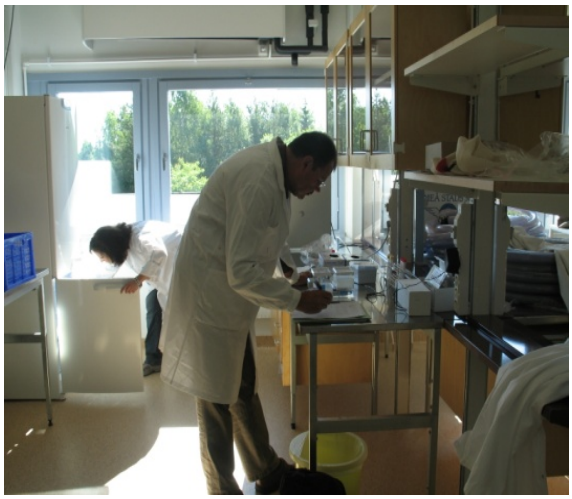
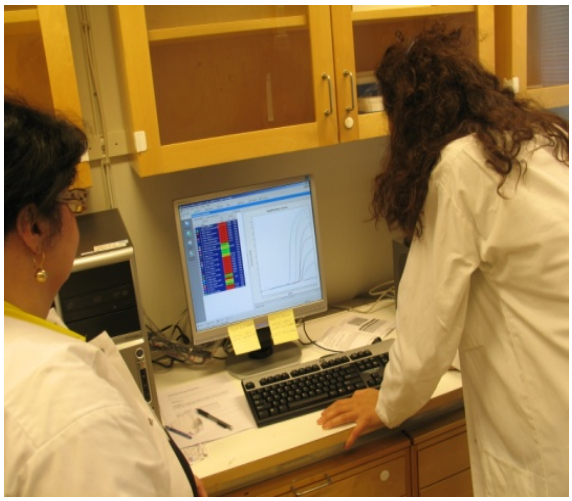
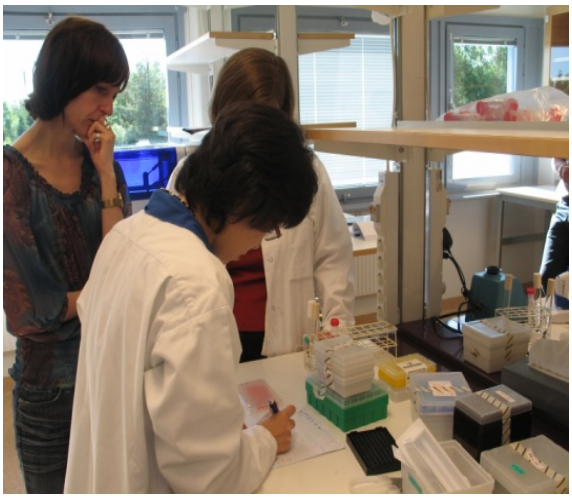
Report on the Tularemia Wetlab Exercise, FOI, Umeå, Sweden



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Background and introduction

Since 2000, the World Health Organization (WHO) has been working with an advisory group of tularemia experts to develop a comprehensive tularemia guidance (www.who.int/csr/resources/publications/deliberate/WHO_CDS_EPR_2007_7/en) and organizing periodic reviews and updates. As an outcome of these activities, it was proposed that a tularemia diagnostic test workshop, jointly hosted by WHO and by tularemia expert laboratories, would be of value to: 1) compare and exchange working knowledge of tularemia disease in endemic countries and, 2) improve the connection among the network of laboratories charged with the responsibility to detect, assess and confirm tularemia disease in humans, animals and its presence in the environment, 3) opportunity to examine different diagnostic techniques and algorithms. Under the revised International Health Regulations of 2005 (IHR), countries are required to develop laboratory core capacities to detect, assess, confirm and report public health events, especially those that may impact travel and trade. Though tularemia is a disease of geographically limited focus, its possible malicious use has been raised as concern for many countries leading to investment and development of more advanced techniques for understanding its pathogenesis, prevention, surveillance and response.

The distribution of tularemia is well-documented in the Northern hemisphere, however, constant sporadic detection and recovery of *Francisella spp*., both pathogenic and non-pathogenic, suggest that there remains gaps in the knowledge of its diversity and survival in the environment and how mammals may become infected. With the heightened awareness of tularemia as potential bio threat agent, laboratories are better prepared to detect *F. tularensis* and have been key to reporting presence of the *F. tularensis* or related species in North America, eastern and central Europe, around the Black Sea and evidence in vectors in the Far East. Taken together, as laboratories become more proficient at the detection of *F. tularensis* and diagnosis of tularemia infections, a workshop is a good gathering place for them to come together and share their knowledge and experiences, this in turn, gives more confidence to the accuracy of results.

In October 17-19, 2005, a practical hands-on (wet lab) exercise on behalf of the Global Health Security Action Group was hosted by the Centers for Disease Control and Prevention at the Division of Vector-Borne Infectious Diseases in Ft. Collins, Colorado, USA. This exercise was very successful in bringing together 7 countries to compare and evaluate their diagnostic tests for the detection of *F. tularensis* and related organisms and it was felt that this should be made available to other countries where tularemia is endemic and/or being discovered or re-discovered.

In June 23-25, 2009, the second practical hands-on (wetlab) exercise was hosted by the Swedish Defence Research Agency (FOI), Umeå, Sweden. This exercise was supported by WHO and the Swedish Ministry of Foreign Affairs. The result of this exercise is summarized in this report.

As the exercise workshop format requires hands-on participation, only a limited number of teams may be hosted at any one time. Therefore a selection of participating laboratories was necessary and based on the following criteria: 1) those nominated by their national authorities, 2) those with proven activities related to tularemia laboratory work (publications, presentations at international meetings and/or, 3) designated as national laboratory for tularemia research, diagnosis or reference testing and 4)

laboratories from countries that did not participate in the first wetlab exercise held in 2005 at Centers for Disease Control and Prevention at the Division of Vector-Borne Infectious Diseases in Ft. Collins, Colorado, USA.

This exercise was very successful in bringing together eight countries to compare and evaluate their diagnostic test for detection of *F. tularensis* and related organisms. As the workshop requires hands-on participation, only a limited number of teams may be hosted at one time. Therefore, it is our hope, that a series of workshops may be organized on a continual basis under the auspices of various volunteer hosting sites in order to make the opportunity available to all qualified laboratory that wishes to participate.

Wetlab aims

- Share diagnostic methods and foster communication by having all participants give a presentation on the methods for tularemia diagnosis in their countries.

- Evaluate the molecular diagnostic methods of each participating country (comparing specificity and sensitivity of PCR analyses).

- Evaluate other commonly used serological diagnostic tests as typically performed by each country (ELISA and/or agglutination techniques).

- Evaluate the capacity of different agar media plates submitted by each country to support growth of *F. tularensis holarctica*.

Participating countries

- Georgia
- Turkey
- Ukraine
- Switzerland
- Portugal
- South Korea
- Norway
- Sweden

Wetlab procedure

Identical blinded samples (DNA, serum, and agar plate cultures) were provided to each country on the first day of the wetlab. The identities of the samples were as follows:

DNA samples

Samples	Identity Subtype (Optional)	Concentration*
Clinical ulcer sample	*F. tularensis* type B	Unknown; actual clinical sample
Blood culture 1	*F.t subs. tularensis* A1, Slovak strain	10^4 CFU/5µl
Blood culture 2	*F.t subs. tularensis* A2, Nevada strain	10^3 CFU/5µl
Respiratory sample	*Haemophilus influenza*	1pg/5µl
Environmental sample	*F. novicida*-like Fx1 (Clarridge *et al.*)	10^3 CFU/5µl
DNA sensitivity titration series, sample 1	*F. tularensis* subsp. *holarctica* strain FSC 200	10^5 CFU /5µl
DNA sensitivity titration series, sample 2	*F. tularensis* subsp. *holarctica* strain FSC 200	10^4 CFU /5µl
DNA sensitivity titration series, sample 3	*F. tularensis* subsp. *holarctica* strain FSC 200	10^3 CFU /5µl
DNA sensitivity titration series, sample 4	*F. tularensis* subsp. *holarctica* strain FSC 200	10^2 CFU /5µl
DNA sensitivity titration series, sample 5	*F. tularensis* subsp. *holarctica* strain FSC 200	10^1 CFU /5µl

*CFU calculation determined prior to DNA purification – likely loss in concentration during purification so CFU in purified DNA is likely lower.

Serum samples

	Results: Absorbance 1:200 (Voller *et al.* ELISA)	
Samples	**IgM**	**IgG**
Sera # 25-01	0.1	3.0
Sera # 25-02	1.2	1.7
Sera # 25-03	0.5	4.1
Sera # 25-04	0.0	0.3
Sera # 25-05	0.1	1.4

Plate culture samples

Samples	Results
Bacterial culture # 1	Negative (*H. influenzae*)
Bacterial culture # 2	Positive *F. tularensis* (LVS)

Section I. Molecular diagnostics - methods and results

PCR Methodologies

Country code	PCR type	Use	Targets	Comments
A	Real time PCR	*Francisella* identification	Idaho Technology Hybridization Probes kit, Target 1 and Target 2	Proprietary; targets unknown
	Conventional PCR	Subtyping	C1-C4 primer set (same target as Ft-M19)	Two different size products for Type A and Type B
B	Real time PCR	*Francisella* identification	ISFtu2 and tul4	
	Real time PCR	Subtyping	30 bp deletion (Ft-M19) melt curve method	Differentiation of *F. tularensis* type B
	Real time PCR	Subtyping	ISFtu2 method	*F. tularensis* Type B specific
C	Conventional PCR	Identification and subtyping	C2-C4 primer set (same targets as Ft-M19)	*F. tularensis* Type B specific
D	Real time PCR	*Francisella* identification	ISFtu2 and fopA	
	Real time PCR	Subtyping	30 bp deletion (Ft-M19) melt curve method	Differentiation of *F. tularensis* type B
E	Conventional PCR	*Francisella* identification	tul4	
	Real time PCR	Subtyping	fopA hybridization probe method	
F	Real time PCR	*Francisella* identification	ISFtu2 and 23 kDa protein	
G	Conventional PCR	*Francisella* identification	tul4	
	Real time PCR	Subtyping	pdpD	*F. tularenisis* Type A specific

Summary of PCR Results

Country code	Primers	Clinical ulcer sample	Blood culture 1	Blood culture 2	Respiratory sample	Environment sample	Sensitivity testing
A	Target 1 and 2	Ft positive	Ft positive	Ft positive	Negative	Ft positive	10^5-10^3 positive
	C1-C4	Type B	Type A	subtype not identified	Negative	subtype not identified	10^5-10^3 positive
B	M19 assay	Ft positive	Ft positive	Ft positive	Negative	Ft positive	10^5-10^3 positive
	ISFtu2 assay	Type B	Negative	Negative	Negative	Negative	10^5-10^3 positive
C*	C2-C4	N/A	N/A	N/A	N/A	N/A	N/A
D	Tul4 and fopA	Ft positive	Ft positive	Ft positive	Negative	Ft positive	10^5-10^3 positive (fopA)
	M19 assay	Type B	Type A	Type A	N/A	Type A	N/A
E	fopA	Ft positive	Ft positive	Ft positive	Negative	Ft positive	10^5-10^4 positive
	N/A	No subtyping perfomed	No subtyping perfomed	No subtyping perfomed	N/A	No subtyping perfomed	
F	ISFtu2 and 23 kDa	Ft positive	Ft positive	Ft positive	Negative	Ft positive	10^5-10^3 positive
		No subtyping perfomed	No subtyping perfomed	No subtyping perfomed	N/A	No subtyping perfomed	
G	tul4	Ft positive	Ft positive	Ft positive	Negative	Ft positive	10^5-10^3 positive
	pdpD	Negative; not subsp *tularensis*	Type A	Type A	N/A	Negative; not subsp *tularensis*	N/A

***** None of the Country C PCR assays worked; suggests an issue with primers or reagent.

Section II. Serology: Methods and results

Country code	Assay	Antigen	Results			
A	Tube agglutination	Heat killed Type B isolate from home country	Sera # 01	1:200 (Positive)		
			Sera # 02	1:200 (Positive)		
			Sera # 03	1:400 (Positive)		
			Sera # 04	1:100 (Intermediate)		
			Sera # 05	1:100 (Intermediate)		
B	Microagglutination	Heat killed Type B isolate from home country + 2 dyes (CVBG and MB) and commercial BD antigen	**Sera**	**CVBG**	**MB**	**BD**
			# 01	Negative	Negative	Negative
			# 02	1:640 (+)	1:80 (+)	1:40
			# 03	1:160 (+)	1:80 (+)	1:40
			# 04	Negative	Negative	Negative
			# 05	1:40	1:40	1:40
C	ELISA	Not available	**IgG**			
			Sera # 01	1:1600 (Positive)		
			Sera # 02	1:1600 (Positive)		
			Sera # 03	>3200 (Positive)		
			Sera # 04	1:400 (Negative/Positive)		
			Sera # 05	>1 :800 (Positive)		
D	FOI ELISA	LPS (no in-house serology method)	**IgG**			
			Sera # 01	>1:3200 (Positive)		
			Sera # 02	>1:3200 (Positive)		
			Sera # 03	>1:3200 (Positive)		
			Sera # 04	=1:1800 (Positive)		
			Sera # 05	=1:1800 (Positive)		
E	Microagglutination and FOI ELISA	In-house prepared killed LVS (for MA) and LPS (FOI ELISA)	**Sera**	**IgM**	**IgG**	**MA**
			# 01	Negative	Positive	Positive
			# 02	Positive	Positive	Positive
			# 03	Positive	Positive	Positive
			# 04	Negative	Negative	Repeat??
			# 05	Borderline	Negative	Negative
F	Microagglutination	CDC-prepared formalin-killed *F. tularensis* SchuS4	Sera # 01	1:32 (Negative)		
			Sera # 02	1:2048 (Positive)		
			Sera # 03	1:256 (Positive)		
			Sera # 04	0 (Negative)		
			Sera # 05	1:64(Positive)		

Country code	Assay	Antigen	Results			
G	Microagglutination and ELISA	In-house prepared isolate from home country (MA assay) and OMP antigen (ELISA)	**Sera**	**IgM**	**IgG**	**MA**
			# 01	Negative	Positive	Negative 1:16
			# 02	Positive	Positive	Positive:1:4096
			# 03	Low Positive	Positive	Positive 1:256
			# 04	Negative	Borderline	Negative
			# 05	Negative	Positive	Positive 1:64

Section III. Agar culture growth: Methods and results

A standardized suspension of LVS and a Type B strain were streaked on all plates and incubated for 24 and 48 hours. Both strains of *F. tularensis* grew on all media.

Country code	Media	Additives	24 hr growth	48 hr growth
A	Chocolate agar (GCII base)	2% haemoglobin	+	++++
B	Brucella medium base 4.3% (BBL)*	8% sheep blood 1% Isovitalex (BD BBL)	+	++
	Brain Heart Infusion Agar 5.2% (Difco)*	0.1% L-Cystein hydrochloride monohydrate (Merck), 0.01% L-Cystin (Merck), 1% glucose, 4% Helicobacter pylori Selective Supplement (Oxoid code: SR147E),	+	++
	Brain Heart Infusion Agar 5.2% (Difco)*	0.1% L-Cystein hydrochloride monohydrate (Merck), 1% glucose 8% human blood (seronegative)	+	+++
	Brain Heart Infusion 3.7% (BHI) (Difco)*	0.1% L-Cystein (Sigma), 1% dextrose (Oxoid), 1.5 % agar (Difco), 4% Helicobacter pylori Selective Supplement (Oxoid code: SR147E), 9% sheep blood	+++	++++

Country code	Media	Additives	24 hr growth	48 hr growth
	Brain Heart Infusion 3.7% (BHI) (Difco)*	0.1% L-Cystein (Sigma), 1% dextrose (Oxoid), 1.5 % agar (Difco), 4% Helicobacter pylori Selective Supplement (Oxoid code: SR147E), 9% sheep blood	+++	++++
C	N/A			
D	Chocolate agar		+	++++
E	Chocolate agar	PolyViteX	++	++++
F	Cysteine heart agar	9 % sheep blood	+	++ (42 hr)
G	Cysteine heart agar (Difco 247100)	2.5% human erythrocyte blood (centrifuged at 300 rpm 10 min)	+++	++++

*depends on the commercial formula

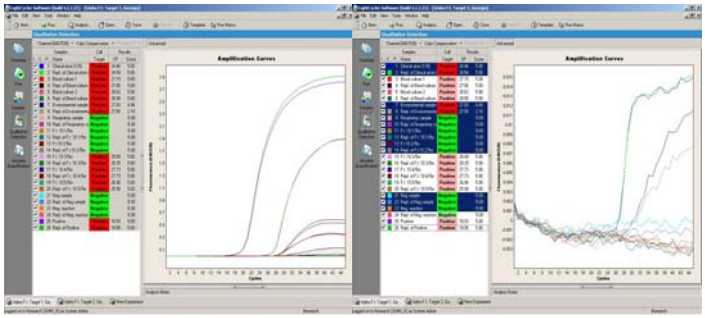
Section IV. Culture identification: Slide agglutionation results

All countries correctly identified the cultures as negative or positive by the slide agglutination method in the WHO manual (WHO, 2007).

Overall summary

- The workshop was very successful; new contacts were fostered and information exchanged.
- The wetlab provided an important opportunity for participants to evaluate their molecular assays on different *F. tularensis* strains as well as *F. novicida*.
- Consistency among the PCR assays was very good; All PCR assays performed correctly identified the *Francisella* positive and negative samples.
- The sensitivity of all PCR assays was comparable.
- Subspecies *holarctica* (type B) (the sole cause of tularemia in all countries participating in the wetlab) was accurately identified using PCR subtyping methods.
- Subspecies *tularensis* (type A) was rarely identified accurately by the PCR subtyping methods
- None of the PCR assays used could differentiate *F. novicida* from *F. tularensis* by PCR subtyping methods. *F. novicida* was identified as *F. tularensis* by all assays used. Specificity should be improved to be able to rule out *F. novicida* as the possible source of infection. Specificity could potentially be improved by use of a secondary subtyping assay or 16S rRNA sequencing.

- Results varied among serology tests performed at the wetlab likely due to the differing methods (agglutination, ELISA), differing antigen preparations, as well as differing cut-offs used. This is an area that could use further analysis and standardization among laboratories.
- Variability was also noted in the growth of *F. tularensis* on the different agar medias. *F. tularensis* grew on all medias, with the heaviest, most rapid growth occurring on 1) cysteine heart agar with human blood 2.5 %, 2) brain heart infusion agar, L-cysteine, L-cystine, dextrose, antibiotic supplement, and 3) brain heart infusion agar, L-cysteine, dextrose, sheep blood (9 %).



Conclusions

This exercise was very successful in bringing together eight countries to compare and evaluate their diagnostic tests for the detection of *F. tularensis* and related organisms.

As the exercise workshop format requires hands-on participation, only a limited number of teams may be hosted at any one time. Therefore, a series of workshops may be organized on a continual basis under the auspices of various volunteer hosting sites in order to make the opportunity available to all qualified laboratory that wishes to participate. We encourage the increased use and funding of this of exercises for promoting development and technology transfer as a mean of increasing security and capacity building in the fields of disease surveillance, detection, diagnosis, and containment of infectious diseases.

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Tularemia wetlab exercise group, at FOI, Umeå, Sweden, 2009. *N.B*, not all participants are shown.

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2. Voller et al., J. Clin. Pathol. 1978, 31:507-520
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