The Diagnosis of Brucellosis in cattle, sheep, goats & pigs

What is needed?

B. Garin-Bastuji
EU / OIE & FAO Brucellosis Expert
ANSES, Maisons-Alfort, France

Brucellosis Workshop
Brucellosis

• Due to *Brucella abortus, melitensis or suis*
  – Gram negative bacteria (*α*-proteobacteriaecae)
  – Mammals facultative intracellular pathogens

• Geographical distribution
  – Mediterranean countries, near- and middle east
  – Distributed world wide

• Clinical signs (non pathognomonic)
  – abortions, sterility, unthrifty offspring
  – orchitis & epididymitis (+hygromas)
  – *joints may be affected, causing lameness and sometimes paralysis (pigs)*
<table>
<thead>
<tr>
<th>Species</th>
<th>Biovars</th>
<th>Preferred natural host</th>
<th>Main geographical area</th>
<th>Pathogenicity for man</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. melitensis</td>
<td>1, 2, 3</td>
<td>Sheep, Goats, Wild ongulates</td>
<td>Mediterranean countries</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Middle &amp; Near East</td>
<td></td>
</tr>
<tr>
<td>B. abortus</td>
<td>1, 2, 3, 4, 5, 6, 7, 9</td>
<td>Bovines, Wild ongulates</td>
<td>Europe, Americas, Africa, Asia</td>
<td>Moderate</td>
</tr>
<tr>
<td>B. suis</td>
<td>1</td>
<td>Suids</td>
<td>Americas, Asia, Oceania</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Suids, Hares</td>
<td>Central &amp; Western Europe</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Suids</td>
<td>USA, China</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Reindeer</td>
<td>USA, Canada, Russia</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Wild rodents</td>
<td>Russia</td>
<td>High</td>
</tr>
<tr>
<td>B. neotomae</td>
<td></td>
<td>Desert wood rat Neotoma lepida</td>
<td>USA</td>
<td>Unknown</td>
</tr>
<tr>
<td>B. ovis</td>
<td></td>
<td>Sheep (males)</td>
<td>Mediterranean countries</td>
<td>No</td>
</tr>
<tr>
<td>B. canis</td>
<td></td>
<td>Dogs</td>
<td>USA, South America Central Europe</td>
<td>Low</td>
</tr>
<tr>
<td>B. ceti</td>
<td></td>
<td>Cetaceans</td>
<td>-</td>
<td>High / Unknown</td>
</tr>
<tr>
<td>B. pinnipedialis</td>
<td></td>
<td>Pinnipeds</td>
<td>-</td>
<td>High / Unknown</td>
</tr>
<tr>
<td>B. microti</td>
<td></td>
<td>Common vole</td>
<td>Central Europe</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
Brucellosis - *the global cycle*

Wild Ruminants, Rodents, Carnivores, Swine

- *B. abortus*
- *B. melitensis*
- *B. suis*
Epidemiology of Brucellosis….

Brucellosis is a "multi-species" infectious and contagious disease…

- different animal species
- different Brucella species

…..to be considered
Abortions
Endometritis
Orchitis in rams
Orchitis in pigs
Wild ruminants – *e.g.* in the EU

Chamois (*Rupicapra rupicapra*)

Alpine ibex (*Capra ibex*)

J. Hars

J. Hars

Abortion is the main sign of brucellosis…

But, most infected females give birth normally...

In both cases, huge and durable excretion of Brucella
Diagnostic tools

• Direct:
  – Detection of the *Brucella* and/or their specific components (Ag, Genes)

• Indirect
  – Measure of the immune response
Diagnosis of Brucellosis….

- No single test able to...
  - identify all infected animals, or
  - certify all free animals

- Tests repetitions needed
- Tests associations (parallel/series) needed

- BUT a test means…a standardised test which also means a validated test and biologicals regularly checked against standards (see OIE update)
Direct Diagnosis

- Bacterioscopy

- Isolation & identification of *Brucella*

- **Antigens: Immuno-enzymology - fluorescence**
  - *Not practicable, no standardisation*
  - *Low specificity, low sensitivity*

- PCR
Bacterioscopy (Stamp)

- Samples to be ground
- Several smears needed
  - **Advantages:** quick and simple
  - **Disadvantage:** presumptive value
    - False negative
    - False positive (*B. ovis*, *Chlamydia*, *Coxiella*,..)
Stamp staining (modified Ziehl-Neelsen)
Direct Diagnosis

- Bacterioscopy

- Isolation & identification of *Brucella*
  
  - *Antigens: Immuno-enzymology - fluorescence*
    - Not practicable, no standardisation
    - Low specificity, low sensitivity

- PCR
Isolation & identification of *Brucella*

- The only unequivocal method
- Identification = definitive diagnosis

- High epidemiological value: biotyping
- Relatively expensive, long lasting
- Bio-hazard: needs expertise, procedures and equipment
- Lack of sensitivity
- Sample sometimes unavailable
  (milk, foeto-maternal materials, genital secretions, lymph nodes, ...)

## Specimens for *Brucella* isolation

<table>
<thead>
<tr>
<th>Live animal</th>
<th>Slaughtered animal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female</strong></td>
<td></td>
</tr>
<tr>
<td>Vaginal discharges</td>
<td>Lymph nodes**</td>
</tr>
<tr>
<td>Milk*</td>
<td>Spleen**</td>
</tr>
<tr>
<td></td>
<td>Udder**</td>
</tr>
<tr>
<td></td>
<td>Uterus**</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td></td>
</tr>
<tr>
<td>Semen</td>
<td>Lymph nodes **</td>
</tr>
<tr>
<td></td>
<td>Spleen **</td>
</tr>
<tr>
<td></td>
<td>Epididymes**</td>
</tr>
<tr>
<td></td>
<td>Sexual accessory glands **</td>
</tr>
</tbody>
</table>

* Cream + pellet
** Ground (stomacher)
Distribution of *Brucella* infection (cattle)

- **Australia** (Hornitsky, 1986)
  - Mam. 79.6%
  - Mam. + Sc. 89.8%
  - Mam. + Sc. + RP 93.9%
  - Mam. + Sc. + RP + Mand. 98.0%
  - Mam. + Sc. + RP + Mand. + Ili 100.0%
  (*# culture + = 86% CFT+ animals*)

- **Northern-Ireland** (1999-2001) 2 dishes/organ (n=342)
  - L.N. Par RP SM RM
  - Pos. 60% 81% 66% 82%
  - Pos. alone 1.7% 6.2% 0.7% 8.9%
<table>
<thead>
<tr>
<th>( \text{Organ} )</th>
<th>172 Sheep &amp; goats Blasco et al. 2002</th>
<th>142 Sheep Marín et al. 1996</th>
<th>40 Goats Marín et al. 1996</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial L.N.</td>
<td>37.4 %</td>
<td>33.8 %</td>
<td>80.0 %</td>
</tr>
<tr>
<td>Scapular L.N.</td>
<td>26.4 %</td>
<td>33.8 %</td>
<td>50.0 %</td>
</tr>
<tr>
<td>Prefemoral L.N.</td>
<td>-</td>
<td>36.6 %</td>
<td>47.5 %</td>
</tr>
<tr>
<td>Iliac L.N.</td>
<td>46.1 %</td>
<td>51.4 %</td>
<td>65.0 %</td>
</tr>
<tr>
<td>Mammary L.N.</td>
<td>69.2 %</td>
<td>81.7 %</td>
<td>82.5 %</td>
</tr>
<tr>
<td>Spleen</td>
<td>28.0 %</td>
<td>36.0 %</td>
<td>25.0 %</td>
</tr>
<tr>
<td>Uterus</td>
<td>17.6 %</td>
<td>19.7 %</td>
<td>25.0 %</td>
</tr>
<tr>
<td>Milk</td>
<td>60.9 %</td>
<td>62.5 %</td>
<td>74.3 %</td>
</tr>
</tbody>
</table>

Distribution of *Brucella* infection (sheep & goats)
**Brucella** on Blood Agar

*Selective media almost always needed*
## Selective media

### Farrell

**Base:**
SDA, BAB or BMB
+ 5 % serum

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nalidixic acid</td>
<td>5 mg</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>25 000 UI</td>
</tr>
<tr>
<td>Natamycin</td>
<td>50 mg</td>
</tr>
<tr>
<td>Polymyxin B (sulf.)</td>
<td>5 000 UI</td>
</tr>
<tr>
<td>Nystatin</td>
<td>100 000 UI</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>20 mg</td>
</tr>
</tbody>
</table>

*(Oxoid SR209A)*

### Modified Thayer-Martin
*(Brown et al. - Marín et al. modification)*

**Base:**
GC medium
Haemoglobin sol. 10 %

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vancomycin</td>
<td>3 mg</td>
</tr>
<tr>
<td>Colistin</td>
<td>7.5 mg</td>
</tr>
<tr>
<td>Nystatin</td>
<td>100 000 UI</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>10 mg</td>
</tr>
<tr>
<td>Amphoterericin B</td>
<td>2.5 mg</td>
</tr>
</tbody>
</table>

*(20 mg)*

*(4 mg)*

*CITA medium*
Comparison of Farrell and m. Thayer-Martin

<table>
<thead>
<tr>
<th>Brucella species</th>
<th>Medium</th>
<th>mean CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>23 B. abortus</td>
<td>Farrell</td>
<td>53.86</td>
</tr>
<tr>
<td>23 B. abortus</td>
<td>m T-M</td>
<td>63.16</td>
</tr>
<tr>
<td>31 B. melitensis</td>
<td>Farrell</td>
<td>74.48</td>
</tr>
<tr>
<td>31 B. melitensis</td>
<td>m T-M</td>
<td>99.50</td>
</tr>
</tbody>
</table>

- 182 infected animals
  - 172 Farrell +
  - 180 m T-M +
  - 182 Farrell + or m T-M +

Simultaneous use of Farrell + mT-M media increase the sensitivity of bacteriological diagnosis

(Marín et al 1996)
Presumptive identification

- Clinical & Epidemiological context
- Growth on Farrell / mT-M (slow > 3-4 days)
- Morphology of colonies (smooth, homogenous, glossy, etc.)
- Gram negative coccobacilli
- Agglutination of anti-Brucella serum
- Catalase +, Oxidase +, Urease +
- No use of sugars

Typing: expert laboratories
Direct Diagnosis

- Bacterioscopy

- Isolation & identification of *Brucella*

- Antigens: Immuno-enzymology - fluorescence
  - Not practicable, no standardisation
  - Low specificity, low sensitivity

- PCR
Direct diagnosis by PCR

- *bscp 31 Kd*
- 16S rRNA
- IS 711/6501
  - Specificity: genus *Brucella*
  - Sensitivity ??

- **No great/long experience in field conditions**
- **Real-time PCR under validation**
### Bacteriology

<table>
<thead>
<tr>
<th>PCR</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>29</td>
</tr>
</tbody>
</table>

**PCR IS711: vaginal swabs**
PCR IS711 : organs

<table>
<thead>
<tr>
<th>Spleen</th>
<th>Bacteriology</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>-</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>L.N.</th>
<th>Bacteriology</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>19</td>
</tr>
</tbody>
</table>

- **PCR is a good complementary test but could not replace bacteriology up to now in all situations….**
Added value of Real time PCR

<table>
<thead>
<tr>
<th>Brucella</th>
<th>IS711 Nb copies</th>
<th>Conventional single PCR *</th>
<th>PCR RT*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IS 711</td>
<td>bscp 31</td>
</tr>
<tr>
<td><strong>B. abortus 544</strong></td>
<td>6 to 8</td>
<td>100</td>
<td>1000</td>
</tr>
<tr>
<td><strong>B. melitensis 16M</strong></td>
<td>7 to 10</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td><strong>B. ovis 63/290</strong></td>
<td>&gt; 20</td>
<td>100</td>
<td>1000</td>
</tr>
</tbody>
</table>

*Lower limit of detection in fg

- **Sensitivity**: ➤
- **Limits contaminations**:

Bounaadja *et al.* (2009)
Direct diagnosis (conclusion)

Isolation (or PCR) & Typing of *Brucella*

- **Advantage**: unequivocal diagnosis
- **Disadvantage**: long and expensive, limited to equipped and experienced labs.

- Not applicable at all stages of an eradication program (too many outbreaks)

- Essential in the last stages:
  - Diagnosis confirmation
  - Trace-back and forward tracing
Diagnostic tools

- **Direct:**
  - Detection of the *Brucella* and/or their specific components

- **Indirect**
  - Measure of the immune response

➢ *Essential in surveillance, control and eradication programmes.*
Brucella =
Facultative intracellular pathogens

Cell response (DTH)
&
Humoral response (antibodies)
Indirect diagnosis

Serological tests
- Early, sensitive but low specificity (RBT/FPA))
- Sensitive but lower specificity (iELISA – pool possible)
- Late, more specific but less sensitive (CFT)
- Specific ≥ but the lowest sensitivity (cELISA)
- Highly sensitive/specific (Milk iELISA > Milk ring test)
  ➢≠ tests: ≠ antibodies detected

Cell tests: Brucellin Skin Test (BST)
highly specific, but not usable in vaccinated animals

➢ Frequent discrepancies between tests
➢ Associations usually needed
Immune response of the infected host - Antibodies

• Foetus
  - congenital infection – no Ab before 1\textsuperscript{st} gestation

• Young
  - low and transitory response

• Adults
  - Response in 1-2 months, sometimes no or low
  - Persistence 6 months or more
  - Fluctuant (calving/abortions) - milk

\textit{Latent infection - abortion, lambing}
\textit{Great individual variations}
\textit{Tests repetition - Discordance - vaccination}
Brucella = Facultative intracellular pathogens

Cell response (DTH) & Humoral response (antibodies)
The cell response

**IN VIVO**

Brucellin allergic skin test (Brucellergene ®)

**In adults:**
- Rapid
- Persistent
- To any *Brucella*

.....including vaccines
Intradermic
Measure of skin thickening
Reading at J+72h

Brucellin AST

Intradermic/subcutaneous
Reading at J+48h
Brucellin AST
Brucellin AST
Immune response of the infected host

Brucella = Facultative intracellular pathogens

↓

Cell response (DTH) &

Humoral response (antibodies)
The S-LPS of *Brucella* – The Major antigen

- The main cause of cross-reactions!!

- **Ab**
  - O-Chain
  - External Membrane
  - Periplasm
  - Internal Membrane
  - Cytoplasm

- **Omp2**
- **CP26**
- **CMI**
Serological tests – old tools

Anti-Brucella post-infection antibodies
Schematic evolution curve
# Immune mechanisms

## Immunoglobulins

<table>
<thead>
<tr>
<th>Tests</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgM</th>
<th>IgA</th>
<th>Sensitised T-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAT</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RBT</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CFT</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>iELISA</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>MRT</td>
<td>+/-</td>
<td>+/-</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>CMI</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Immune response: great individual variability

Possible situations *(Plommet, 1984)*

<table>
<thead>
<tr>
<th>Test</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serology</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brucellin</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ring-Test</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Culture (milk)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Culture (L.N.)</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interpretation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Sensitivity & specificity

- Ability to detect anti-S-LPS antibodies +
- Brucellosis outbreaks detected +
- FPSR herds/flocks detected +
- Sensitivity +
  - CFT
  - SAT
  - RBT/FPA
- Specificity -
  + iELISA
Serological tests – “old” tools

- **SAT (cattle)**
- Rose Bengal (RBT)
- iELISA (serum & bovine milk)
- Milk ring-Test (bovine milk)
  - *Generally used as “screening” tests*
- Complement fixation (CFT)
  - *Generally used as a “confirmatory” test*

.rb, CF & iELISA = the only OIE official tests in S&G
Serological tests – “old” tools (bovine milk)

• **MRT**
  – Sensitivity & specificity if repeated *(cattle only)*
    • false negative: udder infection needed, large tank bulk samples, non-milking animals
    • false positive: colostrum, mastitis, dried-off cows

• **Milk iELISA**
  – Good sensitivity & specificity
Serological tests – old tools (serum)

- All tests
  - Sensitive to antibodies induced by all S-Brucella species and biovars (abortus, melitensis and suis)

- RBT
  - Early detection
  - Lacks sensitivity (in sheep particularly)
  - Lacks specificity (in low prevalence or free areas)
  - Sensitive to vaccine-induced antibodies

- CFT
  - Later but prolonged detection
  - Lacks sensitivity (in recently infected animals)
  - Lacks specificity (but less than RBT)
  - Sensitive (less) to vaccine-induced antibodies
Serological old tools - *How to minimise failings?*

- Modification of RBT (75/25 vs. 25/25) **大大提高** the sensitivity
- Use of complementary tools
  - NH-GDT, less sensitive but more specific of the infection (sub-cutaneously vaccinated flocks)
  - AST, in unvaccinated flocks
  - Culture/PCR in vaccinated flocks in low prevalence or free areas
- Use of epidemiology-based strategy of:
  - performing tests: frequency
  - interpreting tests results (in parallel vs. in series)
Serological old tools - Despite these failings?

• In infected flocks/areas
  - The predictive value of positive results in either test is close to 100%
  - RBT has a very high flock sensitivity
  - The use of both tests in parallel greatly the individual sensitivity
  - Antibodies due to vaccination avoided by the use of the conjunctival route in replacement animals

• In low prevalence or free areas
  FPSR (Y. ent. O:9) could be identified by:
  - The very low proportion of positive results per flock/herd
  - The low levels and duration of antibodies
  - The use of the brucellin skin test

Eradication in cattle reached in many countries
Eradication in S. & G. reached in France (2003), Cyprus and Northern Italy & Spain
Serological tests – « new » tools

- **Protein-iELISA:** very low sensitivity and specificity
- **S-LPS iELISA:** \( \text{sens.}> RBT \& CFT, \text{but spe.} < RBT/CFT \)
  - Standardised in cattle and in S&G
  - And highly sensitive to vaccine induced antibodies
  - Could be used in pools of 10 sera in cattle
  - No validation at large scale in field conditions in S&G
  - Approved in cattle in bulk serum or milk samples
  - Promising as replacing RB

- **C-ELISA:**
  - low sensitivity and specificity in cattle
  - In sheep & goats? First results disappointing

- **Fluorescence polarisation Assay:**
  - OIE & EU official test in cattle (very sensitive but expensive)
  - In sheep and goats?

Pigs ?????? Associations of tests needed for increasing sensitivity and/or specificity
INFECTED UNIT (herd / flock / area)

- Infected/Not infected: I/NI
- Shedding/Not shedding: S/NS
- Test Positive/Negative: P/N

Relative rates of each category depends on:
- Outbreak history
- Control measures

Control means:
- To protect naïve animals (vaccination)
- To identify and eradicate infection more rapidly than it spreads
Control, surveillance & Eradication of animal Brucellosis…

Diagnosis is a critical key…

- Appropriate standardised and controlled biologicals (OIE)
- Appropriate performance (SOPs, ISO 17025)
- Tests associations (series or parallel)
  - to increase the result predictive values
- Test result interpretation…always in relation with:
  - risk-factors
  - status of the herd, the area, the country
Conclusion

New tools needed but…. 
….epidemiology-based strategy essential for sound testing regime design & result interpretation.

« In some cases, it would be more profitable to make better use of existing procedures than to continue to develop new ones."

Merci de votre attention

Dankie vir jou aandag

Thank you