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A novel screening ELISA and a confirmatory Western blot useful for diagnosis and epidemiological studies of tularemia

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SUMMARY

A novel enzyme-linked immunosorbent assay (ELISA) and a confirmatory Western blot (WB) to detect human antibodies against *Francisella tularensis* were evaluated. The ELISA was based on partially purified lipopolysaccharide (LPS), the WB on whole antigen of *F. tularensis*. Positive WB showed a typical LPS ladder. Sensitivity and specificity of the ELISA, as assessed in 104 positive sera and 1149 'normal' sera from healthy young adults, were 99·0% and 97·1% respectively. Sensitivity of the WB was close to 100%, whereas specificity was 99·6%. Antibodies against the LPS of *F. tularensis* were detected in four of the 'normal' sera in both ELISA and WB. The assays were further evaluated using sera of individuals from Norway, Sweden and Kosovo suspected to be infected in tularemia outbreaks. Results revealed that the combination of ELISA and WB is suitable for laboratory confirmation of tularemia as well as for large-scale epidemiological studies.

INTRODUCTION

Tularemia is an infectious disease caused by the small, pleomorphic, heat-labile, Gram-negative, rod-shaped bacterium *Francisella tularensis*. It occurs in the northern hemisphere mainly in two subspecies (types). Type A strains, *F. tularensis* ssp. *tularensis*, have so far been found predominantly in North America; however, strains of this subspecies have also recently been isolated in the Danube region near Bratislava [1]. Type B strains, *F. tularensis* ssp. *holarctica*, are found in Europe, Asia and North America. Before the introduction of antibiotic treatment, the lethality rate was 10–30% for type A infection and <1% for type B infection [2]. The infectious dose for humans is reported to be 10–100 bacteria by uptake via the respiratory tract or skin [3–6].

Tularemia includes a variety of clinical manifestations depending upon the route of infection. The most common course of tularemia, the enlargement of regional lymph nodes with or without the occurrence of skin ulcers, is caused by bites from infected arthropods or other direct contact with the bacteria, often through distinct skin or mucosal lesions. The inhalation of *F. tularensis* may lead to pneumonia whereas the ingestion of infected food or water is associated with the oropharyngeal course of the disease. Infection of the eyes, mainly through contaminated fingers, leads to ocular tularemia. Systemic manifestations without an identified route of infection or entry of the pathogen are called the typhoidal form of tularemia [7]. Outbreaks of various sizes and sporadic cases as well as epidemiological surveillance data have been reported from North America, Europe

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and Asia [8–13]. However, little is known about the prevalence of tularemia in human and animal populations in many countries of central Europe. One reason for that could be the lack of available high throughput screening methods for serological diagnosis of this rather rare pathogen. Currently, agglutination assays and immunofluorescence techniques are commonly used as serological tests for the detection of antibodies to diagnose cases of acute tularemia. The time-consuming handling of large numbers of samples in epidemiological studies, as well as the cross-reactivity and lower sensitivity in terms of long-term antibody surveillance are disadvantages of these assays. The ELISA technique seems to be a suitable tool to overcome these problems. In a previous study, specific IgG antibodies against *F. tularensis* have been demonstrated up to 8 years after infection in 95% of individuals recovered from tularemia [14]. The aim of this study was to evaluate novel ELISA and Western blot (WB) assays for screening of the seroprevalence and confirmatory serodiagnosis of tularemia, using serum collections derived from Germany and from tularemia outbreaks in Sweden, Norway and Kosovo.

METHODS

Bacteria and lipopolysaccharide (LPS) preparation

The live vaccine strain (LVS) of *F. tularensis* (ATCC 29648) was grown for 48 h at 37 °C in a 5% CO_2, humid atmosphere on heart–cysteine–blood agar (Beckton Dickinson, Heidelberg, Germany), harvested into sterile distilled water and adjusted to $OD_{560\ nm} = 1{\cdot}0$. Extraction of the LPS from this suspension of bacteria was performed using Chlamydia extraction buffer (Abbott, Wiesbaden, Germany) for 30 min at 60 °C to a final dilution of 1/2. This 'preliminary antigen preparation' was used in a first investigation of the ELISA. For further purification of the LPS, the antigen suspension was filtered and treated with 3·3 mg/ml proteinase K (Boehringer, Mannheim, Germany) for 2 h at 60 °C in order to digest contaminating proteins. The enzyme was inactivated by boiling for 25 min. Final purification was performed by an overnight dialysis (Slide-A-Lyzer 3·5 K, Pierce, Rockford, USA) in isotonic phosphate-buffered saline (PBS; pH 7·2). To test cross-reactivity, *Salmonella* antigen from Kaufmann–White group N (O:30) and other Enterobacteriaceae were kindly provided by the National Reference Laboratory for Salmonella, Wernigerode, Germany. *Brucella* sp., *Escherichia coli* serotypes O:116 and O:157, *Stenotrophomonas maltophilia*, and *Yersinia enterocolitica* serotype O:9 were from our in-house strain collection (kindly provided by Dr H. Neubauer). All tested bacteria were processed identically to *F. tularensis* strain LVS before use. We investigated possible cross-reactions of the above-mentioned antigens in our ELISA setting with five tularemia hyperimmune sera from our serum collection.

Sera

A total of 1149 anonymized negative sera were obtained at a routine medical check-up from mainly young healthy German adults and denoted as 'prevalence sera'. The sera were collected as remaining volumes after completing the requested medical laboratory investigation. Whereas all sera were tested by ELISA for the presence of IgG and IgM against LPS of *F. tularensis*, 893 sera were additionally tested for IgA. Positive control sera including 75 sera from patients with clinically evident tularemia and 29 sera from seroconverted vaccinees were kindly provided by Dr A. Sjoestedt (FOI/University, Umeå, Sweden). Seven sera from vaccinated volunteers with unknown/uncertain seroconversion were provided by Dr T. Brooks (HPA DSTL, Porton Down, Salisbury, UK). In addition, sera were obtained from tularemia outbreaks in Norway [15] provided by Dr B. A. Berdal (Institute of Microbiology, Armed Forces Medical Services, Oslo), from Sweden provided by Dr A. Sjoestedt (FOI/University, Umeå, Sweden), and from Kosovo collected during a tularemia outbreak investigation during 1999–2000 [13].

ELISA

The LPS solution was diluted 1/100 in carbonate–bicarbonate buffer (pH 9·0, 0·025 M). In general, there was no significant variation from batch to batch of LPS preparations. This was proved by comparing batches using standard positive and negative sera in ELISA. A 96-well microtitre plate (Polysorp, NUNC, Wiesbaden, Germany) was coated with 50 μl antigen at 37 °C for 1 h. The wells were washed once using PBS (pH 7·2, 0·15 M) with 0·05% Tween-20 (PBS/Tween, washing buffer) and blocked with 75 μl 10% goat serum (Sigma, Taufkirchen, Germany) in PBS/Tween. The latter buffer was used as blocking and dilution buffer. After another washing step, either

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Table 1. *Estimation of the specificity of ELISA*

	IgG		IgM		IgA	
Result	OD	% of sera ($n=1149$)	OD	% of sera ($n=1149$)	OD	% of sera ($n=893$)
Negative	<0·26	92·6	<0·90	98·8	<0·90	98·0
Borderline	0·26–0·49	5·6	0·09–0·13	0·7	0·90–0·18	0·9
Positive	>0·49	1·8	>0·13	0·5	>0·18	1·1

Results above the mean OD plus 3 S.D. of all 'seroprevalence sera' were assumed as 'positive', whereas all results between these two values were taken as 'borderline'. The cut-off levels were calculated for each isotype individually including also 'false-positive' sera. Analysis of the results of individual isotypes revealed a specificity of at least 98·2% for IgG, 99·5% for IgM and 98·9% for IgA.

50 μl sample serum or positive and negative controls (diluted 1/500 in dilution buffer) were added and incubated for 1 h at 4 °C. Plates were washed four times and 50 μl horseradish peroxidase conjugated anti-human IgG, IgM, IgA immunoglobulin (Sigma Taufkirchen, Germany) respectively, diluted 1/2000 in dilution buffer, were added and incubated for 1 h at 37 °C. After six rinses with PBS/Tween, substrate reaction was started with 50 μl 66% tetramethylbenzidine (TMB; Seramun, Wölzig, Germany) and stopped after 10 min with 50 μl 0·25 m sulphuric acid. The optical density (OD) of the wells was read at 405 nm using a 'Digiscan' microplate reader (Asys Hytech, Eugendorf, Austria).

Western blotting

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a 4–20% separating Tris–glycine gel (Novex, Frankfurt, Germany). Stock suspensions of LVS samples were inactivated with a final concentration of 1% formalin overnight at room temperature, washed twice with PBS, and adjusted to $OD_{560\ nm}=2{\cdot}5$. Bacteria were diluted 1/2 with sample buffer (Novex) containing 5% mercaptoethanol. After 15 min boiling, the suspension was centrifuged for 20 min at 10000 r.p.m. and electrophoresed at 130 V for 1·5 h. The gel was equilibrated for 10 min in transfer buffer (Novex) and the fractionated material was transferred onto nitrocellulose membrane (0·45 μm) at 30 V for 1 h. The remaining binding sites on the membrane were blocked with 4% skimmed milk in Tris-buffered saline (pH 8·1, 0·2 M) overnight at 4 °C. The dried and cut membrane strips were incubated with sera diluted 1/500, or as indicated, in 10% goat serum/PBS at room temperature for 2 h. After three rinses with washing buffer (YP kit; Microgene, Munich, Germany), the strips were incubated with a polyvalent goat anti-human IgA/IgM/IgG horseradish peroxidase conjugate (Sigma) at room temperature for 1 h. Following another three rinses the membrane was developed with precipitating TMB (Seramun). Positive sera showed a typical LPS ladder at a dilution of 1/500 (Fig.).

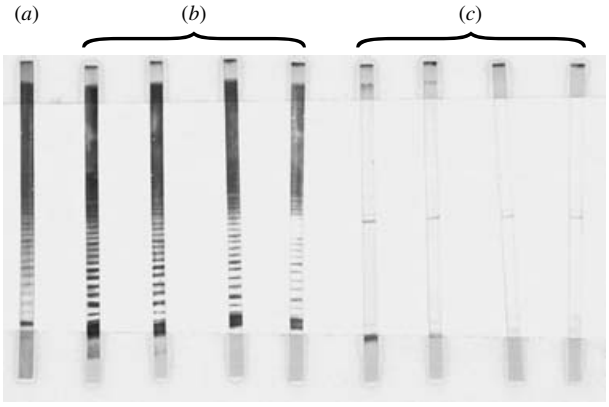

Fig. Typical Western blot band pattern of the LPS antigen of *F. tularensis* in positive control sera and patient sera compared to negative sera. Negative sera developed single bands probably corresponding to heat-shock proteins. (*a*) Positive control serum; (*b*) positive patient sera ($n=4$, seroprevalence investigation); (*c*) negative patient sera ($n=4$, seroprevalence investigation).

ELISA Cut-off-levels

ELISA results below the mean OD plus 1 standard deviation (S.D.) calculated from 1149 'seroprevalence sera' collected in Germany were estimated as 'negative'. Results above the mean OD plus 3 S.D. were assumed to be 'positive', whereas all results between these two values were taken as 'borderline'. The cut-off levels were calculated for each isotype individually (given in Table 1) also including 'false-positive' sera.

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Table 2. *Estimation of the sensitivity of ELISA and Western blot*

	Patients ($n=75$)					Vaccinees ($n=29$)			
	IgG	IgM	IgA	IgG+IgA +IgM	WB*	IgG	IgA	IgM	WB
Negative (*n*)	11	8	3	1	0	0	0	0	0
Borderline (*n*)	14	4	7	1	1	0	0	0	0
Positive (*n*)	50	63	65	73	73	29	29	29	29
Sensitivity (%)†	85·3	89·3	96·0	98·7	98·7	100·0	100·0	100·0	100·0

One patient negative by ELISA for all isotypes and Western blot was also not confirmed serologically by an independent laboratory. Therefore, the sensitivity of combined ELISA and Western blot was close to 100·0%. The two negative and borderline sera could result from not (yet) seroconverted patients.

* Confirmed by Western blot.

† Calculated for ELISA including 'borderline'.

This ranking gave a satisfactory discriminatory power between positive and negative sera (see Table 1).

RESULTS

A first investigation trial with 1000 sera using the 'preliminary antigen preparation' revealed a relatively high background OD of 0·3–0·4 in ~40% of sera. The antigen used initially was extracted from the LVS strain without further digestion of contaminating proteins or dialysis of the antigen suspension. Neither dilution of the antigen from 1/100 to 1/800 nor of the conjugate from 1/2000 to 1/32000 resulted in a decrease of the background activity. Given a previously described interference with heat-shock proteins [16] and possibly other proteins, the antigen was further treated with proteinase K and dialysed, in order to purify the LPS solution. In subsequent assays, background activities were thereby reduced in nearly all sera to an OD below 0·1 (data not shown).

Sensitivity and specificity of ELISA and WB

The sensitivity of ELISA was assessed using 104 sera from clinically evident tularemia patients or vaccinees 6–12 months after immunization with LVS by scarification (Table 2). All but one sera were found positive or borderline (one serum) when combining the results for IgG, IgM and IgA revealing a sensitivity of 98·7%. The one negative serum which was also negative by WB was not confirmed by another independent laboratory (data not shown). Taking all the results together, 103 out of 104 sera were detected by ELISA revealing a sensitivity of at least 99·0%.

The WB, using a whole bacterial lysate of *F. tularensis* LVS as antigen, was applied in order to confirm or rule out a tularemia infection in cases of 'borderline' and 'positive' ELISA results (Table 2). A LPS-specific ladder, ranging from 15–98 kDa (Fig.) was found in all confirmed positive control sera and in none of the ELISA-negative sera at a dilution of 1/500, rendering the WB reliable as confirmatory and reference test. Therefore, the sensitivity of the WB was assumed as 100%. The same characteristic WB pattern was seen in four 'prevalence sera' positive for IgG, IgM or IgA. According to the results of the positive control sera, test sera showing the LPS-specific band pattern at a dilution of $\geqslant 1/500$ were considered as 'positive'. Four additional bands at 10, 15, 65 and 80 kDa were still present when tested against most positive and negative control sera after treatment of the antigen with proteinase K and overnight dialysis. These bands were presumed to represent the heat-shock proteins Gro-EL and Gro-ES [16] which were probably incompletely digested or not digested by poteinase K.

Three out of seven vaccination sera from the United Kingdom had low IgG and IgM ELISA titres whilst two sera remained negative, although the respective immunizations had been performed 1 year before blood was taken for antibody determination (data not shown). Negative or low-reacting sera in ELISA were confirmed negative by WB. Those 'positive control' and vaccination sera that failed to be positive by ELISA also remained negative with WB.

The specificity of the ELISA was calculated from the results using normal control sera (Table 3). If all of the sera found positive in the ELISA are considered as false-positive (36 out of 1149), specificity

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Table 3. *Detection of antibodies against* F. tularensis *in human sera*

Serum collection	*n*	IgG positive		IgM positive		IgA* positive		ELISA† positive		WB confirmed		Prevalence
		n	%	*n*	%	*n*	%	*n*	%	*n*	%‡	%
Germany	1149	21	1·8	6	0·5	10	0·9	36	3·2	4	11·1	0·3
Norway outbreak§	16	10	62·5	10	62·5	n.t.	—	10	62·5	10	100·0	62·5
Sweden outbreak¶	100	51	51·0	64	64·0	65	65·0	73	73·0	73	100·0	73·0
Kosovo outbreak‖	481	174	36·2	165	34·3	162	33·7	177	36·8	176	99·4	36·6

* Number of sera tested for IgA was 893.
† The number of ELISA-positive sera considers at least one positive result for different serotypes tested.
‡ Per cent of positive ELISA.
§ Sera from an outbreak in Norway, caused by lemming carcasses in a water well [2].
¶ Sera from an outbreak in central Sweden [23].
‖ Sera collected during a tularemia outbreak in Kosovo 1999–2000 [18].
n.t., Not tested.

should be at least 97%. Since four sera were confirmed as positive in the WB, the actual specificity was 97·2%.

The specificity of the WB was estimated using 104 ELISA-negative sera from the probably low-endemic area Germany. Two of these sera were found 'positive' by WB, whereby a specificity of 98·1% was calculated for WB.

Cross-reactivity

Cross-reactions with *F. tularensis* have been previously described for antibodies against *Brucella* spp., *E. coli* serotypes O:116 and O:157, *Salmonella* serotypes of the Kaufmann–White group N (O:30), *Stenotrophomonas maltophilia* and *Y. enterocolitica* serotype O:9 [17, 18] when using the agglutination test or the indirect immunofluorescence technique. Five high titres sera from tularemia patients were investigated for reactivity to the above-mentioned antigens in our ELISA. Cross-reactivity did not occur with any of the tested antigens.

Application of ELISA and WB for seroprevalence studies

In 1149 healthy adults, screened for IgG, IgM and IgA antibodies against the LPS surface antigen of *F. tularensis*, four (0·3%) were found positive using the combined ELISA and WB diagnostic approach (Table 3).

In IgG ELISA, 1064 sera (92·6%) were below the negative cut-off level and were, therefore, supposed to be 'negative' (Table 1). By contrast, 64 sera (5·6%) were tested as 'borderline' and 21 sera (1·8%) were 'positive'. Positive IgM or IgA values corresponding to the 'IgG-positive' sera did not occur. A total of 1135 sera (98·8%) were 'negative' for IgM, whereas eight (0·7%) were 'borderline' and six (0·5%) were 'positive' with ELISA. From 893 sera tested for IgA, 875 (98·0%) were 'negative', eight (0·9%) 'borderline' and 10 (1·1%) 'positive'. To confirm 'positive' and 'negative' ELISA results, 170 wb were performed. In total, four positive WB sera were detected in ELISA-positive sera (0·3%). All other tested sera showing 'borderline' results in the different isotype-specific ELISAs were not confirmed by WB.

Furthermore, we addressed the question why sera, presenting OD values above the ELISA cut-off level for positivity, were negative by WB. All these sera showed reactivity to the 10, 15, 65 and 80 kDa bands in WB. Presuming contamination of proteins from the heat-shock class in the proteinase K-digested LPS solution, we further tested 19 ELISA reactive sera with another LPS preparation by phenol water extraction from *F. tularensis* LVS [19]. Background activities were thereby reduced but not completely removed in nearly all 19 sera (data not shown).

Evaluation of ELISA and WB using sera from tularemia outbreaks or endemic areas

In addition to the German sera, we tested sera from tularemia outbreaks in Sweden [20], Norway [15], and Kosovo [13] in order to evaluate the diagnostic capacity of our serological methods (Table 3). From northern Norway, we obtained 16 sera from nine individuals with suspected waterborne tularemia, who

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became ill after drinking water from a reservoir probably contaminated by dead lemming carcasses [15]. Both water and lemming samples were positive for the *F. tularensis* antigen either by the rapid immunochromatography test, ELISA or PCR [15]. Serological investigation by our institute showed positive ELISA and WB results in 10 sera corresponding to seven individual cases, whilst two persons (six sera) remained 'negative' in both tests.

A total of 100 sera of patients with clinically suspected tularemia from central Sweden were investigated by both ELISA and WB. According to the predefined cut-off levels, we found 73 sera positive for IgG and/or IgA and/or IgM by ELISA. All these sera were confirmed as 'positive' by WB. Two additional sera which were assumed as 'borderline' for IgA were positive by WB (Table 3). The combination of all three antibody isotype results revealed the highest level of sensitivity. Two individuals with clinically evident tularemia confirmed by PCR-positive ulcer swabs, developed predominantly IgA antibodies, and would have been missed if not tested for this immunoglobulin isotype. According to the Swedish patient database, all patients tested positive by our WB had clinical tularemia whilst the negative results matched patients without typical symptoms of *F. tularensis* infection (data not shown).

In a study of a tularemia outbreak investigation in Kosovo during 1999–2000 [13], we studied 481 sera from suspected tularemia cases or exposed individuals (Table 3). A total of 177 sera were positive by ELISA, of which 176 were confirmed positive by WB. Almost all sera showed high ODs by ELISA and in most positive sera antibodies were detected for all three isotypes against the *Francisella* LPS.

DISCUSSION

Epidemiological studies on tularemia should not be based on clinical evidence alone, because a considerable number of infections are asymptomatic or are not diagnosed by the physician. Epidemiological investigations, as well as the diagnosis of acute disease in the routine laboratory, currently rely on the agglutination test or the indirect immunofluorescence technique, that are disadvantageous in terms of being time consuming, and having cross-reactivity, sensitivity, and specificity problems. Here we report a novel serological approach utilizing an ELISA as screening and a WB as a confirmatory assay respectively.

The screening ELISA with semi-purified LPS preparation as the target antigen was highly sensitive and specific for the detection of IgG, IgA and IgM antibodies against *F. tularensis*. Pretreatment of the antigen with proteinase K and overnight dialysis markedly reduced background activities when the same LPS concentrations were used. The sensitivity level of the screening ELISA was close to 100%. Negative ELISA results found in post-vaccination sera from the United Kingdom are probably due to an inadequate response to immunization (data not shown). The specificity level of the ELISA was ~97%, when all positive 'seroprevalence' sera were assumed as false-positive.

The WB has proved to be an appropriate confirmatory test in the case of positive screening of ELISA results, with 100% sensitivity and 98·1% specificity. Due to the unique and highly specific LPS antigen of *F. tularensis*, the typical LPS ladder allows a reliable diagnosis of tularemia.

Four individuals (0·34%) from a total of 1149 healthy adults had IgG antibodies against the LPS of *F. tularensis* as assessed by a positive screening ELISA and confirmed by WB. For comparison, the occurrence of antibodies against *F. tularensis* in 1072 Finnish blood donors from a rural area, screened by an agglutination test was reported to be 0·7% [9]. According to these results, the prevalence of tularemia in Germany seems to be somewhat lower than Finland. This could be an indication for differences in the distribution of natural foci or due to differences in selection of participants in the two surveys, for example by age or rural/urban setting.

It is not clear how the tularemia pathogen circulates in Germany and how it can occasionally be transmitted to humans. Alternatively, it cannot be determined from this anonymized study where the seropositive individuals had been exposed to *Francisella* and whether they had an elevated risk for a subclinical episode or even a clinical manifestation of tularemia. This should be the aim of directed investigations in future.

High OD ELISA results with negative WB findings may be caused by cross-reactivity. Cross- reactions between *F. tularensis* and *Brucella* spp. and between the latter and *Y. enterocolitica* serotype O:9 have been found in agglutination tests [21, 22]. The immunofluorescence technique detecting *F. tularensis* is cross-reactive with *Pseudomonas* spp. [23, 24] and *E. coli* [23, 25]. The antibody screening ELISA described was not cross-reactive with any of the above-mentioned

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antigens, as determined by using positive and slightly elevated sera. Cross- reactivity in our assay was apparently more likely to be caused by proteins, such as the 10-, 15-, 65- and 80-kDa bands in the WB, which several pathogens might have in common. Even if enzymic digestion of the LPS extract seems to be a suitable tool to decrease the amount of contaminating proteins, the 10-, 15-, 65- and 80-kDa heat-shock proteins may be resistant to proteinase K digestion, since the respective bands remained visible in the WB after treatment. Therefore, high ODs in the screening ELISA might result from antibodies against those heat- shock proteins. They are associated with the respective protein components in the WB and can frequently be found in positive and negative tularemia sera. Cross-reaction due to the LPS fraction, as previously reported [9], is unlikely in our assay. In another investigation we have found the LPS fraction of *F. tularensis* to be unique and highly specific for this bacterium and, therefore, it induces highly specific antibodies. Indeed monoclonal antibodies specific for LPS of *F. tularensis* did not recognize LPS of potentially cross-reacting bacteria [26].

The combination of ELISA and WB either ruled out or confirmed tularemia in clinically suspected cases from outbreak areas, rendering both tests as reliable tools for clinical diagnosis. Sera from outbreak areas that were negative by ELISA and WB can either originate from exposed, but not infected individuals or from patients who did not seroconvert. In the Kosovo outbreak, a number of negative sera from suspected tularemia patients were found to be positive for mumps antibodies and indeed a mumps epidemic was reported at the same time as the tularemia outbreak (data not shown).

The agglutination test has been reported to be a useful tool for the early and specific diagnosis of tularemia [27]. Along with the serological diagnosis of acute tularemia, our study focused particularly on the ability to detect serum antibodies even years after infection. Although suitable for elucidating acute tularemia, the microagglutination assay failed to be sufficiently sensitive to determine immunity after tularemia in our study (data not shown).

In conclusion, tularemia can be diagnosed with high reliability in cases of acute disease, also years after infection, by means of a screening ELISA combined with a confirmatory WB. The LPS of *F. tularensis* is unique and specific for the detection of LPS antibodies and cross- reactions did not occur. The presence of enzootic regions in Germany as well as serological evidence of immunity in presumably exposed populations are subjects for further investigation.

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REFERENCES

1. **Gurycova D.** First isolation of *Francisella tularensis* subsp. *tularensis* in Europe. Eur J Epidemiol 1998; **14**: 797–802.
2. **Tärnvik A, Sandström G, Sjöstedt A.** Epidemiological analysis of tularemia in Sweden 1931–1993. FEMS Immunol Med Microbiol 1996; **13**: 201–204.
3. **Hornick RB, Eigelsbach H.** Aerogenic immunization of man with live tularemia vaccine. Bacteriol Rev 1966; **30**: 532–538.
4. **McCrumb FRJ, Snyder MJ, Woodward TE.** Studies of human infections with *Pasteurella tularensis*: comparison of streptomycin and chloramphenicol in the prophylaxis of clinical disease. Trans Assoc Amer Phys 1957; **70**: 74–80.
5. **Saslaw S, Eigelsbach HT, Wilson HE, Prior JA, Carhart S.** Tularemia vaccine study, I: intracutaneous challenge. Arch Intern Med 1961; **107**: 121–133.
6. **Saslaw S, Eigelsbach HT, Prior JA, Wilson HE, Carhart S.** Tularemia vaccine study, II: respiratory challenge. Arch Intern Med 1961; **107**: 134–146.
7. **Rohrbach BW.** Tularemia. J Am Vet Med Assoc 1988; **193**: 428–432.
8. **Berdal BP, Mehl R, Meidell NK, Lorentzen-Styr A-M, Scheel O.** Field investigations of tularemia in Norway. FEMS Immunol Med Microbiol 1996; **13**: 191–195.
9. **Koskela P, Herva E.** Immunity against *Francisella tularensis* in northern Finland. Scand J Infect Dis 1982; **14**: 195–199.
10. **Dahlstrand S, Ringertz O, Zetterberg B.** Airborne tularemia in Sweden. Scand J Infect Dis 1971; **3**: 7–16.
11. **Ohara Y, Sato T, Homma M.** Epidemiological analysis of tularemia in Japan. FEMS Immunol Med Microbiol 1996; **13**: 185–189.
12. **Taylor JP, Istre GR, McChesney TC, Satalowich FT, Parker RL, McFarland LM.** Epidemiologic characteristics of human tularemia in the southwest-central States, 1981–1987. Am J Epidemiol 1991; **133**: 1032–1038.

13. **Reintjes R, Dedushaj I, Gjini A, et al.** Tularemia outbreak investigation in Kosovo: case control and environmental studies. Emerging Inf Dis 2002; **8**: 69–73.
14. **Bevanger L, Maeland JA, Kvan AI.** Comparative analysis of antibodies to *Francisella tularensis* antigens during the acute phase of tularemia and eight years later. Clin Diagn Lab Immunol 1994; **1**: 238–240.
15. **Berdal BP, Mehl R, Haaheim H, et al.** Field detection of *Francisella tularensis*. Scand J Infect Dis 2000; **32**: 287–291.
16. **Ericsson M, Tarnvik A, Kuoppa K, Sandstrom G, Sjöstedt A.** Increased synthesis of DnaK, GroEL, and GroES homologs by *Francisella tularensis* LVS in response to heat and hydrogen peroxide. Infect Immun 1994; **62**: 178–183.
17. **Heizmann W, Botzenhart K, Doller G, Schanz D, Hermann G, Fleischmann K.** Brucellosis: serological methods compared. J Hyg 1985; **95**: 639–653.
18. **Moyer NP, Evins GM, Pigott NE, et al.** Comparison of serological screening tests for brucellosis. J Clin Microbiol 1987; **25**: 1969–1972.
19. **Westphal O, Lüderitz O, Biste F.** About the extraction of bacteria using phenol water [in German]. Z Naturforsch 1952; **71**: 148–155.
20. **Sjöstedt A, Eriksson U, Berglund L, Tärnvik A.** Detection of Francisella tularensis in ulcers of patients with tularemia by PCR. J Clin Microbiol 1997; **35**: 1045–1048.
21. **Francis E, Evans AE.** Agglutination, cross-agglutination, and agglutinin absorption in tularemia. Public Health Rep 1926; **41**: 1273–1295.
22. **Karlsson KA, Hurvell B, Thal E.** On the detection of *Yersinia enterocolitica* by the immunofluorescent method. Symposium on *Yersinia*, *Pasteurella* and *Francisella*, Malmö, In: Contributions to microbiology and immunology, vol. 2. Basel: Karger, 1973: 71.
23. **Franek J, Procházka O.** Fluorescent antibody demonstration of Pasteurella tularensis. Folia Microbiol 1965; **10**: 77–84.
24. **Yager RH, Spertzel OR, Jaeger RF, Tigertt WD.** Domestic fowl: source of high titre *P. tularensis* serum for fluorescent antibody technique. Proc Soc Exp Biol Med 1960; **105**: 651–654.
25. **Karlsson KA, Söderlind O.** Studies of the diagnosis of tularemia. Symposium on Yersinia, Pasteurella and Francisella, Malmö. In: Contributions to microbiology and immunology, vol. 2. Basel: Karger, 1973: 224.
26. **Grunow R, Splettstösser W, McDonald S, et al.** Detection of *Francisella tularensis* in biological specimens using a capture enzyme-linked-immunosorbent assay, an immunochromatographic handheld assay, and a PCR. Clin Diagn Lab Immunol 2000; **7**: 86–90.
27. **Sato T, Fujita H, Ohara Y, Homma M.** Micro-agglutination test for early and specific serodiagnosis of tularemia. J Clin Microbiol 1990; **28**: 2372–2374.