Microbiological diagnosis of *Brucella* spp.

and Austrian epidemiology of brucellosis (*B. suis* biovar 2)

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Workshop “Dangerous Pathogens” and Leptospirosis, 29 May 2009
1. Epidemiology of *B. suis* biovar 2 (Hofer)

2. Conventional methods of diagnosis (Hofer)

3. Molecular methods of diagnosis (Revilla-Fernández)
Brucella species found in Austria

*Brucella melitensis* (imported cases in humans)

*Brucella abortus* (1986 last cases in cattle)

*Brucella ovis* (sheep in Western Austria)

*Brucella canis* (imported case in a dog)
*Brucella species found in Austria*

**Brucella suis** Biovar 2
widespread in Austria in hares and wild boars
(endoemic in 5 federal countries)

**Brucella microti**
New described species in
common vole (2000, Czech Republic)
red fox (2007, Lower Austria)

**Brucella sp.**
red fox (2009), new species, not yet officially described
Reservoir of *Brucella suis* biovar 2

*Lepus europaeus*  
European brown hare  
reservoir of *Brucella* and *Francisella*

*Sus scrofa*  
Wild boar  
Increasing number of wild boars can cause increasing outbreaks of brucellosis in domestic swine
Brucella suis biovar 2 infection in hares („Hare brucellosis“)

Chronic infection (Bodily condition may be surprisingly unaffected)

Formation of nodules, varying in size from that of a millet seed to a cherry or even larger (these often become purulent) particularly in testicle, uterus, liver and spleen

Sporadically found in Lower and Upper Austria, Salzburg, Styria and Burgenland

Photograph: T. Steineck, FIWI-VUW
Brucella suis biovar 2 infection in wild boars

Isolation from 2 wild boars:
In district Güssing 2007/08 from lymph node respectively both prostate gland and seminal vesicle

Conclusion:
Wild boars may excrete brucella are a reservoir of B. suis biovar 2 in Austria!

Serological detection in 4 wild boars: 2004/06 (by FIWI-VUW and AGES) in the districts Waidhofen/Thaya and Schärding, in both districts outbreaks occured in domestic swine 2003/2004!
Endemic regions can be detected serologically by investigation of blood from red foxes (become infected preying on hares)

In activated endemic regions of tularemia 1997/98 brucellosis could be diagnosed by culture in 2 hares and by serological investigation in 1 hare and 12 foxes

Natural foci of brucellosis seem to be congruous with foci of tularemia

Serological investigation 1997/98
Brucella suis biovar 2 can be isolated from red foxes in endemic regions. "Brucella suis" biovar 2 can be isolated from mandibular lymph nodes (arrow) with no visible lesions (latent infection).

Besides testing for rabies, additional screening of red foxes for the presence of Brucella should be conducted in endemic regions.
Recently described *Brucella* species that has originally been isolated from diseased common voles (*Microtus arvalis*) in South Moravia, Czech Republic in 2000.

Our findings demonstrate that *B. microti* is prevalent in a larger geographic area covering the region of South Moravia and parts of Lower Austria. Foxes could have become infected by ingestion of infected common voles.

Recent isolation from two foxes in Lower Austria
Lymph nodes with no visible lesions
Species not yet officially described, cooperation with Dr. Holger Scholz, Munich (Bundeswehr Institute)

Finding in two foxes indicates a natural focus!

Host and reservoir of this new species unknown!

Infection of foxes followed preying on mice, hares, wild boars, another wildlife species?
Latent brucellosis infection of foxes can indicate active natural foci

Brucella suis biovar 2 isolations from red foxes

Summer 07
1 out of 54 foxes from the district of Neusiedl/See

Autumn/winter 07/08
1 out of 13 foxes from the district Oberpullendorf

Spring/summer 08
1 out of 5 foxes from the district Deutschlandsberg
Brucella suis biovar 2 outbreaks in domestic pig (swine brucellosis)

Lower Austria 2003
Outbreak in several farms in the district Waidhofen/Thaya (notifiable disease)
20 – 50 % of the pregnant sows showed abortion

Upper Austria 2004
Outbreak in 1 farm in the district Schärding

Methods of transmission
Outdoor rearing and gutting wild boars or brown hares
To date, *B. suis* Biovar 2 has rarely been reported as the cause of human brucellosis (low virulence).

Infection has been reported in two **immuno-compromised hunters**, who had been extensively exposed through gutting or skinning boars or hares in France.

**Is brucellosis due the biovar 2 of *Brucella suis* an emerging zoonosis in France?**

Two case reports in wild boar and hare hunters.


Detection of *Brucella suis* by culture

**Agar medium**
Columbia agar base
Addition of 10% sheep blood
Oxoid supplement
Colonies usually visible after 2 or 3 days
(Most *Brucella* show a slow growth)

**Identification of *Brucella* culture**
Slide agglutination test with monospecific anti-*Brucella*-serum
*B. suis* identified by agglutination with anti-*Brucella* (A) serum

Infected placenta of a domestic swine stained with a modified Ziehl-Neelsen method (Red stained *Brucella* intra- and extracellular)
Biochemical identification of *Brucella suis* biovar 2

**Production of Urease**  
*B. suis* shows a very strong reaction (positive in a few minutes)

**Effect of dyes on growth**  
*B. suis* is Thionin - resistant but basic Fuchsin - sensitive

**Production of Sulphuretted hydrogen**  
*B. suis biovar 2* shows no reaction (lead acetate paper remains white)
**B. microti** (newly described species)

- Weak Urease reaction
- Fast growing *Brucella* species
- Usually agglutination with anti-*Brucella* (M) serum
- Non-motile and coccoid rod-shaped
- Biochemically identified as *Ochrobactrum* by using the API 20NE test

1 week old culture of *B. microti*
Molecular methods for the diagnosis and differentiation of *Brucella* sp.

Sandra Revilla-Fernández  
Institute for Veterinary Disease Control, Mödling

Workshop “Dangerous Pathogens” and Leptospirosis, 29 May 2009
Brucella sp. Taxonomy

- 3 complete genomes sequenced
- >90% genes share 98-100% nucleotide homology
- 6 recognised Species: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae*
- 2 proposed Species: *B. ceti*, *B. pinnipedialis* (marine mammals)
- 1 new strain: *B. microti*

- genetic similarity with other plant and animal pathogens and symbiotics (Agrobacterium, Rhizobium, Bartonella, Ochrobactrum)
Diversity of molecular methods

- Detection: - classical PCR
  - real-time PCR
  - 16S rRNA Sequencing

- Species Differentiation:
  - Southern Blot
  - PCR + RFLP
  - SNP analysis
  - Pulse-field gel electrophoresis
  - Multiplex-PCR: Brucella-Ladder, B.suis-Ladder
  - AMOS-PCR

- Strain Differentiation: MLVA and VNTR-Typing (most discriminative!)

“There is not single test by which a bacterium can be identified as Brucella. A combination of growth characteristics, serological, bacteriological an/or molecular methods is usually needed“ (OIE, chapter 2.4.3)
## Applied methods

<table>
<thead>
<tr>
<th></th>
<th>Target gene</th>
<th>Specificity</th>
<th>Publication</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Brucella</em> Real-time PCR</td>
<td><em>Brucella</em> sp.</td>
<td>Bogdanovics et al., 2004</td>
</tr>
<tr>
<td>2</td>
<td><em>Brucella</em>-Ladder multiplex-PCR</td>
<td><em>Brucella</em> sp. and farm animal vaccines</td>
<td>García-Yoldi et al., 2006 OIE Manual</td>
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<tr>
<td>3</td>
<td><em>Brucella suis</em>-Ladder multiplex-PCR</td>
<td><em>Brucella suis</em> biovars 1 to 5</td>
<td>García-Yoldi et al., 2007</td>
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<td>4</td>
<td><em>Brucella</em> PCR (B4/B5)</td>
<td><em>Brucella</em> sp. (also with hybrid. probes)</td>
<td>Baily et al., 1992 Al-Dahouk et al., 2007</td>
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<td>5</td>
<td><em>Brucella canis/suis</em> differentiation</td>
<td><em>B. suis</em> 1,3,4,5 and <em>B. neotomae</em> not differentiated</td>
<td>Vizcaíno et al., 1997</td>
</tr>
<tr>
<td>6</td>
<td><em>B. microti</em> PCR</td>
<td><em>B. microti</em></td>
<td>Scholz et al., 2008</td>
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<tr>
<td>7</td>
<td>Sequencing</td>
<td><em>Brucella</em> sp./ other Species</td>
<td>Harris and Hartley, 2003</td>
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<tr>
<td>8</td>
<td>LC real-time PCRs</td>
<td>IS711</td>
<td>Redkar et al., 2001</td>
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<td>9</td>
<td><em>Brucella</em> MLVA typing (Dr. Scholz, Munich)</td>
<td>16-MLVA Markers 1 panel (8): Sp. identification 2 panel (8): high discriminative</td>
<td>Le Flèche et al., 2006 Al-Dahouk et al., 2007</td>
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</table>
Laboratorial procedure (1)

- Organs, placenta, swab
  - positive culture
  - DNA isolation
  - Brucella ladder PCR
    - Brucella sp. real-time PCR
      - negativ
      - fox, hare, pig

phenotypic profiling
**Brucella Ladder Multiplex PCR**

(García-Yoldi et al., 2006)

<table>
<thead>
<tr>
<th>Gene</th>
<th>B.a.</th>
<th>B.m.</th>
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<th>B.c.</th>
<th>B19v</th>
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<td>omp31 (1071bp)</td>
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<td>rpsL (218bp)</td>
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<td>CRP (152bp)</td>
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Gene not included in the principal panel, published in OIE Manual.
Laboratorial procedure (2)

- DNA isolation
- positive culture
- Brucella sp. real-time PCR
  - Brucella ladder PCR
    - B. abortus
    - B. melitensis
    - B. ovis
    - vaccine strains
  - B. suis
    - B. suis ladder PCR
      - B. suis biovar ?
      - B. suis 1, 2, 3, 4, 5
  - negativ
  - B. suis

- fox, hare, pig
- Organs, placenta, swab
- phenotypic profiling

Vaccine strains of Brucella include:
- B. abortus
- B. melitensis
- B. ovis
Diagnostic of *Brucella melitensis* / *B. suis* 2

-*B. melitensis*: 5 human imported cases:

-*B. suis* biovar 2: wild boar, hares and red foxes:

Brucella- real-time PCR

![Real-time PCR graph](image1)

Brucella- Ladder PCR

![Ladder PCR gel](image2)

Brucella suis- PCR

![PCR gel](image3)
Diagnostic of *Brucella microti* in red foxes

- all them identified as *Brucella suis* sp.
- By typing of *Brucella suis* it could not be identified as any of the known *B. suis* biovars
- 16S rRNA Sequencing of *B. microti* reveals a *Brucella* sp.
New Brucella sp. (2 red foxes, October 08)

- Brucella real-time PCR: positiv
- Brucella Ladder PCR: atypical DNA pattern!

16S rRNA sequencing: *B. abortus/ Ochrobacterum* sp. (97% homology)

- but *Ochrobacterum* sp. is not detected by the Multiplex PCR!
- MLVA in process (Scholz, Bundeswehr Munich)
Laboratorial procedure (4)

- positive culture
  - DNA isolation
  - Brucella ladder PCR
    - B. abortus
    - B. melitensis
    - B. ovis (vaccine strains)
  - Brucella sp. real-time PCR
    - B. suis
    - B. suis ladder PCR
    - B. suis biovar ?
    - B. suis 1, 2, 3, 4, 5
  - new Brucella sp.?
  - B. microti PCR
    - B. microti
    - classification/ MLVA typing

- negativ
  - Ochrobactrum PCR
    - and/or
    - 16S rRNA sequencing
  - Bacterial Species determination

fox, hare, pig
Organs, placenta, swab
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