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Short communication

Diagnosis of tularemia using piezoelectric biosensor technology

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Abstract

A piezoelectric immunosensor for indirect diagnosis of tularemic infection in mouse serum was developed. *Francisella tularensis* LVS antigen was covalently immobilized on the sensing surface using cystamine and glutaraldehyde for activation and modification of the gold electrode. The normal mouse serum (NMS) and serum prepared from mice immunized by *Escherichia coli* were used as negative controls providing signal of 28 Hz during a 5 min interaction. The tularemic infectious (immunized) mouse serum (IMS) as sample resulted in the signal above 75 Hz (fifth day after infection). The control sensor containing bovine serum albumin as sensing element provided a signal below 5 Hz with NMS as well IMS. The effects of dilution degree and purification of sera were tested. To improve resolution of the method, sample pretreatment steps such as precipitation with ammonium sulphate and immunoglobulin extraction on CBind™ L and MEP HyperCel columns were tested. R.S.D. of measurements was 2.3% for NMS and 2.4% for IMS, respectively. The developed method allows to indicate the presence of anti-tularemic antibodies shortly (1–3 days) after infection, one analysis is completed in 10 min.

Keywords: Piezoelectric immunosensor; *Francisella tularensis*; Serum; Biowarfare agent

1. Introduction

Francisella tularensis is the causative agent of tularemia; it is one of the most infectious pathogenic bacteria requiring inoculation or inhalation of only 10–50 organisms [1] to cause the disease. This infection is spread by rabbits (rabbit fever) and rodents. Frequent ways of human infection are biting by the infected animal and inhalation of the airborne bacteria [2]. The other possible ways include glandular, ulceroglandular, oculoglandular, oropharyngeal and typhoidal infections.

F. tularensis belongs to the highly infectious pathogens and is enrolled also on the official list of biological warfare agents. There are several subspecies of *F. tularensis* described in the literature with somewhat confusing nomenclature. *F. tularensis* subspecies *tularensis* (type A; also referred to as subspecies *nearctica* by investigators in the former Soviet Union) demonstrates citrulline ureidase activity; it is highly infectious and virulent and is found primarily in North America. *F. tularensis* subsp. *holarctica* (type B; formerly referred to as *palaearctica* or *palearctica* in some sources) is less virulent and it does not demonstrate citrulline ureidase activity [3,4].

Several methods for *F. tularensis* detection exist, although a rapid diagnostic test for tularemia is not widely available. One of the classical methods is culture isolation from clinical specimens with subsequent identification by the immunofluorescence assay or morphology of colonies. The cysteine enriched broths are used for cultivation; colonies on this medium are typically 1 mm in diameter after 24–48 h of incubation at 37 °C. This method is time consuming and requires special handling. Antigen detection assays (direct methods) include polymerase chain reaction using primers directed against genes encoding outer membrane proteins such as fopA or the 17-kDa lipoprotein; the sensitivity of this method is between 10^2 and 10^4 CFU/mL [5,6]. Enzyme linked immunoassays [7], respectively, enzyme linked immunosorbent assay based tests provide detection limits at 10^4–10^6 CFU/mL [5]. Some works also describe the identification of *F. tularensis* subspecies using comparative proteome analysis [8]. The indirect methods such as agglutination, microagglutination [7,9] or enzyme linked immunoassays detect immunoglobulins M and G [5,7]. Several biosensors for pathogens were described [10], though the detection of *F. tularensis* was rare. The LAPS-based biosensors for *F. tularensis*

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exhibited detection limit of 3.4×10^3 cells during an incubation time of 1 h [11] and the piezoelectric biosensor achieved limit of detection 5×10^6 CFU/mL [12].

Piezoelectric biosensors represent an economic alternative suitable for detection of pathogenic microorganism. These devices allow direct label free monitoring of target molecules and microorganisms in real time [13,14]. In this contribution, we are extending our previous experiments focused on the direct detection of the pathogen [12] towards monitoring production of anti-tularemic antibodies in mouse as a model organism for the infection studies.

2. Materials and methods

2.1. Bacteria and antigen preparation

F. tularensis LVS (ATCC 29684) and *Escherichia coli* (ATCC 9637) were cultured under aerobic conditions at 37 °C. Cells were harvested after 24 h and suspended in 50 mM phosphate buffer pH 7.0 at a density of 10^{10} CFU/mL (turbidimetric assay). The suspension was centrifuged and resuspended either in phosphate buffer (for immunization) or in deionized water (for antigen preparation).

Antigen was prepared by repeated freezing/thawing cycles (eight times) using liquid nitrogen. The undisrupted microbes were eliminated by centrifugation. Thus obtained antigen solution was diluted to the total protein content of 0.15 mg/mL. All experiments with vital *F. tularensis* microbes were performed in the certified (level 3) microbiological facilities.

2.2. Reagents

Cystamine, glutaraldehyde (25%), Triton X 100, 4-chloro-1-naphthol and Bradford Total Protein Kit were obtained from Sigma (St. Louis, MO, USA); Dulbecco modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (Long Island, NY, USA); rabbit anti-mouse serum labeled with horseradish peroxidase was from DAKO (Glostrup, Denmark). All other chemicals were of the highest purity available. Deionized water prepared with a MilliQ system (Millipore) was used throughout. Persteril (Peroxides, Sokolov, Czech Republic) was used for dezinfection of laboratory utilities.

2.3. Animal inoculations and serum preparation

The specific-pathogen-free BALB/c female mice were purchased from ANLAB Prague, Czech Republic. Mice were housed in a certified facility at the Center of Biological Defense, Těchonín. Mice were inoculated subcutaneously with 0.1 LD_{50} of *F. tularensis* LVS or with the equivalent dose of *E. coli* and were divided into 13 groups (six for *F. tularensis* – immunized mouse sera – IMS, six for *E. coli* – control mouse sera – CMS, and one for normal mouse serum – NMS). The sera were collected on days: 1, 3, 5, 7, 10 and 14. Pooled blood from three mice per group was clotted for 30 min at 4 °C and centrifuged at $375 \times g$ for 20 min.

2.4. Dot blot assay

A strip of nitrocellulose membrane (pore size 0.45 μm, Bio-Rad Laboratories, Richmond, CA, USA) was incubated for 1 h at 37 °C with the solution of LVS antigen (1 mg/mL) in phosphate-buffered saline pH 7.2 (PBS); 1 mL was applied per strip of the membrane with 96 slots. The strip was rinsed briefly in PBS and incubated overnight at 4 °C in the culture medium DMEM with 10% FBS to block the residual binding sites on the membrane. The strip was washed for 10 min in PBS and allowed to dry on a filter paper. The samples of sera were spotted onto the nitrocellulose membrane. Two micro liters of sample was dropped on the strip at 1:10, 100, 200, 400 and 800 dilutions in PBS. At the same time, the control positive sera diluted 1:50 in PBS was applied. The strip was further incubated for 1 h at 24 °C followed by washing and drying. Then, the strip was incubated for 1 h at 24 °C with a rabbit anti-mouse serum labeled with horseradish peroxidase diluted 1:100 in 10% FBS and the DMEM culture medium. The strip was washed in PBS and the color was developed by adding 0.6 mg/mL 4-chloro-1-naphthol in PBS with 0.02% hydrogen peroxide. The final reaction was stopped by washing the strip with distilled water. When at least some spots were present, with reactivity stronger or equal to the positive control, the result was considered as positive. A trace reaction or the absence of any reaction was considered as a negative result.

2.5. Sensors and measurement setup

The piezoelectric quartz crystals (10 MHz, optically polished surface, 5 mm gold electrodes) were obtained from International Crystal Manufacturing (ICM, Oklahoma City, OK, USA). The crystal was washed by dipping into acetone for 30 min. A self-assembled monolayer was prepared by spreading 10 μL of 20 mg/mL aqueous cysteamine solution over the electrode area for 2 h at laboratory temperature. After washing with water, thus obtained amino groups were activated with 10 μL glutaraldehyde (3% in water) for 2 h. The electrode was again washed and finally coated with 10 μL of the antigen solution for 7 h at 4 °C. Finally, the surface was blocked for 2 h with 10 mg/mL bovine serum albumin and washed with water. Thus obtained biosensor was stored in the refrigerator.

The experiments were performed with the piezoelectric system in the flow-through arrangement. The sensor with immobilized antigen was placed in a thin-layer flow-through cell with the internal volume of 10 μL. The peristaltic pump PCD 21 M (Kouřil, Kyjov, Czech Republic) was connected with the silicone tube CV1 (Kouřil) to the flow-through cell. The flow rate of the peristaltic pump was adjusted to 50 μL/min. The initial background frequency (signal) was allowed to stabilize for 2–5 min. The detection setup consisted of the Lever Oscillator (ICM) and the universal counter UZ 2400 (Grundig, Fuerth, Germany) connected to PC through the serial port for transfer of data using the own program LabTools.

The analyzed sample—pretreated mouse serum, immune (IMS), control (CMS) or normal (NMS) was pumped through the cell for 5 min. A zone of buffer followed (2–5 min) to sta-

bilize the response. As a signal for evaluation, the difference of frequency in buffer zones before and after sample introduction was used. Regeneration of the sensing surface was achieved during a 5 min flow of 50 mM NaOH with 0.1% Triton X 100.

2.6. Pretreatment of samples

To isolate IgG fraction from the analyzed sera, several procedures were tested including extraction and precipitations. For solid phase extraction (SPE), two materials were evaluated. The MEP HyperCel particles were from BioSepra (Marlborough, MA, USA) and the CBind^{TM} L particles (immobilized protein L) were obtained from Fluka (Buchs, Switzerland). The MEP HyperCel column was loaded with serum sample (up to one volume of the column) and subsequently washed by 10 volumes of 50 mM Tris/HCl pH 8.0. The globulin fraction was eluted using 50 mM acetate pH 4.0. Finally, the column was regenerated with 1 M NaOH for 30 min and two column volumes of 2 M urea. The use of CBind^{TM} L included loading of the sample (one column volume), washing with 20 volumes of 20 mM phosphate buffer with 150 mM NaCl, pH 7.2. Glycine buffer, 100 mM pH 2.2 was used for the elution. The column was cleaned with 20 mM Tris/HCl pH 7.5 containing 6 M guanidine hydrochloride. For both extractions, the pooled eluates were dialyzed against 50 mM phosphate buffer at 4 °C for 1 h.

The saturated ammonium sulphate was used for precipitation of the serum IgG fraction. It was slowly dripped into the serum sample to the final saturation of 40%. The mixture was shaken for 1 h at 4 °C and centrifugated for 20 min at 3000 × *g* at 4 °C afterwards. The supernatant was discarded and the pellet was resuspended in 50 mM phosphate buffer pH 7.0 to obtain the initial volume of the sample.

3. Results and discussion

3.1. Immunosensor assay development

Initially, the mice sera obtained during days 1–14 of infection as well as sera from positive controls were investigated for the presence of antibodies against *F. tularensis* LVS using the commonly used dot blot assay. Distribution of antibody titers (dilutions from 1:10 to 1:800) indicated (Table 1) that no reactions appeared in the first three intervals (1, 3, 5 days after infection). Individual differences were observed in the time of infection and the dilution of the antibodies from 7 to 14 days. The highest immunoglobulin titer (≥200) was found at the 14th day.

Table 1
Titers of antibodies in mouse sera after infection with *F. tularensis* measured by the dot blot technique

Days after infection	Titer of serum
1	–
3	–
5	–
7	≥10
10	≥10
14	≥200

The positive controls (diluted 1:50) were always evaluated as positive during all days.

Further experiments were carried out using the immunosensor. The interaction of the sera (both positive and negative) diluted 5-times with the sensing surface containing covalently immobilized tularemic antigen are shown in Fig. 1. The negative control – NMS serum from normal non-infected mouse – provided quite large response of 59 Hz within the 5 min binding interval. The rather high background response obtained due to the non-specific adsorption of the NMS control was a complication. However, a significant increase of response was obtained in the case of serum samples taken from infected mice on different days after inoculation. Even 1 day after inoculation, the response was 73 Hz, and 128 Hz for the serum from day 14.

The influence of sample dilution degree was tested in order to improve resolution of the immunosensor. The binding experiments were carried out using the sample providing the strongest immunity response (Fig. 2). The scale 0 (undiluted), 1:5, 1:10, 1:20 and 1:40 was selected for dilution using phosphate buffer pH 7.0. The serum collected on the 14th day after inoculation was used as IMS. The IMS and NMS were measured with the piezoelectric immunosensor and the ratio $\Delta f_{IMS}/\Delta f_{NMS}$ was always calculated from mean values ($n = 3$). As expected, the ratio gradually improved from 2.3 at dilution degree 0 and sera diluted more than 1:10 provided the best resolution of IMS and NMS signals, as the concentration of ballast proteins decreased. The dilution degree of 1:10 was chosen as optimum—a compromise providing good resolution of infected/normal sera and reasonably high signals for a short 5 min-lasting measurement.

Several sample pretreatment methods were tested in order to improve assay resolution and shorten the time of analysis. The original serum, serum precipitated with ammonium sulphate and immunoglobulin fraction extracts obtained using CBind^{TM} L and MEP HyperCel were compared for influence on the IMS:NMS ratio of signals. Each sample was diluted 10 times; the serum collected on the 14th day after inoculation was

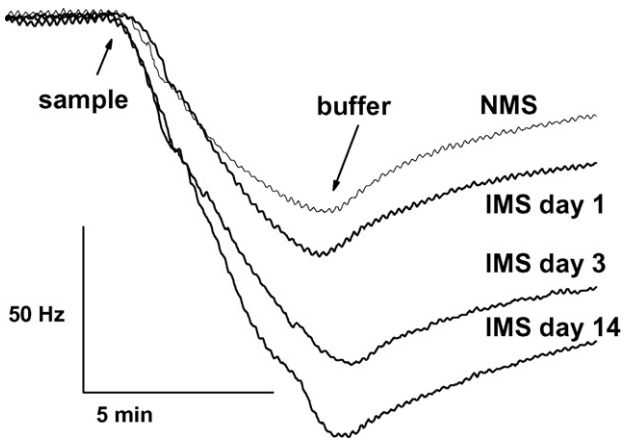

Fig. 1. The example of interaction traces (resonant frequency vs. time plots) obtained in the course of serum samples binding on the piezoelectric immunosensor with covalently immobilized tularemic antigen. The control (NMS, thin line) and infected (IMS, thick lines) mouse sera were diluted 5-times using the carrier buffer.

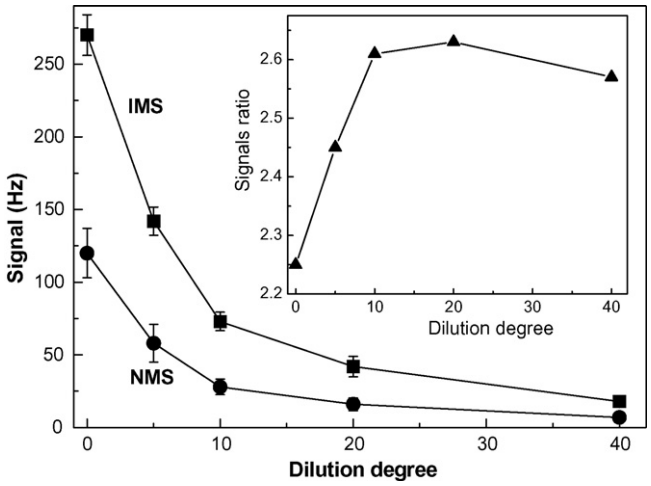

Fig. 2. The effect of serum dilution degree on response of the piezoelectric immunosensor. The signal (Δf) of IMS ((■), collected 14th day after inoculation) and NMS (●) is presented. Dilution scale is from 0 (undiluted) to 40 (diluted 1:40 by phosphate buffer pH 7.0). The inset graph presents the corresponding ratio of signals for IMS and NMS samples ((▲), $\Delta f_{IMS}/\Delta f_{NMS}$).

used throughout. As shown in Table 2, both extraction procedures did not exhibit significant influence on the signals ratio in comparison with the non-pretreated serum (the ratio equal to 2.61); the change was to 2.66 and to 2.64 for MEP HyperCel and CBind^{TM} L, respectively. Considering that extraction is a time consuming step increasing the time of analysis for more than 100 min and the effect on the resolution was minimal, the extraction methods were abandoned. A better result was obtained using the ammonium sulphate precipitation, the ratio of signals IMS:NMS improved significantly from 2.61 to 3.76. A disadvantage of precipitation was the required time of analysis adding more than 2 h to the simple measurement of the non-pretreated serum. On the other hand, a higher sensitivity represents an important advantage for detection of positive sera early at the beginning of infection. However, one has to consider the difficulty in reproducibility of this manual method. Therefore, the analysis of non-pretreated serum samples appears most promising due to the short time of assay and no complicated additional steps.

The extraction and precipitation methods provide good explanation of the NMS signal character. MEP HyperCel and CBind^{TM} L were designed for purification of immunoglobulins and in the serum sample persist immunoglobulins with different class specificity. On the other hand, precipitation with ammonium sulphate provided mostly IgG fraction. Thus, the non-specific signal of NMS was probably mainly caused by the native immunoglobulins as IgM, which are removed during the precipitation. In addition, the remaining part of the background signal can be attributed to the presence of early-phase IgA exhibiting rather broad specificity, physiologically important for the immediate reaction with bacterial antigens in vivo. This, however, contributes also to the high background response with NMS in our measurements.

Table 2
Comparison of serum pretreatment methods

Pretreatment	Δf_{IMS} (Hz)	Δf_{NMS} (Hz)	$\Delta f_{IMS}/\Delta f_{NMS}$ ratio	Analysis time (min)
None	73	28	2.61	10
Precipitation, $(NH_4)_2SO_4$	79	21	3.76	160
Extraction, CBind^{TM} L	66	25	2.64	120
Extraction, MEP HyperCel	85	32	2.66	120

Signals (Δf) from the piezoelectric immunosensor with immobilized *F.tularensis* antigen for IMS (collected 14th day after inoculation) and NMS. The ratio of both signals ($\Delta f_{IMS}/\Delta f_{NMS}$) is shown, too. The analysis time does not include the collection of blood.

3.2. Monitoring of the infection progress

The crude non-purified sera collected from the infected mice in intervals of 1, 3, 5, 7, 10 and 14 days after inoculation were tested by the piezoelectric immunosensor. NMS from healthy mice and CMS from mice immunized by *E. coli* were used as negative controls. Each sample was diluted 10 times. The overall progress of antibody production is presented in Fig. 3. The infection process was evident even on the first day after inoculation according to the signal of 33 Hz for IMS while NMS provided only 28 Hz. The rapid increase of signal approached maximum on the fifth day after inoculation (87 Hz) and the further increase was lower. The highest signal was obtained on the 14th day after inoculation (95 Hz; a different value compared to the corresponding value of 73 Hz in Table 2 was due to the use of a different immunosensor).

The signal increase observed on the fifth day after inoculation corresponds with the first manifested symptoms of disease [15]. No relevant difference between signal of NMS and CMS was observed. The control sensor containing immobilized bovine

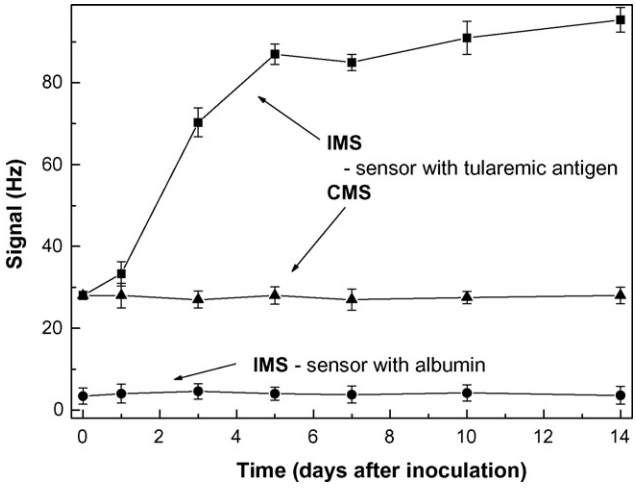

Fig. 3. Monitoring of time increase of antibody levels in mice sera (10-times diluted) after inoculation with *Francisella tularensis* (IMS, (■), (●)) and *Escherichia coli* (CMS, (▲)). The specific response was obtained for IMS using the piezoelectric sensor modified with tularemic antigen (■). For negative control experiments, levels in IMS were followed also using the sensor modified with albumin (●) and the levels of anti *E. coli* antibodies were followed using the sensor with tularemic antigen (▲). The beginning (day 0) of the curves corresponds to normal mice. The error bars indicate estimated standard deviations ($n = 3$).

serum albumin as sensing element provided signals below 5 Hz with all the tested sera – CMS, NMS as well as IMS.

The obtained results were evaluated using the *t*-test (IMS versus NMS, both measured on the specific immunosensor with immobilized LVS antigen, $n = 3$). The results measured 1 day after inoculation can be classified as positive with the probability level of 0.95; results from the following days (third and higher) were detected with the probability level of 0.99. The R.S.D. values for the NMS and IMS (day 14) samples were 2.3 and 2.4% for intra-day measurements ($n = 5$).

4. Conclusions

A novel method for the indirect detection of *F. tularensis* was developed based on the measurement of anti-tularemic antibodies in serum samples of infected mice. The piezoelectric immunosensor with covalently immobilized tularemic antigen was compared with the standard dot blot method. The dot blot was only positive during the late phase of infection – in the last three of the six intervals, whereas piezoelectric biosensor provided positive signals in all intervals. Those results demonstrate significantly that both methods are able to detect the later phases of infection. The piezoelectric biosensor is able to detect the onset of the infection process very early, even 1 day after injection of the bioagent. The advantage of this method is a simple direct arrangement with low cost of analysis. The proposed concept of the immunosensor seems to be suitable also for screening of human sera for potential infection with tularemia, but no such positive samples were available in our country.

The current efforts are mainly focused on the direct detection of harmful bioagents as bacteria, viruses, toxins and other pathogens and biosensors play quite important role. However, for the highly toxic bioagents causing infection in only a very small dose (low concentration, few microorganisms only), the primary detection could potentially fail and the bioagents will remain undetected. In this case, the highly sensitive secondary detection of the infection becomes extremely important. In this contribution, we have shown that the piezoelectric immunosensor represents a promising alternative tool suitable for rapid detection of the onset of infection.

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