Microbiological diagnosis of *Francisella tularensis*

and Austrian epidemiology of tularemia

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Institute for Veterinary Disease Control, Mödling

Workshop “Dangerous Pathogens” and Leptospirosis, 29 May 2009
1. Epidemiology (Hofer)

2. Conventional methods of diagnosis (Hofer)

3. Molecular methods of diagnosis (Revilla-Fernández)
History of tularemia in Austria

1935 First diagnosis in a human in Lower Austria (infection by skinning a hare)

1936/37 and 1945/46 2 epidemics, each with about 200 notified cases (most human cases were caused by contact with hares)

1959/60 More than 700 verified cases of pneumonia in a sugar factory in the district of Bruck/Leitha (inhalation of aerosol by washing contaminated beets when mice were observed in masses after very dry weather conditions in autumn)
Human cases from 1950 - 1997

Decrease of the number of human cases in the last decades
In activated natural foci an increase of the number of **hare** and **human** tularemia cases can be recorded (1994/95, 1997/98)

In **epidemic years** diagnosed human cases can reach the number of 26 (1994)

In **interepidemic years** diagnosed human cases mostly amount less than 5 (1999 – 2008)

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</thead>
<tbody>
<tr>
<td>Hare</td>
<td>20</td>
<td>18</td>
<td>5</td>
<td>33</td>
<td>37</td>
<td>27</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>Human</td>
<td>26</td>
<td>16</td>
<td>9</td>
<td>16</td>
<td>19</td>
<td>2</td>
<td>5</td>
<td>1</td>
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</table>
Epidemic years

Cyclical increase of the common vole *Microtus arvalis* (dry weather conditions)

Outbreak in brown hares in autumn (widespread deaths)

Frequent infection of humans during the hunting season (skinning of hares) with increasing notified cases of tularemia in late autumn
Most **human cases (red)** were diagnosed from Oct. until Feb., when many septic cases in hares were observed. An increasing number of **hare cases (black)** was also recorded from Feb. until May.
Where is the main reservoir of *Francisella tularensis* in Austria?

*Lepus europaeus*
- European hare
- Brown hare
- Field hare

- Infection carrier
- Source of human infection
- Latent or chronic (interepidemic)
- Acute septicemia (epidemic years)
How healthy are „healthy“ hares?

1 week old culture
Inoculation with a section plane of the organs

Latent infection
Culture of kidney, spleen, liver from hares without macroscopic pathological alterations

Results
Single colony of F. tularensis from the organs of 2 out of 98 hares

Hunting bag examination Nov. 2007
TULMON Study (VUW-FIWI, BMLV, AGES)
Chronically diseased hares excrete *Francisella* via urine!

**Chronic tularemia**
- Necrotic lesions in kidney and lung
- Enlarged spleen

**Chronic infection**

40 hares (apparently healthy) shot in December 2008 were examined. 10 hares showed characteristic pathologic findings. Culture of 5 hares yielded *Francisella* (small quantity of colonies, agar plate must be inoculated with the section plane of the organs).

Hunting bag examination Dec. 2008
TULMON Study
(VUW-FIWI, BMLV, AGES)
Epidemic outbreaks in hares („hare pest“)

Acute tularemia
Enlarged spleen

Septic diseased hares
often show an enlarged spleen exclusively!

Chronical pathologic alterations
can be found additionally in outbreaks

Epicarditis, pneumonia, nephritis
(purulent - necrotic) being most frequent and necrotising orchitis
(not reported in hares before 1999)

Photograph: T. Steineck, FIWI-VUUW
Acute tularemia - Enlarged spleen

Photograph: T. Steineck, FIWI-VUW
Tularemia - Necrotising orchitis

Photograph: T. Steineck, FIWI-VUW
Tularemia outbreak in hares 1994/95

From Oct. 1994 until Dec. 1996 62 cases in hares from 13 districts in 3 federal countries were diagnosed.

Most cases (hare and human) were observed 1994 in the district of Mistelbach.

Endemic region is mainly the North-eastern part of Austria adjoining endemic regions of Czech Republic, Slovakia, Hungary.
Tularemia outbreak in hares 1997/98

1997 42 cases in hares from **10 districts** were diagnosed

1998 45 cases in hares from **16 districts** were diagnosed

Remarkable activation of the natural foci in the south of Burgenland and the North of Lower Austria 1998
Red foxes prefer preying on brown hares

Latent tularemia infection in red foxes (Vulpes vulpes)

Serological investigation 1997/98

Activated natural foci of tularemia can be detected serologically by investigation of blood from red foxes

It is remarkable, that in the same area foxes also showed antibodies against Brucella (Hares are also a very important reservoir for Brucella suis biovar 2)
Francisella tularensis can be isolated from red foxes!

In activated endemic regions, Francisella tularensis 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 Francisella should be conducted in endemic regions in Austria.
Latent tularemia infection of foxes indicates natural foci

Francisella isolation from red foxes

Autumn/winter 07/08
4 out of 51 foxes from the district Waidhofen./Thaya

Spring/summer 2008
1 out of 11 foxes from the district Mistelbach

June 07 - March 09
10 out of 494 foxes from 4 districts of Burgenland
(only 5 cases are shown in the graph)
Subspecies *tularensis* in Austria!

New serious epidemiological situation in Austria!

1990 **Subsp. tularensis** (high virulence for humans!) was isolated from an *Ixodes ricinus* tick nearby Graz (Styria) in a laboratory in Bratislava.

Gurycova D.: „Characterisation and Classification of different strains of *Francisella tularensis* isolated in Central Europe.“

Schu S4 laboratory strain is the most likely source of the European isolates of F. tularensis subsp. tularensis and indicate that anthropogenic activities, such as movement of strains or animal vectors, account for the presence of these isolates in Europe.

Given the highly pathogenic nature of this subspecies, the possibility that it has become established wild in the heartland of Europe carries significant public health implications.


Antimicrobial susceptibilities of Austrian *Francisella tularensis holarctica* biovar II strains. Tomaso H, Al Dahouk S, Hofer E, Splettstoesser WD, Treu TM, Dierich MP, Neubauer H.

50 strains from hares and human patients were examined
24 antimicrobial agents were determined using Etests
All isolates were sensitive to *tetracyclines*, *aminoglycosides*, *quinolones*, chloramphenicol and rifampicin
Resistance was observed in all isolates against *erythromycin*, *penicillins* and aztreonam

Data can be applied for the detection and comparison of resistance developement and for the guidance of therapy
Culture of *Francisella tularensis*

4 – 5 days old culture acute-septic tularemia characteristically dark agar medium around the colonies

Cystine Heart – Agar
Sheep blood 10 %
Ampicillin 100 µg/ml
Polymyxin B 100 µg/ml

Confluent growth after 2 - 3 days incubation in septic cases *(inoculate with a loop)*

Single colonies after 4 – 5 days in chronic cases with small quantity of bacteria *(inoculate with a section plane)*
Identification of *Francisella tularensis*

**Extremely small!**
Usually not visible in a Gram stain, visible in a Giemsa or IF stain in organs with a high number of bacteria (septic diseased hares)

**Extremely coccoid!**
Culture material in a light microscope (phase contrast) or electron microscope

**Rapid identification**
Slide agglutination test with culture material (specific antiserum)
Characterisation of *Francisella tularensis*

Subsp. *holarctica* biovar I and II utilize Glucose but not Glycerin

With agardiffusion test (15 µg Ery) only

Subsp. *holarctica* biovar II shows resistance (no inhib. zone)

Only phenotype found in hares and humans in Austria till now
Molecular methods for the diagnosis and differentiation of *Francisella tularensis*

Sandra Revilla-Fernández
Institute for Veterinary Disease Control, Mödling

Workshop “Dangerous Pathogens” and Leptospirosis, 29 May 2009
Francisella tularensis - Taxonomy

• 7 complete genomes sequenced (approx. 1,89 Mb)

• 4 recognised Subspecies:
  - F. tularensis subsp. tularensis
  - F. tularensis subsp. holarctica
  - F. tularensis subsp. mediasiatica
  - F. tularensis subsp. novicida

• close genetic relationship despite marked variation in virulence in mammals

• F. tul. tul. has greater diversity than F. tul. holar. despite a less broader geographical prevalence

Francisella Genome Research: http://www.francisella.org/
Diversity of molecular methods

- Detection methods:
  - classical PCR
  - real-time PCR
  - PCR + RFLP
  - 16S rRNA

- Relative resolution of DNA-based typing methods

"a combination of the specific PCR together with one method generating subspecies-specific patterns is suitable as a rapid and relatively simple strategy for discrimination of Francisella species and subspecies."


(Johansson et al., 2004, APMIS 112)
## Applied methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Target gene</th>
<th>Specificity</th>
<th>Publication</th>
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<tbody>
<tr>
<td>1</td>
<td><em>Francisella tularensis</em> LC Real-time PCR with hybridisation probes</td>
<td><em>tul4 (lpnA)</em> 17 kDa protein</td>
<td>conserved in <em>Francisella tularensis</em></td>
</tr>
<tr>
<td>2</td>
<td><em>Francisella tularensis</em> SYBR Green real-time PCR</td>
<td>Ft-M19</td>
<td>differentiation of <em>F. tularensis subsp. holarctica/ tularensis</em></td>
</tr>
<tr>
<td>3</td>
<td>DNA Sequencing</td>
<td><em>tul4</em> protein 16s rRNA</td>
<td>confirmation, not discriminative</td>
</tr>
<tr>
<td>4</td>
<td><em>Francisella</em> MLVA analysis (in process) cooperation AGES-MoD</td>
<td>sequencing of 6 VNTR Markers of <em>F. tularensis</em></td>
<td>Phylogenetical clustering, diversity, strain linkage</td>
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</table>

### Other typing methods used within international cooperation

<table>
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<th>Method</th>
<th>Target gene</th>
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<th>Publication</th>
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<tr>
<td>5</td>
<td><em>Francisella</em> MLVA analysis (in process) cooperation AGES-FLI Jena</td>
<td>sequencing of 6 VNTR Markers of <em>F. tularensis</em></td>
<td>Phylogenetical clustering, diversity, strain linkage</td>
</tr>
<tr>
<td>6</td>
<td>DNA sequencing cooperation AGES-Bundeswehr Munich</td>
<td>5 housekeeping genes 2 protein genes</td>
<td>Phylogenetical clustering for <em>tularensis/holarctica</em></td>
</tr>
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</table>

DNA-typing methods allow safe strain characterisation from killed bacterial preparations!!
Laboratorial Procedure

human, fox, hare tissue, wound

phenotypic profiling

bacterial culture

DNA isolation

High Pure Template PCR Kit

Francisella tul4 real-time PCR

positiv

Differentiation Ft-M19 real-time PCR

F. tul. tularensis
F. tul. holarctica

MLVA-VNTR typing

negativ

16S rDNA sequencing

Bacterial Species determination

Gel Electrophoresis

sequencing

Ft- M19 real-time PCR

Francisella tul4 real-time PCR

DNA isolation

High Pure Template PCR Kit

F. tul. tularensis
F. tul. holarctica

MLVA-VNTR typing

negativ

16S rDNA sequencing

Bacterial Species determination

Gel Electrophoresis

sequencing

Francisella tul4 real-time PCR

negativ

16S rDNA sequencing

Bacterial Species determination

Gel Electrophoresis

sequencing

Francisella tul4 real-time PCR

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16S rDNA sequencing

Bacterial Species determination

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Bacterial Species determination

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sequencing

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negativ

16S rDNA sequencing

Bacterial Species determination

Gel Electrophoresis

sequencing
Francisella tularensis: detection

- Positive cases (2004-2008):
  - wild hares (organs, culture)
  - 2 human samples (lymph node, FFPE)
  - red foxes (culture)

- Results:
  - tul4 gene real-time PCR

- fox 2005
- fox 2007A
- fox 2007E
- Francisella tularensis +
- negative control
**F. tularensis subsp. holarctica:** Subspecies differentiation

Real-time PCR and melting curve analysis of Ft-M19 fragments:

The assay rapidly identifies the two main subspecies since a 30-bp sequence is deleted only in isolates of subspecies *holarctica*.

DNA-fragments of approximately 101-bp are amplified only from isolates of subspecies *holarctica*, whereas fragments of 131-bp are amplified only from isolates of subspecies *tularensis*. 
MLVA-VNTR analysis of Austrian isolates 1997 til 2009 (1)

- Cooperation AGES-MoD/ARWT (Plicka)

- 23 *F. tul. holar.* strains from hare, fox and human samples (limited geographical distribution)

- Project start: May 09

- MLVA-VNTR: Sequencing of 6 discriminative VNTR-loci of *F. tularensis* and phylogenetical analysis

- Future analysis of repeat units of the VNTR loci will be validated with GeneMapper Analysis software and ABI DNA Sequencer

(Johansson et al., 2004, APMIS 112)
Preliminary results:

• MLVA analysis of Ft-M3 locus of 23 Austrian isolates of F. tul. holarctica, which is the most discriminative, generated at least 3 different alleles.

• The little genetic diversity found in F. tul. holarctica may be in accordance with previous global analysis.

• Interspecies transmission between hare and fox is shown.

• The genetic relationship to strains from other Austrian regions remains under study.
MLVA-VNTR typing of Austrian isolates 2005 til 2009

- Cooperation AGES- Dr.Tomaso (FLI, Jena)

- 49 strains of *F. tularensis* subsp. *holarctica* sent in February 09 for MLVA-VNTR-Analysis (hare, fox, human samples) from a wider geographical distribution

- Preliminary analysis of Ft-M3 and Ft-M6 sequences reveals linkage of the Austrian strains (99-100% homology)

- Analysis still in process, not published
Acknowledgements:

We thank all laboratory staff and contributors from the Institute for Veterinary Disease Control in Mödling (K. Reisp), the Austrian Ministry for Health (A. Höflechner-Pöltl), Austrian Ministry of Defence (H. Plicka) and the VUW-FIWI (T. Steinek) for their excellent work and assistance.

Thank you very much for your attention.
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