Microbiological diagnosis of *Brucella* spp.

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

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Institute for Veterinary Disease Control, Mödling

Workshop “Dangerous Pathogens” and Leptospirosis, 29 May 2009
Brucellosis

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
(endemic 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.
*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.

1 week old culture of *B. suis* biovar 2
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
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)

Chromosomes I and II of B. suis strain 1330 (3.31 Mb)
Paulsen et.al., PNAS, 2002
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>
</tr>
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<tbody>
<tr>
<td>1</td>
<td><em>Brucella</em> Real-time PCR</td>
<td><em>per gene</em></td>
<td><em>Brucella</em> sp.</td>
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<tr>
<td>2</td>
<td><em>Brucella</em>-Ladder multiplex-PCR</td>
<td>6 different polymorphisms</td>
<td><em>Brucella</em> sp. and farm animal vaccines</td>
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<td>3</td>
<td><em>Brucella suis</em>-Ladder multiplex-PCR</td>
<td>MLVA of 6 VNTR markers</td>
<td><em>Brucella suis</em> biovars 1 to 5</td>
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<td>4</td>
<td><em>Brucella</em> PCR (B4/B5)</td>
<td>bcsp31 (31 KDa)</td>
<td><em>Brucella</em> sp. (also with hybrid. probes)</td>
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<td>5</td>
<td><em>Brucella</em> canis/suis differentiation</td>
<td>Omp31 PCR+RFLP</td>
<td><em>B. suis</em> 1,3,4,5 and <em>B. neotomae</em> not differentiated</td>
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<tr>
<td>6</td>
<td><em>B. microti</em> PCR</td>
<td>specific genomic island</td>
<td><em>B. microti</em></td>
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<tr>
<td>7</td>
<td>Sequencing</td>
<td>16S rRNA</td>
<td><em>Brucella</em> sp./ other Species</td>
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<tr>
<td>8</td>
<td>LC real-time PCRs</td>
<td>IS711</td>
<td>Exclusive for <em>B. abortus, B. melitensis, B. suis</em>1</td>
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<tr>
<td>9</td>
<td><em>Brucella</em> MLVA typing (Dr. Scholz, Munich)</td>
<td>16-MLVA Markers</td>
<td>1 panel (8): Sp. identification 2 panel (8): high discriminative</td>
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Laboratorial procedure (1)

fox, hare, pig

Organs, placenta, swab

phenotypic profiling

positive culture

DNA isolation

Brucella ladder PCR

Brucella sp. real-time PCR

negativ

Brucella Ladder Multiplex PCR  
(García-Yoldi et al., 2006)

### Brucella Ladder Multiplex PCR

(García-Yoldi et al., 2006)

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<th>Gene</th>
<th>B.a.</th>
<th>B.m.</th>
<th>B.s.</th>
<th>B.o.</th>
<th>B.c.</th>
<th>B19v</th>
<th>Rev1v</th>
<th>B.n</th>
<th>Vaccine</th>
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<td>wboA (1682bp)</td>
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<td>omp31 (1071bp)</td>
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<td>Pol A (794bp)</td>
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<td>eryC (587bp)</td>
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<td>CRP (152bp)</td>
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</table>

**B.mi.**

——— gene not included in the principal panel, published in OIE Manual

Laboratorial procedure (2)

Organs, placenta, swab

DNA isolation

positive culture

phenotypic profiling

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

Brucella sp. real-time PCR

negativ

fox, hare, pig
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**

**Brucella- Ladder PCR**

**Brucella suis- PCR**
Diagnostic of *Brucella microti* in red foxes

- All of 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.

**Brucella- Ladder PCR**

**Brucella suis- PCR**

**Brucella microti- PCR**

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**AGES**

Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

New Brucella sp. (2 red foxes, October 08)

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

- 16S rRNA sequencing: *B. abortus* / *Ochrobactrum* sp. (97% homology)
- but *Ochrobactrum* sp. is not detected by the Multiplex PCR!
- MLVA in process (Scholz, Bundeswehr Munich)
Laboratorial procedure (4)

- Fox, hare, pig
  - Organs, placenta, swab
    - DNA isolation
      - Phenotypic profiling
      - Positive culture

- Brucella ladder PCR
  - Babortus
  - Bmelitensis
  - Bovis
  - 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

- Ochrobactrum PCR and/or
  - 16S rRNA sequencing

- Negativ

- Bacterial Species determination
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