AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

Microbiological diagnosis of *Francisella tularensis* and Austrian epidemiology of tularemia

Erwin Hofer
Institute for Veterinary Disease Control, Mödling

Workshop “Dangerous Pathogens” and Leptospirosis, 29 May 2009

Tularemia

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

1. **Epidemiology** (Hofer)

2. **Conventional methods** of diagnosis (Hofer)

3. **Molecular methods** of diagnosis (Revilla-Fernández)

History of tularemia in Austria

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

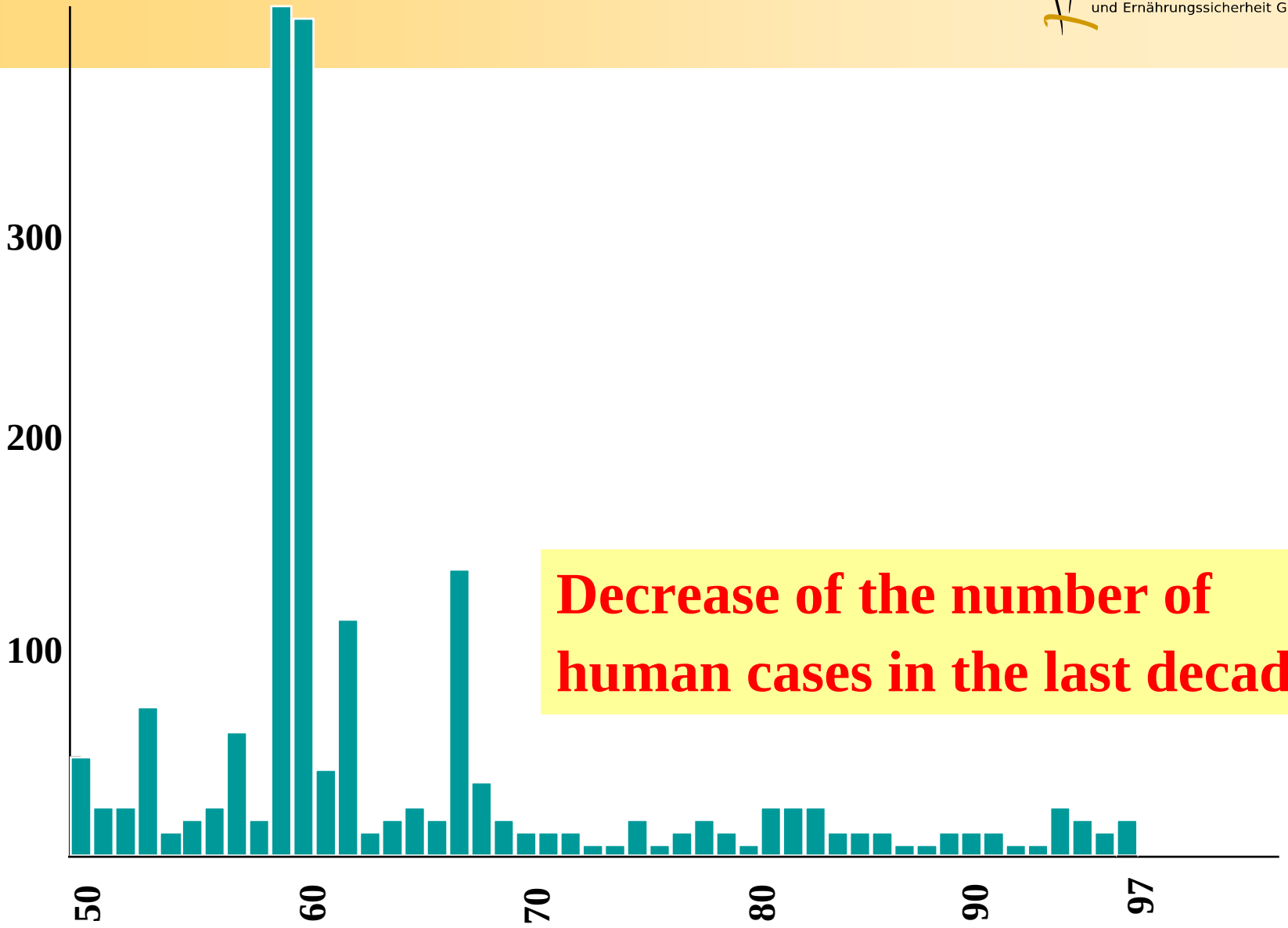
1935 First diagnosis in a human in Lower Austria (infection by skinning a hare)

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

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH



Decrease of the number of human cases in the last decades

Diagnosed tularemia cases 1994 - 2001

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

Cases	1994	1995	1996	1997	1998	1999	2000	2001
Hare	20	18	5	33	37	27	16	11
Human	26	16	9	16	19	2	5	1

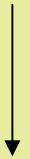
In activated natural foci an increase of the number of **hare** and **human** tularemia cases can be recorded (1994/95, 1997/98)

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)**

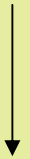
Epidemic years

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

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



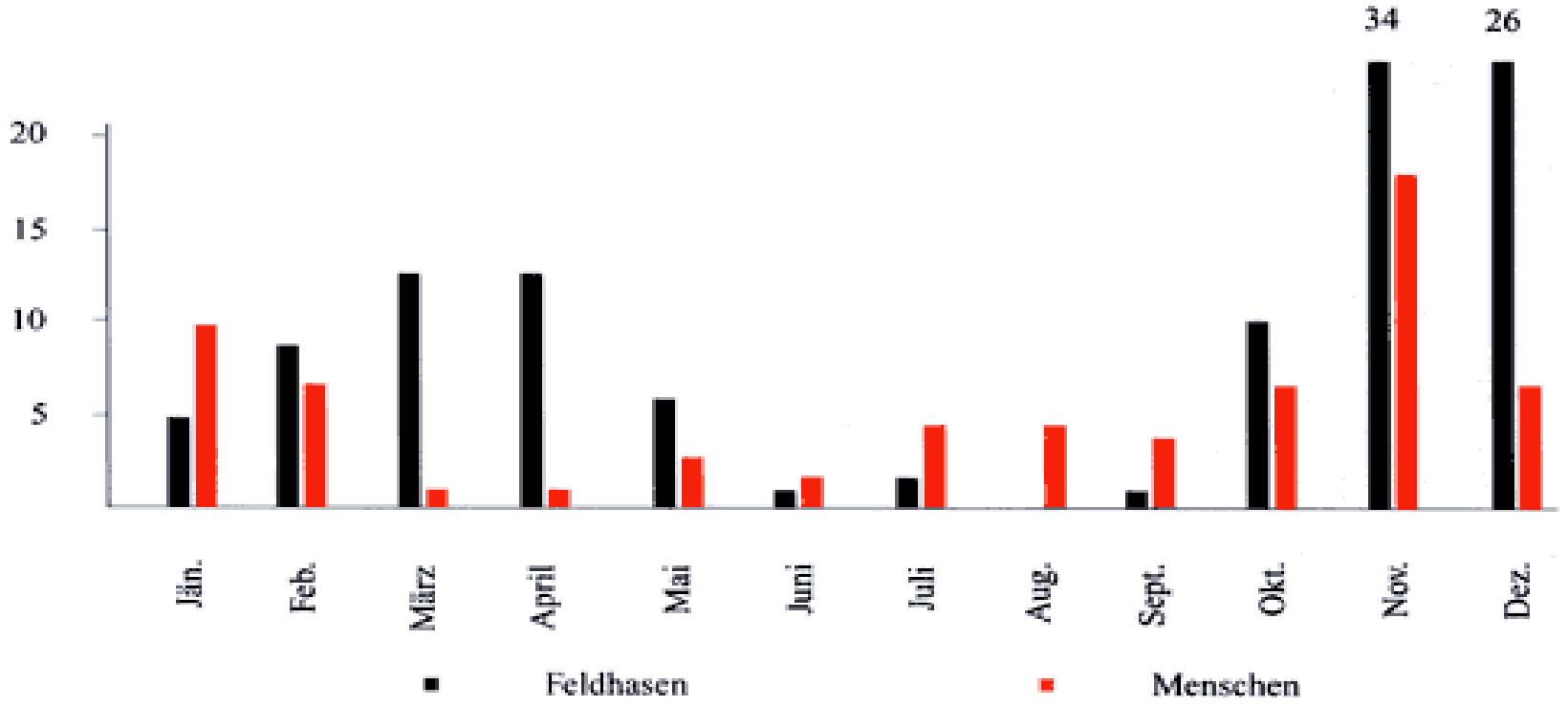
Outbreak in **brown hares** in autumn (widespread deaths)



Frequent infection of **humans** during the hunting season (skinning of hares) with increasing notified cases of tularemia in late autumn

Tularemia cases from Oct. 1994 until March 1998

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH



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 ?

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

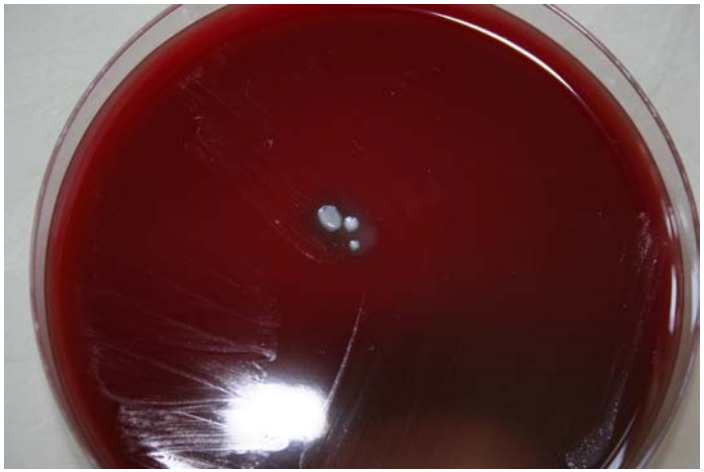
Lepus europaeus
European hare
Brown hare
Field hare

- **infection carrier**
- **source of human infection**
- **latent or chronic** (interepidemic)
- **acute septicemia** (epidemic years)

How healthy are „healthy“ hares ?

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

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

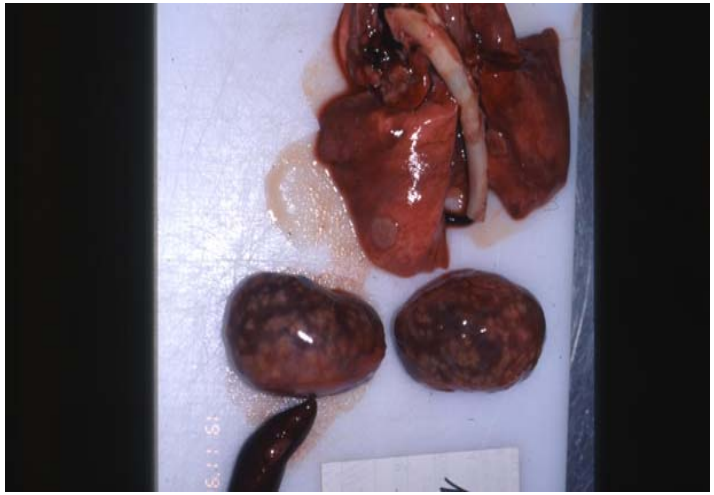
Hunting bag examination Nov. 2007
TULMON Study
(VUW-FIWI, BMLV, AGES)

Chronically diseased hares excrete *Francisella* via urine !

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

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“)

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

Acute tularemia
Enlarged spleen

Photograph: T. Steineck, FIWI-VUW

Septic diseased hares

often show an enlarged spleen exclusively !

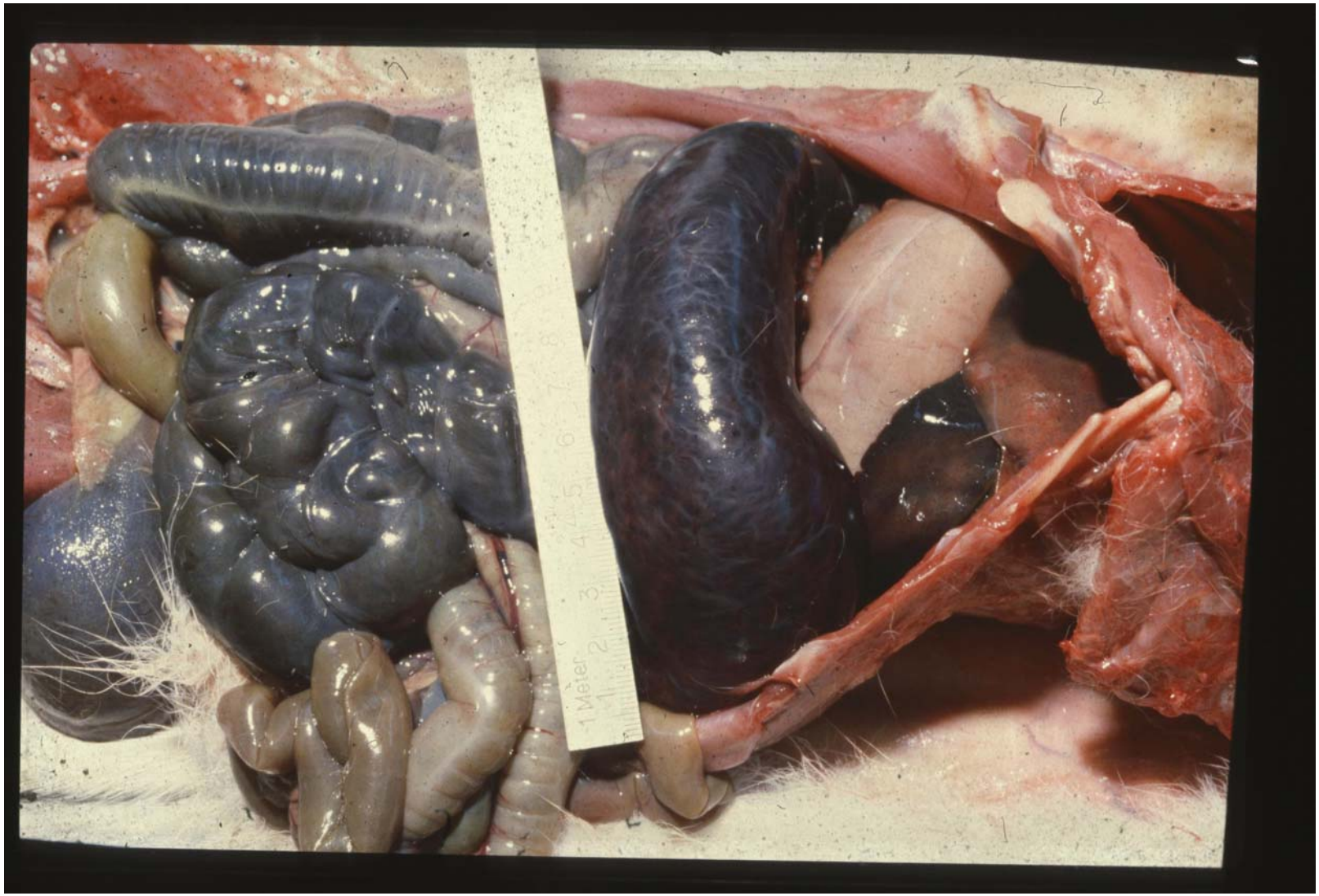
Chronical pathologic alterations
can be found additionally in outbreaks

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

Acute tularemia - Enlarged spleen

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

Photograph: T. Steineck, FIWI-VUW



Tularemia - Necrotising orchitis

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

Photograph: T. Steineck, FIWI-VUW

Tularemia outbreak in hares 1994/95

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

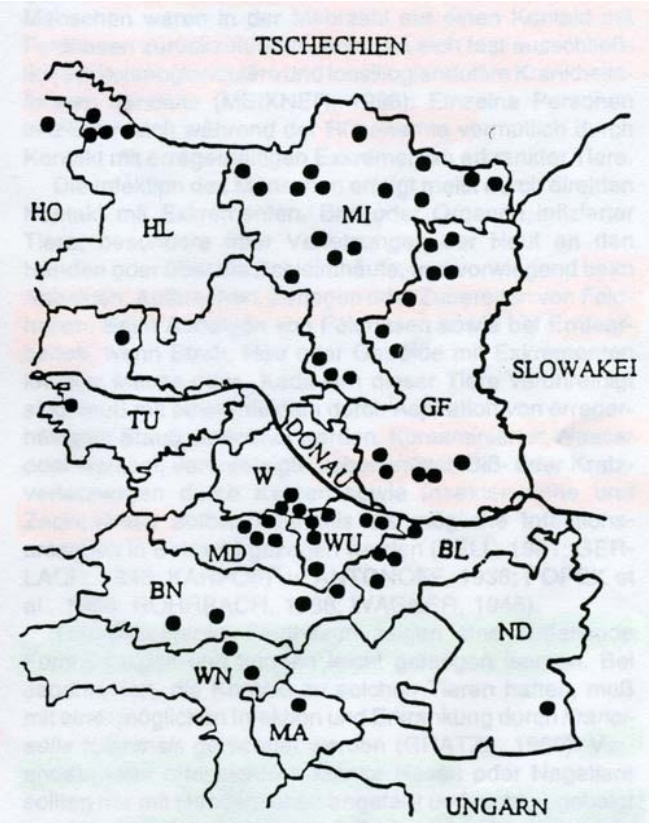


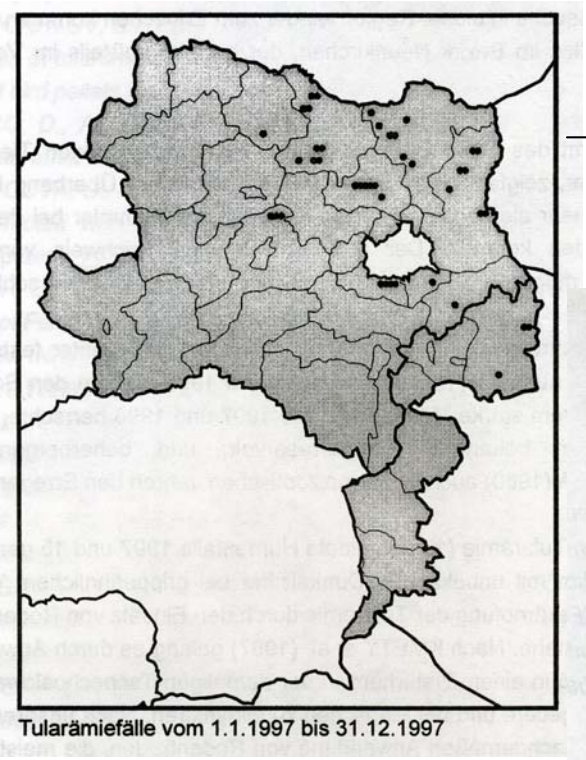
Abb. 5: Geographische Verbreitung der nachgewiesenen Tularämiefälle beim Feldhasen von Oktober 1994 bis Dezember 1996; W = Wien, WN = Wiener Neustadt, MA = Mattersburg, ND = Neusiedl am See, BN = Baden, BL = Bruck/Leitha, WU = Wien-Umgebung, MD = Mödling, TU = Tulln, GF = Gänserndorf, MI = Mistelbach, HL = Hollabrunn, HO = Horn

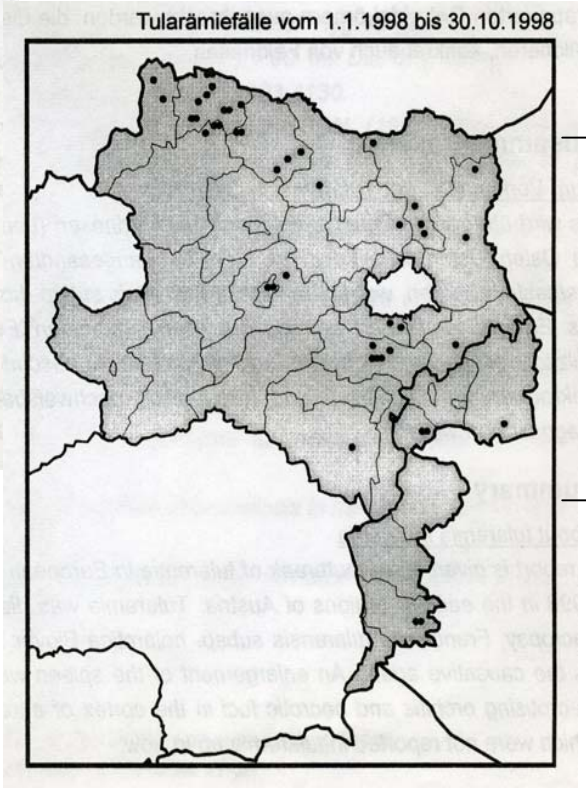
From Oct.1994 until Dec. 1996 **62 cases in hares** from 13 districts in 3 federal countries were diagnosed

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

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

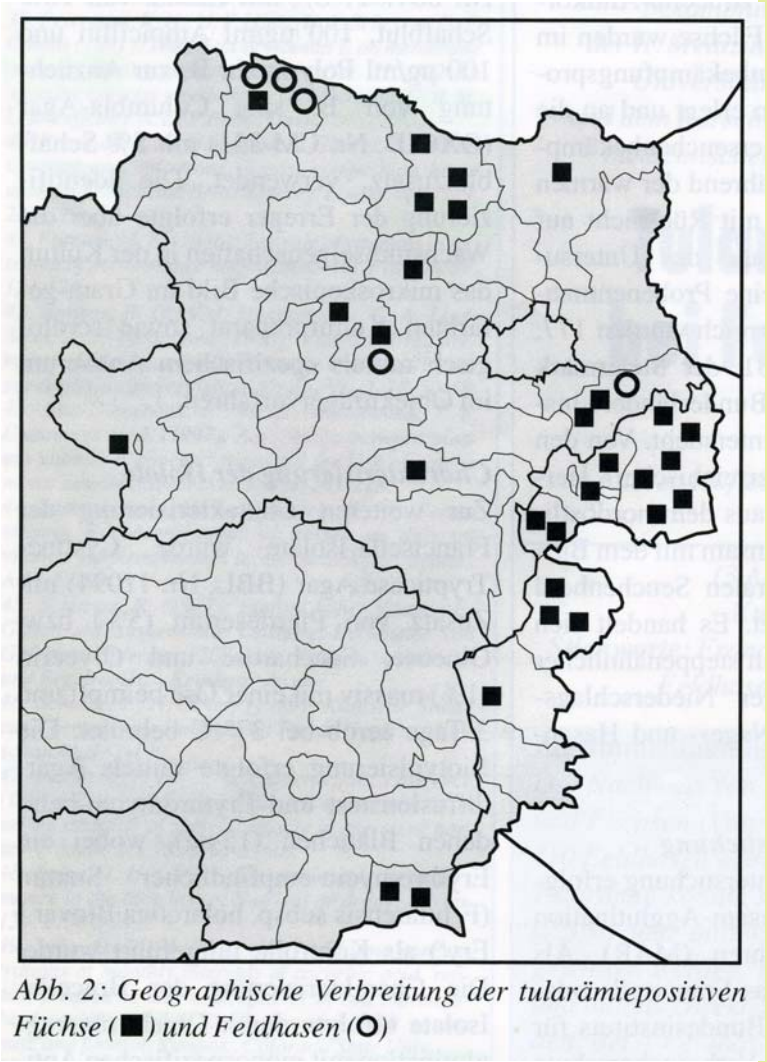


Abb. 2: Geographische Verbreitung der tularämiepositiven Füchse (■) und Feldhasen (O)

Latent tularemia infection in red foxes (Vulpes vulpes)

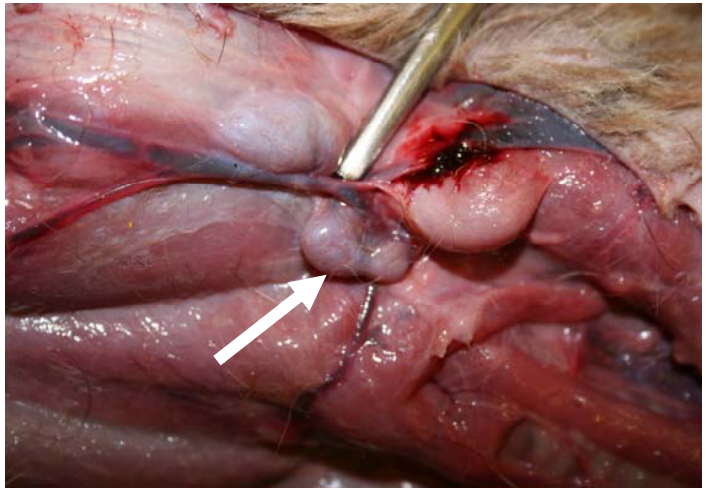
Serological investigation 1997/98
Tierärztl. Umschau 55, 264-268 (2000)

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 !

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

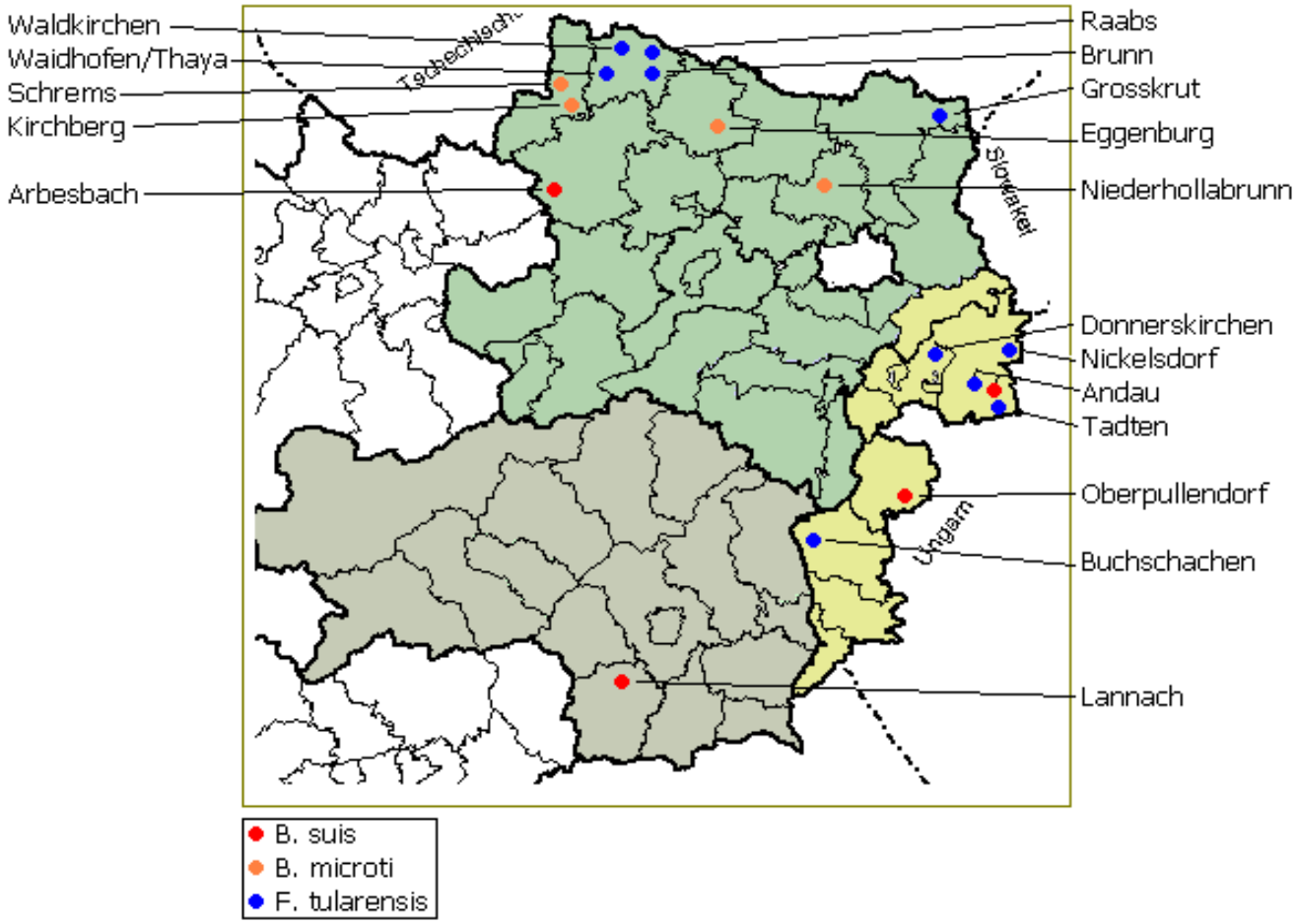


In **activated endemic regions** *Francisella tularensis* can be isolated from **mandibular lymph nodes** (arrow) with no visible lesions (latent infection)

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

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH



***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 !

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

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.“

Second International Conference on Tularemia,
Hradec Kralove, Czech Republic, Vojenske Zdravotnicke Listy – Supplementum, Rocnik LXVI, c. 1 (1997) 24.

Subspecies *tularensis* in Austria !

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

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.

Citation: Chaudhuri RR, Ren C, Desmond L, Vincent GA, Silman NJ, et al (2007) Genome Sequencing Shows that European Isolates of Francisella tularensis Subspecies tularensis Are Almost Identical to US Laboratory Strain Schu S4. PLoS ONE 2(4): e352. doi:10.1371/journal.pone.0000352

Antimicrobial susceptibilities

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

Int J Antimicrob Agents. 2005 Oct;26(4):279-84.
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*

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

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

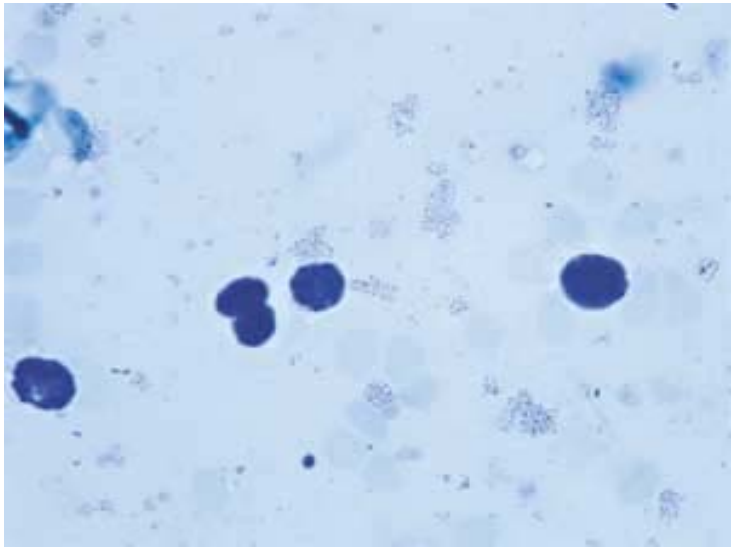
Cystine Heart – Agar
Sheep blood 10 %
Ampicillin 100 µg/ml
Polymyxin B 100 µg/ml

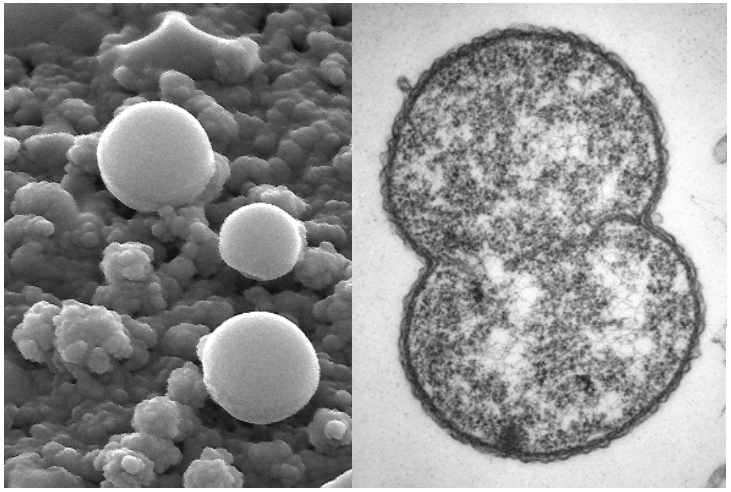
Confluent growth after 2 - 3 days incubation in septic cases (inoculate with a loop)

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

Identification of *Francisella tularensis*

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH





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!

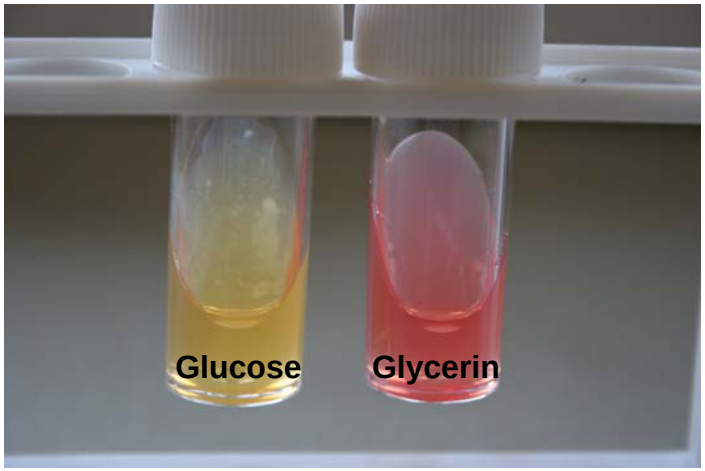
Culture material in a light microscope (phase contrast) or electron microscope

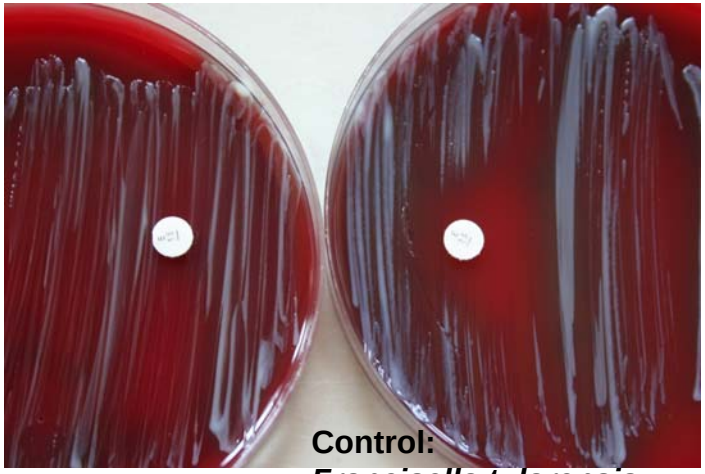
Rapid identification

slide agglutination test with culture material (specific antiserum)

Characterisation of *Francisella tularensis*

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH





Control:
Francisella tularensis **subsp. *holarctica* Biovar I**

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

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

Molecular methods for the diagnosis and differentiation of *Francisella tularensis*

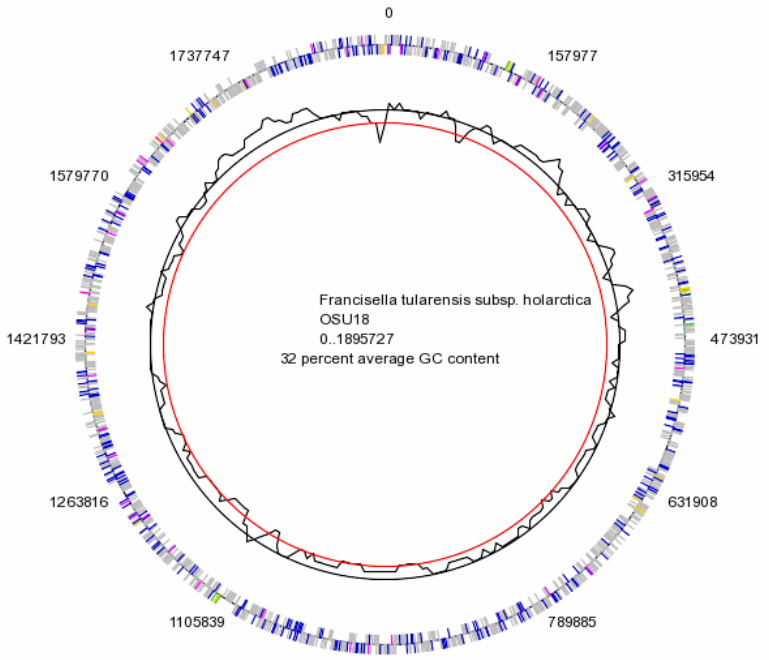
Sandra Revilla-Fernández
Institute for Veterinary Disease Control, Mödling

Workshop “Dangerous Pathogens” and Leptospirosis, 29 May 2009

Francisella tularensis- Taxonomy

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

- 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/

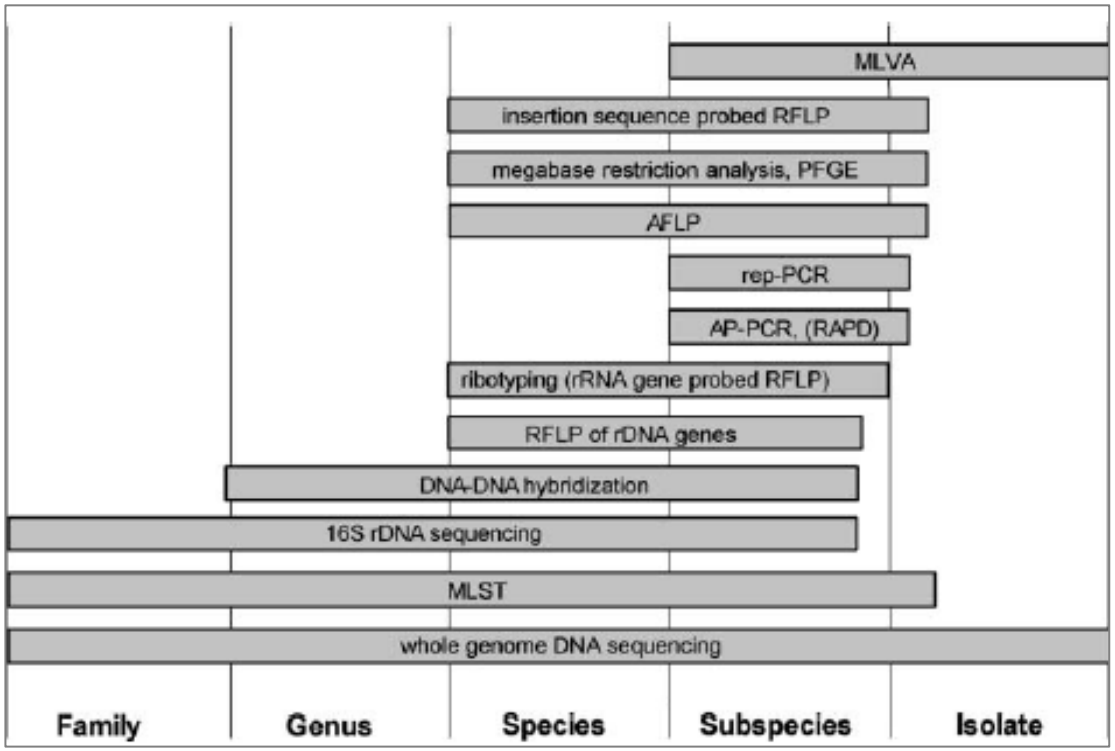
Diversity of molecular methods

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

- **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., 2000: J.Clin.Microbiol. 38)



(Johansson et al., 2004, APMIS 112)

Applied methods

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

		Target gene	Specificity	Publication
1	***Francisella tularensis*** **LC Real-time PCR with hybridisation probes**	***tul4 (IpnA)*** **17 kDa protein**	**conserved in** ***Francisella tularensis***	**In-house Oligos (Plicka, MoD/ARWT) not published**
2	***Francisella tularensis SYBR Green*** **real-time PCR**	**Ft-M19**	**differentation of** ***F. tularensis subsp. holarctica/ tularensis***	**In-house Oligos (Plicka, MoD/ARWT) not published**
3	**DNA Sequencing**	***tul4*** **protein 16s rRNA**	**confirmation, not discriminative**	
4	***Francisella*** **MLVA analysis (in process) cooperation AGES-MoD**	**sequencing of 6 VNTR Markers of** ***F. tularensis***	**Phylogenetical clustering, diversity, strain linkage**	**Johansson et al., 2004 Byström et al., 2005**
Other typing methods used within international cooperation				
5	***Francisella*** **MLVA analysis (in process) cooperation AGES- FLI Jena**	**sequencing of 6 VNTR Markers of** ***F. tularensis***	**Phylogenetical clustering, diversity, strain linkage**	**Johansson et al., 2004 Byström et al., 2005**
6	**DNA sequencing cooperation AGES-Bundeswehr Munich**	**5 housekeeping genes 2 protein genes**	**Phylogenetical clustering for** ***tularensis/holarctica***	**Nübel et al., 2006**

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

Laboratorial Procedure

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

human, fox, hare
tissue, wound

phenotypic profiling

bacterial culture

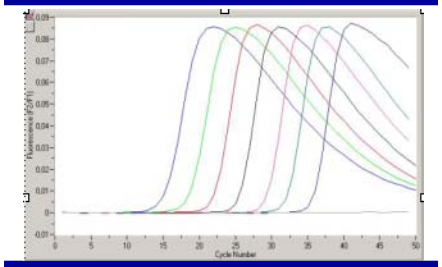
DNA isolation

High Pure Template PCR Kit

Francisella tul4 real-time PCR

positiv

negativ



Differentation Ft-M19 real-time PCR

F. tul. tularensis
F. tul. holarctica

Gel Electrophoresis

16S rDNA sequencing

sequencing

Bacterial Species determination

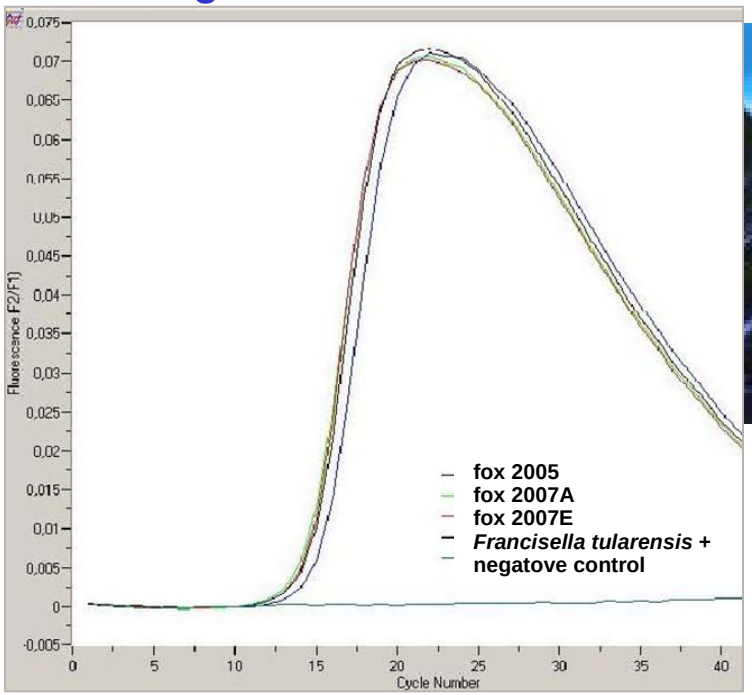
MLVA-VNTR typing

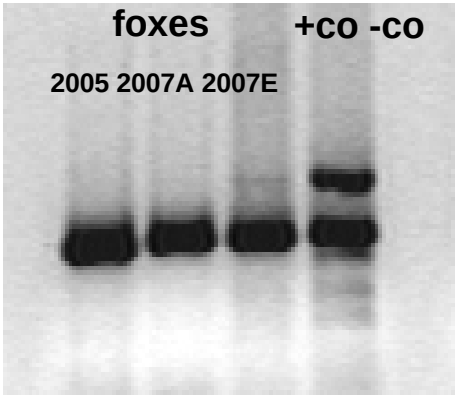
Francisella tularensis : detection

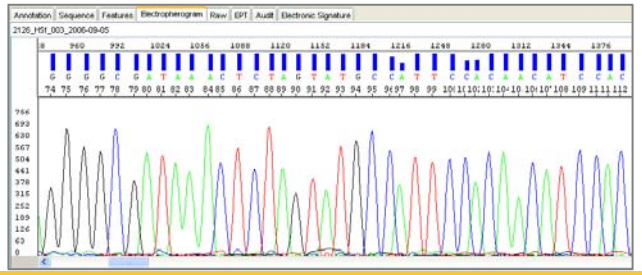
AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

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

tul4 gene real-time PCR

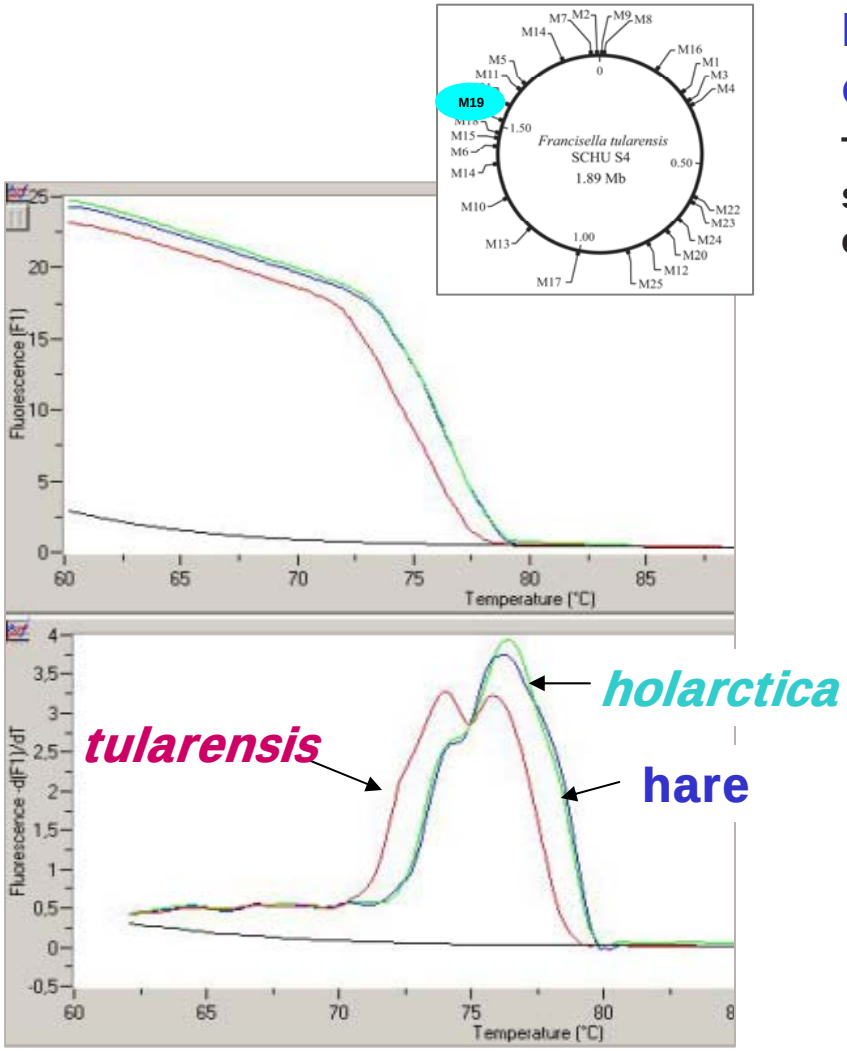






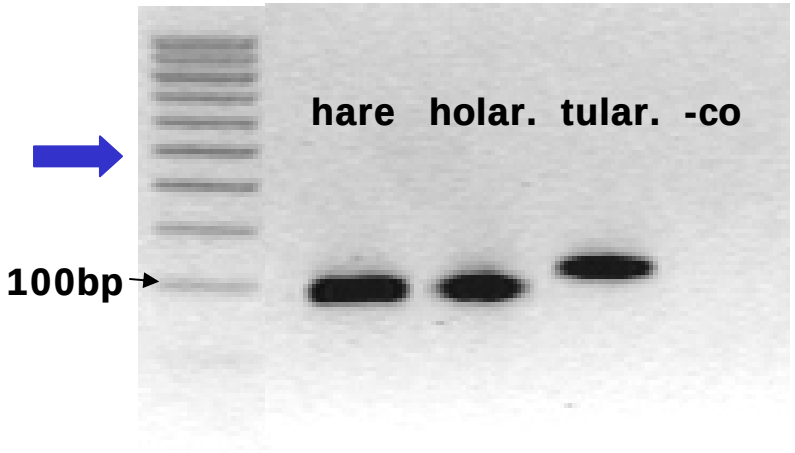
F. tularensis subsp. *holarctica*: Subspecies differentation

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH



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*.



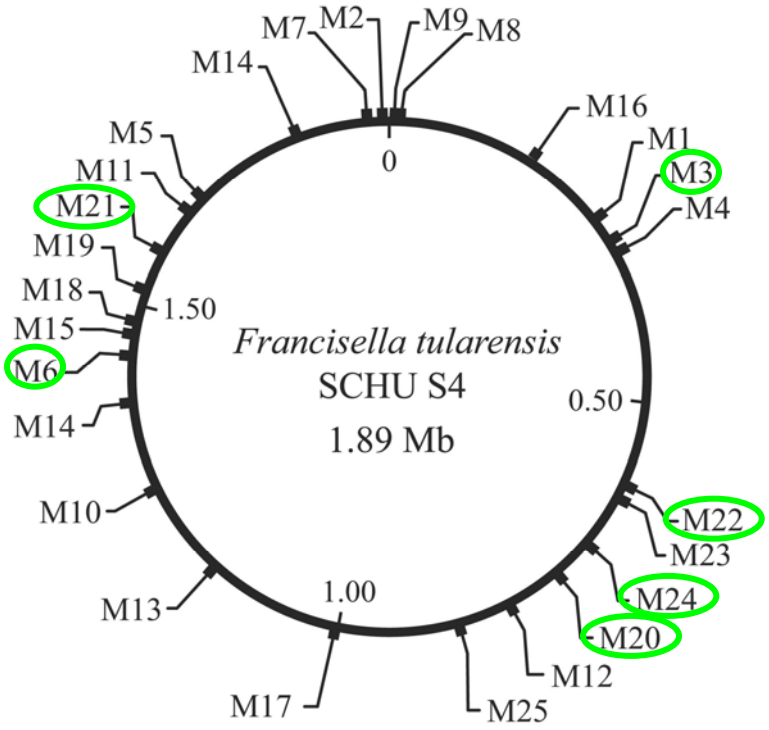
DNA-fragments of approximately 101-bp are amplified only from isolates of subspecies *holarctica*, whereas fragments of 131-bp are amplified only from isolates of subspecies *tularensis*.

MLVA-VNTR analysis of Austrian isolates 1997 til 2009 (1)

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

❖ **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)

MLVA-VNTR analysis of Austrian isolates 1997 til 2009 (2)

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

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

AGES
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

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

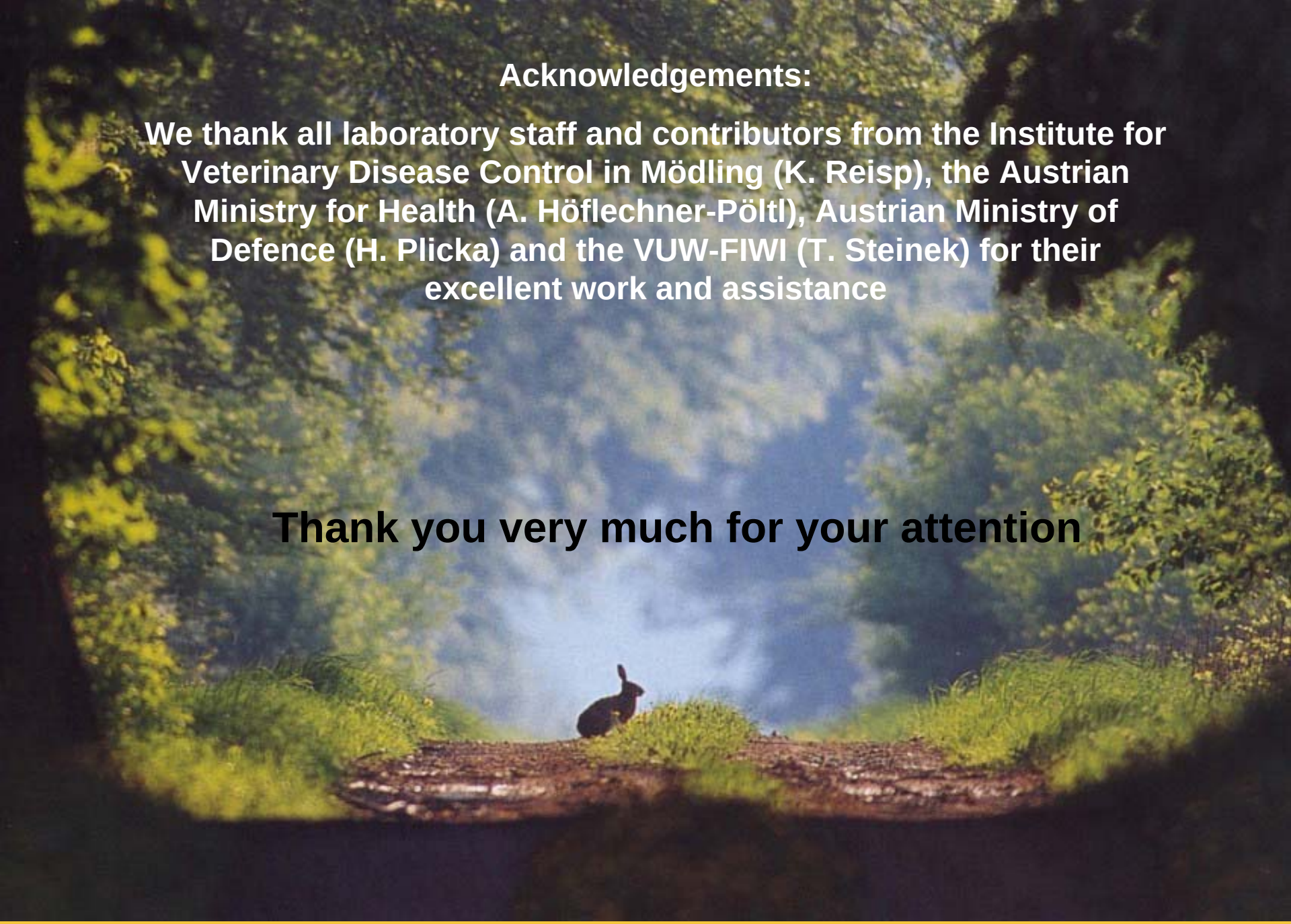
- **49 strains of *F. tularensis* subsp. *holarctica* sent in Februar 09 for MLVA-VNTR-Analysis (hare, fox, human samples) from a wider geographical distribution**

- **Preliminary analysis of Ft-M3 and Ft-M6 sequences reveales 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

AGES

Österreichische Agentur für Gesundheit
und Ernährungssicherheit GmbH

Health. Nutrition. Safety.
Our Concern.

www.ages.at