Full Research Paper

Piezoelectric Biosensor for a Simple Serological Diagnosis of Tularemia in Infected European Brown Hares (*Lepus europaeus*)

Miroslav Pohanka [1,]*, **František Treml** [2], **Martin Hubálek** [3], **Hana Banďouchová** [4], **Miroslava Beklová** [4] **and Jiří Pikula** [4]

1 Center of Biological Defense, Těchonín, Czech Republic. E-mail: rau@atlas.cz
2 Department of Infectious Diseases and Epizootiology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. E-mail: tremlf@vfu.cz
3 Institute of Molecular Pathology, University of Defence, Hradec Králové, Czech Republic. E-mail: hubalek@pmfk.cz
4 Department of Veterinary Ecology and Environmental Protection, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. E-mail: pikulaj@vfu.cz

* Author to whom correspondence should be addressed. E-mail: rau@atlas.cz

Abstract: Piezoelectric biosensor was used for diagnosis of infection by *Francisella tularensis* subsp. *holarctica* in European brown hares. Two kinds of experiments were performed in this study. First, sera from experimentally infected European brown hares (*Lepus europaeus*) were assayed by piezoelectric biosensor and the seventh day post infection was found as the first one when statistically significant diagnosis of tularemia was possible; all other sera collected from hares later than on day 7 following the infection were found tularemia positive. Typing to classify the field strain of *F. tularensis* used for the experimental infection was confirmed by proteome study. Second, sera from 35 European brown hare specimens sampled at hunting grounds and tested as tularemia positive by slow agglutination allowed diagnosis of tularemia by the piezoelectric biosensor. All these sera of naturally infected hares were found as tularemia positive, too. Efficacy of the piezoelectric biosensor for the serological diagnosis of tularemia is discussed.

1. Introduction

Tularemia is caused by the Gram-negative, facultative intracellular, non-motile and non-spore forming coccobacillus, *Francisella tularensis*. It is a zoonotic disease that may easily be transmitted to humans [1]. Tularemia occurs over the Northern Hemisphere in endemic areas of natural foci [2-4]. Small mammalian species seem to be the most important reservoir of *F. tularensis*, while blood-sucking arthropods play a role as vectors the agent from one host to another. E.g. one study reported that more than 2% of *Dermacentor reticulatus* ticks are harbouring viable *F. tularensis* cells [5]. Furthermore, the epidemiological study performed in Central Europe proved presence of antibodies against *F. tularensis* in 17% of wild boars hunted during years 1993 – 1994 [6]. Tularemia may spread by direct contact with infected individuals and/or indirect contact through inhaling contaminated micro-particles or drinking water containing this bacterium. Siret et al. referred an example of confirming high virulence of tularemia; the infection was spreading by furs from a tularemia infected dog during common dinner [7].

Taxonomical investigations confirmed the existence of four *F. tularensis* subspecies, i.e. *tularensis, holarctica, mediaasiatica* and *novicida*. Further studies analyzed evolutionary relationships [8, 9]. The subspecies *tularensis* (formerly biotype A; in some sources presented as *nearctica*) occurs only in North America, although one isolate from continental Europe was referred to as subsp. *tularensis* [10]. Metabolic fermentation of glycerol and L-citrulline was historically taken as a distinguishing parameter [11]. Nowadays, analysis of 16S rRNA is probably the most frequently used procedure for taxonomical determination of isolates [12]. The subspecies *holarctica* (formerly biotype B or subspecies *palaeartica*) is the second fully virulent *F. tularensis* representative; however, case-fatality rate is lower than in patients infected by the subsp. *tularensis* [13]. Presence of the subsp. *holarctica* in nature was, e.g., studied in rodent populations in China and nearly 5% of rodents were tested as tularemia positive [14]. The last two subspecies, i.e.*mediaasiatica* and *novicida* are less important due to low virulence.

Although traditional detection techniques and assays for *F. tularensis* seem to be numerous [15], biosensors were also referred to as a useful tool for various pathogen assays as well as their serological diagnosis [16, 17] or toxin detection [18]. Biosensors were designed for detection of non-pretreated cells [19], or even found suitable for detection of only one living cell [20]. Piezoelectric biosensors have widely been used and their performance for studies of affine interactions was extensively referred [21]. The most typical piezoelectric biosensors are based on quartz crystal microbalances (QCM) and current improvements of QCM have initiated lowering of detection limits and increased sensitivity [22]. QCM piezoelectric biosensors could be used for detection of whole *F. tularensis* cells [23, 24], and, furthermore, serological diagnosis of tularemia [25] and characterization of antibodies against *F. tularensis* [26] were easily feasible with piezoelectric biosensors.

The presented work is dedicated to designing a piezoelectric biosensor for real serum sample examination. All measuring protocols were simplified for better method applicability for pertinent field tests. Sera were obtained from European brown hares representing the natural reservoir of tularemia and the most important source of infection of humans. Results were compared with those ones obtained using the widely available slow agglutination test.

2. Results and Discussion

2.1 Determination of F. tularensis subspecies

In the first time of this study, we tested the taxonomy of the *F. tularensis* isolate used. Glass slide agglutination was used in the first round of testing. A commercial strain of *Escherichia coli* (Czech Collection of Microorganism) suspended in the same manner as *F. tularensis* served as a negative control. The agglutination serum provided a significant precipitate when suspension of the tested *F. tularensis* sample was assayed; furthermore, the tested *E. coli* sample provided no precipitate. The agglutination test thus confirmed that the tested sample was *F. tularensis*; however, we were not able to confirm the subspecies taxonomy. For this reason, we realized a proteomic study for subspecies determination.

The proteomic study represents a time and cost consuming method; however, the necessary information for subspecies determination can be obtained on a good information level. The obtained isolate was classified as *F. tularensis* subsp. *holarctica*. Following proteins were found as the most obvious markers according to the Phenyx 2.1 database: DNA-binding protein HU-beta, major membrane protein precursor (TUL4), peroxiredoxin (EC 1.11.1), 30S ribosomal protein S5, chaperone GroES, sigma-54 modulation protein and specific sequences in GroEL protein: K/GRNVVLDKSFGAPTITK/D, K/AVTAGMNPMDLKR/G, K/ALDGLTGENDDQNHGIALLRK/A.

2.2 Agglutination test of real serum samples

Real sera from hares naturally infected by *F. tularensis* were obtained and tested as described in the experimental section. A total of 35 sera from hares tested tularemia positive using a rapid agglutination test at hunting grounds were obtained. All these tularemia suspected samples were found as tularemia positive using the slow agglutination method later in the laboratory. Table 1 shows the measured titers for *F. tularensis* and their percentage in this collection of samples.

Table 1. Agglutination test results of 35 tularemia suspected sera. Titers with the proper number of individuals and percentage of the given titer are included in the table. N means the number of individuals attaining the given titre.

| Titers	1: 40	1: 80	1: 160
N	19	14	2
%	54.3	40.0	5.7

2.3 Performance of biosensor on serum from experimentally infected hares

A total of five hares were experimentally infected with *F. tularensis* and blood samples were collected at regular intervals. All serum samples were consequently measured. The preincubation 10 min was used throughout measuring protocol. This preincubation interval is sufficient for maximal saturation of biosensor surface according the previous works where investigation of real binding curves in a flow through arrangement was presented [25, 26]. Outputting signals were calculated

according to the equation 1. The value of f_0 was typically around 9,998,000 Hz, f_a frequencies obtained by immunized sera performance were typically around 150 Hz lower. The real binding curves were not recorded because of biosensor performance as dipstick with signal output into a frequence counter only. The data of Δf are presented as Figure 1. The experimental values were processed by non-linear fitting (Boltzmann function) in Origin 6.1(OriginLab Corporation). Limit of detection (LOD) computed as signal/noise=3 level was included into the graph as a dash line. The biosensor was able to distinguish the immune system response from the 7th day post infection.

The advanced phase of infection can be obviously diagnosed with the piezoelectric biosensor. No value exceeded the zone of LOD so interpretation of data is unambiguous; furthermore, no relevant decrease of output signals was found later during the infection. This part of experiments was made on experimentally infected hares; nevertheless, good interpretation of these data implies supposed good performance in the following part.

Figure 1. Assay of sera from tularemia infected hares. The assay was performed by piezoelectric biosensor. The graph includes outputting signal Δf (Hz) vs. days after experimental infection. The time interval 0 (x-axis) corresponds to the serum from healthy hares (obtained prior to the experimental infection). Standard deviation (n=5) is represented by the error bars. The dash line indicates the limit of detection level (signal/noise = 3).
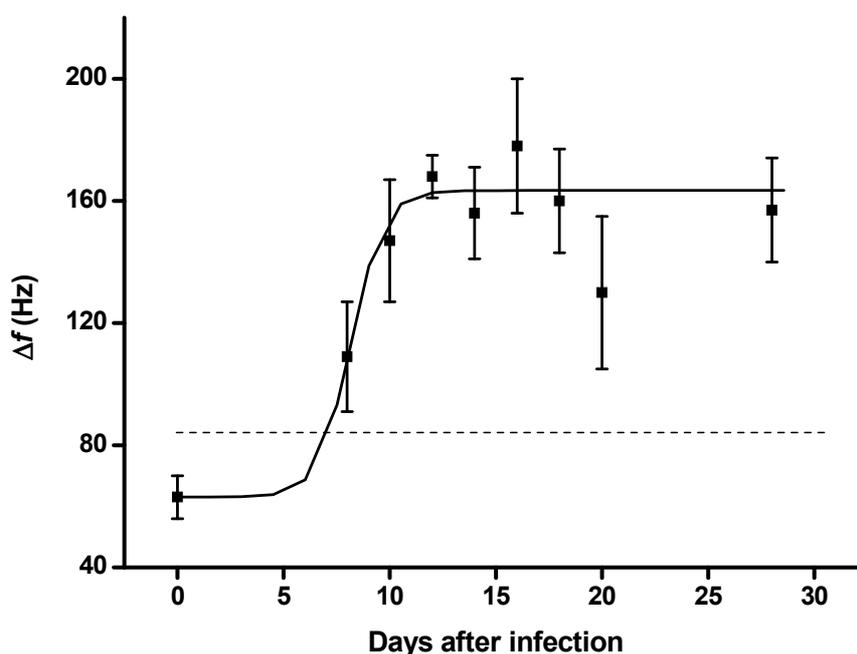
2.4 Assay of real serum samples

Real serum samples from hares were analyzed by the piezoelectric biosensor in the same way as the ones from experimentally infected hares and the data were compared with titers obtained by slow agglutination. Data from both methods are summarized in the following table.

As shown in Table 2, all sera were found tularemia positive using both methods. Agglutination titers from healthy hares were equal or less than ten. The same sera analyzed by piezoelectric

biosensor provided the signal of 63±7 Hz; a similar signal was obtained when hyper-immunized control serum against serum proteins (Sevapharma) was analysed. Both methods could be applied for tularemia diagnosis. Some discrepancies occurred, e.g., when the high titer (~160) serum No. 8 provided the signal of 117 Hz; on the other side, low titer (~40) sera such as No. 15 and 28 provided signals of 208 and 213 Hz, respectively. These discrepancies can be explained by the different principle of signal vizualization. The piezoelectric biosensor is sensitive to the mass bound on the electrode surface. E.g., a sample including IgG provides approximately five times lower signal in comparison with another sample with the same molar concentration of IgM. The piezoelectric biosensor or other methods based on mass studies such as solid phase extraction are more sensitive in the first phase of infection than classical methods such as the enzyme-linked immunosorbent assay; these results were already observed in some other studies [25, 27].

Table 2. Tabular summarization of output signals Δf (Hz) obtained using
the piezoelectric biosensor together with agglutination titers.

Sample No.	Δf (Hz)	titer	Sample No.	Δf (Hz)	titer	Sample No.	Δf (Hz)	titer
1	183	80	13	118	40	25	149	80
2	145	160	14	98	40	26	139	40
3	130	80	15	208	40	27	144	40
4	138	40	16	175	80	28	213	40
5	160	80	17	145	40	29	109	80
6	139	80	18	133	40	30	203	80
7	155	40	19	120	40	31	177	40
8	117	160	20	105	40	32	125	40
9	148	40	21	96	80	33	124	80
10	185	40	22	118	40	34	148	40
11	136	80	23	171	80	35	160	40
12	122	80	24	162	80			

2.5 Conclusions concerning biosensor performance

The piezoelectric biosensor with immobilized antigen from *F. tularensis* was constructed and performed for tularemia positive as well as negative hare sera. The whole experiment was compared with the classical immunological serological method, i.e., slow agglutination. This biosensor was able to significantly diagnose all tested samples and even experiments on laboratory infected hares proved antibodies with adequate level of statistical significance early in the course of infection. The biosensor was found suitable for practical examination of samples; especially, all experiments and the whole device configuration was designed in a purposeful as well as low cost way of minimum consumption of reagents and samples. These facts could appoint the piezoelectric biosensor as a useful tool for serological diagnosis of tularemia in the near future.

3. Experimental Section

3.1 Cultivation and antigen production

Livers from infected hares (South Moravian region, Czech Republic) were homogenized in a glass tube and aseptically spread over plates with McLeod agar with Iso VitaleXTM (Becton-Dickinson) and bovine hemoglobin supplements. Cultivation was realized in a thermostatic box adjusted up 37.0ºC under humid atmosphere for at least one day. Grown colonies were harvested immediately after cultivation and were suspended into phosphate buffered saline (PBS), deionized water or physiological solution for further processing.

Antigen was prepared from cells during freezing/thawing cycles. Partial extraction of the lipid fraction was found as suitable for immunogenic antigen enrichment prior to the freezing cycle. One ml of cell suspension in deionized water with addition of Triton X 100 0.01% was mixed with 1 ml of acetone and let to incubate at 4.0 ºC for two hours. After that, cells were washed by three-time centrifugation (4,000 × g for 10 minutes) in deionized water. Freezing was realized in liquid nitrogen, following thawing under laboratory temperatures. The undisrupted cells were removed by centrifugation (4,000 × g for 10 minutes).

3.2 Bacterial isolate identification

Two methods were considered for isolate identification. Agglutination on a glass slide was used for fast identification of the isolate; however, taxonomical determination of subtype was not possible by this method. For this purpose, a proteomic study was realized. Agglutination was realized in the typical manner when 10 µl of cell suspension (approximately 10^9 CFU/ml) was injected onto a glass slide and consequently mixed with the same volume of agglutination serum against *F. tularensis*. Following it, immunoprecipitation process was observed in daylight.

The proteomic study and mass spectrometry was selected as a suitable procedure for isolate taxonomy investigation. The sample was three times washed by PBS (centrifugation 4,000 × g for 10 minutes) and finally suspended into 1 ml of 8 M urea with two proteases inhibitor, i.e., Complete mini (Roche) and benzoase (Sigma). The suspension was frozen processed by French press (AB Biox) in two rounds. Undisrupted cells were removed from the mixture by fast centrifugation (14,000 × g for 10 min); 200 µl of the supernatant were mixed with 1 ml of 50 mM ammonium bicarbonate buffer and digestion was set up by adding 1 µg trypsin. The mixture was incubated in a box adjusted at 37 ºC overnight; after digestion, the mixture was desalted on HLB cartridge (Waters). The dried eluate was suspended into 50 µl of 2% acetonitrile and 0.1% (v/v) formic acid in water. Three µl of in this way obtained sample were processed on AtlantisTM C18 column (75 µm × 150 mm; Waters) and CapLC system (Waters) interfaced through nanospray with Q-TOF UltimaTM API (Waters). Peptides were eluted by 80 min gradient of acetonitrile. Data were processed using the ProteinLynx script in MassLynx software (Waters) and compared with *Francisella* proteins database in Phenyx 2.1 (GeneBio, Switzerland).

3.3 Experimental animals and their infection; collection of blood samplesfrom naturally infected European brown hares

Hares for experiments were obtained from the European Brown Hare Breeders´ Association of the Czech Republic. The animals were outbred and one year old. Experimental animals were kept individually in special cages for rabbits/hares. They were fed granules for rabbits without supplementation of anticoccidials, high quality hay and provided with drinking water *ad libitum*. Experimental infections were performed by a suspension of *F. tularensis* cells washed down from the culture growing on blood agar with cystine using physiological saline solution. No adjuvans was employed. After a thorough mixing we measured the absorbance of the suspension at 605nm of wavelength using a spectrophotometer (Unicam Helios Gamma&Delta, Spectronic Unicam, United Kingdom) in order to determine the number of bacterial cells per volume units according to the Brown´s method. The obtained number was only approximate and served to estimate the dilution necessary to get the dose required. The exact infectious dose was then determined by culture and counting colony forming units in the suspension administered to the experimental animals. The resulting dose administered to hares amounted to 10^4 bacterial cells. It was injected using a hypodermic needle subcutaneously in the dorsal trunk area. Experimental animals were blood sampled prior to the infection and then every other day. Blood was collected from the jugular vein using an insulin syringe (1ml/40UI, Braun Petzold GmbH, Germany).

Another collection of blood samples used in this study was obtained from naturally *F. tularensis* infected hares that were shot during the 2006 hunting period in South Moravia, Czech Republic. These blood samples were collected by cardiac puncture and processed to obtain serum in the laboratory on the day of collection.

3.4 Agglutination

Blood sera of European brown hares were examined using a commercially available *F. tularensis* antigen for slow agglutination (Bioveta Ivanovice na Hane, Czech Republic). Standard procedures to determine the titre were followed as recommended by the producer to perform microagglutination in plastic well plates.

3.5 Biosensor preparing and measuring setup

The piezoelectric 10 MHz QCMs were purchased from International Crystal Manufacturing (ICM). The crystals had smoothed surface with circular gold electrodes (5 mm diameter) on the opposite sides. Ever new one was washed in acetone for half an hour and dried. 20 µl of cystamine 10 mg/ml modified the gold electrode within 2 hours of incubation. Glutaraldehyde 3% in water activated amino-groups of cystamine self-assembled monolayer (SAM) within another 2 hours of incubation. Finally, the antigen prepared in freezing/thawing cycle (concentration approx. 1 mg/ml) in the amount of 20 µl was injected per electrode and let react in fridge overnight. Free aldehyde groups of glutaraldehyde were saturated by incubation with bovine serum albumin (10 mg/ml).

The measuring device was consisting of the above described biosensor, the lever oscillator (ICM), and frequency counter (Grundig). The described arrangement is displayed as Figure 2.

The typical measuring cycle was as follows:

- Biosensor was dipped into deionized water and frequency f_0 was measured
- 20 µl of serum sample were injected over the biosensor electrode and let incubate for 10 min
- The electrode was washed consequently by PBS, 0.5% Triton X 100, PBS
- The biosensor was dipped into deionized water again and frequency f_a was determined
- Regeneration of the immobilized antigen was realized by incubation of the biosensor in 0.1 M glycine buffer of pH 2.2 with 0.5 % Triton X 100; the biosensor was washed by PBS after regeneration.

Outputting signal Δf was computed according to the following equation (1):

$$\Delta f = f_0 - f_a$$

Figure 2. Piezoelectric biosensor in the lever oscillator (A); on the picture B there is a detailed view of QCM piezoelectric biosensor.
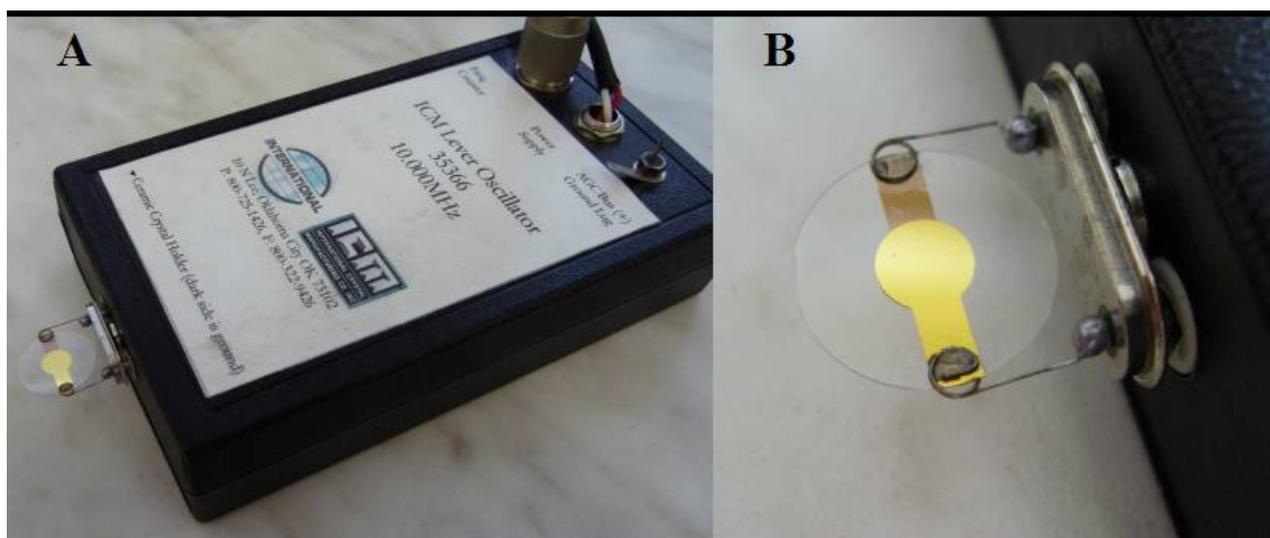
Acknowledgements

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