tr>
<tr>
<td>6</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; – PBS&lt;sub&gt;OD&lt;/sub&gt; ≥ 0.1</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; – PPDa&lt;sub&gt;OD&lt;/sub&gt; ≥ 0.1</td>
<td>Romania, Greece</td>
</tr>
<tr>
<td>7</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; – PBS&lt;sub&gt;OD&lt;/sub&gt; ≥ 0.05</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; &gt; PPDa&lt;sub&gt;OD&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Ireland</td>
</tr>
<tr>
<td>8</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt;/PBS&lt;sub&gt;OD&lt;/sub&gt; ≥ 2</td>
<td>PPDb&lt;sub&gt;OD&lt;/sub&gt; – PPDa&lt;sub&gt;OD&lt;/sub&gt; ≥ 0.05</td>
<td>Italy</td>
</tr>
</tbody>
</table>
Cut-off values in the IFN-γ

<table>
<thead>
<tr>
<th>Number</th>
<th>blPPD vs. PBS (cut-off A)</th>
<th>blPPD vs. aPPD (cut-off B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Spain, Portugal, Poland*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Germany*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>United Kingdom</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Austria*</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Hungary</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Romania, Greece</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Ireland</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Italy</td>
<td></td>
</tr>
</tbody>
</table>

Cut-off values in the IFN-γ (tanto por uno) de reactores
Scientific Opinion on the use of a gamma interferon test for the diagnosis of bovine tuberculosis

ToR1. Suitability of the IFN-γ test for inclusion amongst the official tests for the purpose of granting and retaining an officially tuberculosis free herd status.

C1. PPD-based IFN-γ could be included among the official test. In some populations the Sp may not be as high as the SIT.

R1. IFN-γ harmonised protocol in the EU →

ToR2. Suitability of other, possible newer tests, if any.

C2. There are no other tests suitable.

ToR3. Advise the Commission on which further validation studies are necessary to evaluate other tests.

C3. Uncertainty regarding the specificity of the PPD-based IFN-γ.

R2. Validation of the IFN-γ test: factors (environmental mycobacteria, prevalence, age, TB history, production system), performance of IFN-γ with defined antigens, serial use of IFN-γ and SITT →
<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SITT</td>
<td>63.2-100% (83.9%)</td>
<td>75-99% (96.8%)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>73-100% (92%)</td>
<td>85-99% (90%)</td>
</tr>
</tbody>
</table>

- Periodic diagnosis: most of the infections are recent (no lesions, ↓ load of bacteria → compromise culture).
- Culture and/or macroscopic compatible lesions are not a reliable indicator in animals recently infected.
- [Culture positive = confirmed infection] but [culture negative ≠ no infection].

Current diagnostic strategy:
- SITT: ↑ Se and Sp at herd level
- IFN-γ: ancillary test in confirmed herds with a good Se at individual level

- Herd level: good specificity
- Individual level: good sensitivity

It is not perfect.....but it is the less imperfect strategy!!!!!
EXAMPLE 1: Geographical area with high prevalence of TB

<table>
<thead>
<tr>
<th>Number of confirmed herds by culture</th>
<th>N analyzed animals</th>
<th>Animals SIT +ve</th>
<th>Animals IFN-γ +ve</th>
<th>Animals +ve to both tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>54</td>
<td>7364</td>
<td>226</td>
<td>871</td>
<td>150</td>
</tr>
</tbody>
</table>

1. Herds

54 herds +ve to bacteriological culture

- Herds with reactors to SITT: 35/54 (64.8%)
- Herds with reactors to IFN-γ: 53/54 (98.1%)
- Herds with reactors to SITT+IFN-γ: 34/54 (62.9%)

19 herds with ONLY reactors to IFN-γ: (35.2%)
2. Animals

1120 animals for bacteriological culture and 251 animals culture positive.

- Animals detected by SITT: 58/251 (23.1%)
- Animals detected by IFN-γ: 218/251 (86.5%)
- Animals detected by SITT+IFN-γ: 44/251 (17.5%)

174 animals detected ONLY by IFN-γ: (69.3%)
EXAMPLE 2: Co-infection tuberculosis and paratuberculosis

- Tuberculosis and paratuberculosis infection confirmed by culture
- > 600 animals
- Complex epidemiology: *M. bovis* (6 spoligotyping profiles)
  *M.a.p* (Type II and III)

<table>
<thead>
<tr>
<th></th>
<th><em>M.a.p</em> +ve</th>
<th><em>M.a.p</em> -ve</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. bovis</em> +ve</td>
<td>16</td>
<td>30</td>
<td>46</td>
</tr>
<tr>
<td><em>M. bovis</em> -ve</td>
<td>94</td>
<td>76</td>
<td>170</td>
</tr>
<tr>
<td>Total</td>
<td>110</td>
<td>106</td>
<td>218</td>
</tr>
</tbody>
</table>

Diagnostic test

<table>
<thead>
<tr>
<th>Diagnostic test</th>
<th>Tbc+ animals (n=46)</th>
<th>Ptb+ animals (n=110)</th>
<th>Tbc+ / Ptb+ (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ detection test</td>
<td>78.3 (63.6-89)</td>
<td>-</td>
<td>50 (24.6-75.3)</td>
</tr>
<tr>
<td>Ptb serology</td>
<td>-</td>
<td>75.4 (66.3-83.2)</td>
<td>50 (24.6-75.3)</td>
</tr>
</tbody>
</table>

Apparent sensitivities with confidence intervals (95%) of the IFN-γ detection test and the paratuberculosis serology in the groups formed based on culture results.
EXAMPLE 2: Co-infection tuberculosis and paratuberculosis

- Tuberculosis and paratuberculosis infection confirmed by culture
- > 600 animals
- Complex epidemiology: *M. bovis* (6 spoligotyping profiles)
  *M.a.p* (Type II and III)

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<tr>
<th></th>
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<th><em>M.a.p</em> -ve</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td><strong>M. bovis</strong> +ve</td>
<td>16</td>
<td>30</td>
<td>46</td>
</tr>
<tr>
<td><strong>M. bovis</strong> -ve</td>
<td>94</td>
<td>76</td>
<td>170</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>110</td>
<td>106</td>
<td>218</td>
</tr>
</tbody>
</table>

Diagnostic test | All animals (n=218) | Tbc+/Ptb- (n=30) | Tbc+/Ptb+ (n=16) | Tbc-+/Ptb+ (n=94) |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ test</td>
<td>78.3 (60.4-86.4)</td>
<td>93.3 (77.9-99.2)</td>
<td>50 (24.6-75.3)</td>
<td>-</td>
</tr>
<tr>
<td>Ptb serology</td>
<td>57.3 (47.5-66.7)</td>
<td>-</td>
<td>50 (24.6-75.3)</td>
<td>79.8 (70.2-87.4)</td>
</tr>
</tbody>
</table>

Apparent sensitivities with confidence intervals (95%) of the IFN-γ detection test and the paratuberculosis serology in the groups formed based on culture results.
1. Clinical signs.
2. Cellular immune response (SIT, IFN-γ)
3. Postmortem analysis (lesions, pathology)
4. Bacteriological culture

COUNCIL DIRECTIVE
of 26 June 1964
on animal health problems affecting intra-Community trade in bovine animals and swine
(64/432/EEC)
ANNEX B

TUBERCULOSIS

1. IDENTIFICATION OF THE AGENT

The presence of *Mycobacterium bovis* (*M. bovis*), agent of bovine tuberculosis, in clinical and post-mortem specimens may be demonstrated by examination of stained smears or immunoperoxidase techniques and confirmed by cultivation of the organism on primary isolation medium.

Pathological material for the confirmation of *M. bovis* should be taken from abnormal lymph nodes and parenchymatous organs such as lungs, liver, spleen, etc. In the cases where the animal does not present pathological lesions, samples from the retropharyngeal, bronchial, mediastinal, supramammary, mandibular and some mesenteric lymph nodes and liver should be collected for examination and culture.

Identification of isolates may be usually carried out by determining cultural and biochemical properties. The polymerase chain reaction (PCR) may also be employed for the detection of the *M. tuberculosis* complex. DNA analysis techniques may prove to be faster and more reliable than biochemical methods for the differentiation of *M. bovis* from other members of the *M. tuberculosis* complex. Genetic fingerprinting allows distinguishing between different strains of *M. bovis* and will enable patterns of origin, transmission and spread of *M. bovis* to be described.

The techniques and media used, their standardisation and the interpretation of results must conform to that specified in the OIE Manual of Standards for Diagnostic Tests and Vaccines, Fourth Edition, 2000, Chapter 2.3.3 (bovine tuberculosis).

OIE Terrestrial Manual

CHAPTER 2.4.7.

BOVINE TUBERCULOSIS

1. Identification of the agent

In cattle, clinical evidence of tuberculosis is usually lacking until very extensive lesions have developed. For this reason, its diagnosis in individual animals and an eradication programme were not possible prior to the development of tuberculin by Koch in 1890. Tuberculin, a concentrated sterile culture filtrate of tubercle bacilli grown on glycerinated beef broth and, more recently, on synthetic media, provides a means of detecting the disease.

Immunological responses to *M. bovis* infections in cattle continue to be studied in attempts to develop improved or alternative diagnostic methods, as skin testing sometimes has practical drawbacks. The gamma interferon test is increasingly being used as a diagnostic blood test for tuberculosis in cattle and for other animals (e.g. goats, buffalo) and is available commercially. The lymphocyte proliferation test and the IgG1 enzyme-linked immunosorbent assay (ELISA) have proven to be useful as ancillary serial (to enhance specificity) and parallel (to enhance sensitivity) tests in farmed red deer.

The presence of *M. bovis* in clinical and post-mortem specimens may be demonstrated by examination of stained smears or tissue sections and confirmed by cultivation of the organism, on primary isolation medium. Collection containers should be clean and preferably sterile (use of sample containers that are contaminated by environmental mycobacteria may result in the failure to identify *M. bovis* infection due to the rapid growth of the environmental mycobacteria); where feasible, single-use plastic, disposable containers, 50 ml in capacity, may be used for a variety of specimen types. Specimens that are to be sent to the laboratory must be cushioned and sealed to prevent leakage, and properly packaged to withstand breakage or crushing in transit. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) for shipping specimens from a suspected zoonotic disease must be followed. The requirements are summarised in Chapter 1.1.1 Collection and shipment of diagnostic specimens. Prompt delivery of specimens to the laboratory greatly enhances the chances of cultural recovery of *M. bovis*. If delays in delivery are anticipated, specimens should be refrigerated or frozen to retard the growth of contaminants and to preserve the mycobacteria. In warm ambient conditions, when refrigeration is not possible, boric acid may be added (0.5% [w/v] final concentration) as a bacteriostatic agent, but only for limited periods, no longer than 1 week.
Workflow in the tuberculosis laboratory

**Macroscopic analysis**
- Compatible lesions of tuberculosis

**Microscopic analysis**
- Auramine or Ziehl-Neelsen stains (tissue or colony)

**Bacteriological culture**
- Culture positive
- Identification by PCR
- Molecular characterization
  - DVR-spoligotyping, VNTRs
...other diagnostic tests?

- Not official.
- Research activity.
- At least the same performance than actual ones (SIT, IFN-\(\gamma\), culture)
- Take into consideration the applicability in an eradicacion program/surveillance program.

Serology?
DNA extraction?
Change protocols?
Improvement of SIT?
Characterization?
Other antigens?
...other diagnostic tests?

Improvement of SIT?

Objective: increase performance of the technique.
- Objectivity of reading: new devices?
- Increase detection of infected animals (Se).
...other diagnostic tests?

Serology?

- **Objective**: to detect animals with advanced disease that could be missed by the intradermal tests and the IFN-γ assay (anergic).
- **Initial studies**: sensitivity (18-73%) - specificity (88 - 96%).
- **Boosting effect**: increase of the serological response after performing the intradermal test.
- **Different antigens**: **MPB70 and MPB83** most relevant in the MTB complex.
- **New systems**: multiantigen, other platforms (MAPIA, rapid-test, FPA, etc.).
- **Other animals species**.
Thank you

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