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# ELISA Detection of *Francisella tularensis* using Polyclonal and Monoclonal Antibodies

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**ABSTRACT**

The mouse monoclonal and polyclonal antibodies were produced for the detection of intracellular pathogen and potential warfare agent *Francisella tularensis*. Antibody titers obtained were 1:640 for polyclonal antibodies and 1:320 for monoclonal antibodies. Both antibodies were used in the indirect enzyme-linked immunosorbent assay (ELISA) found to detect *F. tularensis* whole cells. The limit of detection was 5.4×106 CFU/ml for polyclonal antibodies and 6.9×106 CFU/ml for monoclonal antibodies. The value sample could be distinguished from any concentration of another gram-negative bacterium: *Escherichia coli*.

**Keywords:** Tularemia, *Francisella tularensis*, ELISA, biowarfare agent, immunodetection, enzyme-linked immunosorbent assay, antibodies, pathogen detection

## 1. INTRODUCTION

The potential biological warfare (BW) agent *Francisella tularensis* is a causative agent of tularemia in humans, with several manifestations and different severity based on the route of entry of the pathogen. The last large-scale epidemic of tularemia was observed in 1966-67 in Sweden[1]. Due to low-infection dose, up to ten cells[2] and natural spreading through small rodents and mosquitoes combined with simple cultivation, Center for Disease Control and Prevention in Atlanta enrolled *F. tularensis* as the one of the most risky BW agents it was enlisted into category A between six most important BW agents. Importance of *F. tularensis* as BW agents in comparison with others BW agents was evaluated by a mathematical model[3]. The naturally occurring tularemia infection exhibits the mortality[4] rate less than 3 per cent for ulceroglandular form. The infection is curable with chemotherapy using streptomycin[5], gentamicin[6] or fluoroquinolone derivates[7].

The enzyme-linked immunosorbent assay (ELISA) has been proved as a reliable technique for the diagnosis of several serious diseases and detection of causative agents. Sera from patient suffering with anthrax were evaluated using ELISA[8] method; the whole *Bacillus anthracis* cells were detected using ELISA and compared with PCR and flow cytometry[9]. Agglutination test and ELISA were used for serological testing of sera from patients suffered from brucellosis[10] and antibodies against *Brucella melitensis* were detected in sheep milk[11]. Murine serum from mice, experimentally infected with Yersinia pestis, was evaluated both for specific antibodies and antigens[12], and plague was diagnosed. Antibodies specific to outer lipopolysaccharide (LPS) antigen O157 from *Escherichia coli* were detected by blocking ELISA[13]. Maltseva[14] *et al.* used ELISA for serological screening for orthopoxviruses. ELISA is frequently used for diagnosis of tularemic infection and also for direct or indirect detection of *F. tularensis*. Evaluation of patients' sera by ELISA was made by Carlsson[15] *et al.* for serotyping; wherein LPS antigen from *F. tularensis* outer membrane and polyclonal antibody labeled by horseradish peroxidase were used. Antibodies against *F. tularensis* were examinated in serum from wild boars[16], and human sera from Norway, Sweden and Kosovo[17]. The comparison of ELISA with other immunological methods for patients' sera has also been reported[18]. Presence of F. tularensis cells was detected by ELISA in tissue samples from European brown hares and results were compared with immunochromatographic handheld assay as also by PCR [19]. Detection by biosensors was also proposed as a method for the detection of BW agents including *F. tularensis*[20], and application of biosensors allowed detection of whole *F. tularensis* cells[21] as well as serological diagnosis of tularemia[22]. Historical aspect of *F. tularensis* as potential BW agent was also reviewed[23].

As ELISA is a very routine method in middle-lend and larger-lend laboratory facilities designated for defence and/or epidemiologic purposes, our preserving effort is aimed at optimisation of ELISA method parameters. Improving of ELISA applicability in a manner for the use of antibodies generated in the authors Laboratory was the aim of the present study. One of the most important BW agent, *F. tularensis*, was chosen as a model.

## 2. EXPERIMENTAL DETAILS

### 2.1 Preparation of Bacteria and Antigen

As model strain of *F. tularensis* LVS (ATCC 29684)

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was used. This strain expresses reduced virulence in humans but remains fully virulent in rodent models of infection. Bacteria were cultured on McLeod agar (Thayer-Martin agar base, Merck Eurolab, Stockholm, Sweden; Bacto agar, Oxoid, Basingstoke, United Kingdom) supplemented with bovine hemoglobin (Bovine hemoglobin, BD, Franklin Lakes, NJ USA) at 36.9°C and collected after 24 hour period. Cells were suspended in saline solution and bacterial content was determined by re-cultivation. *F. tularensis* antigen was prepared by 8 cycles of repeated freezing in liquid nitrogen and thawing of bacterial suspension as published previously[22,24-25]. Finally antigen solution was adjusted to 0.15 mg/ml after determining by Bradford Total Protein Kit (Sigma, St. Louis, MO, USA). Another gram-negative bacterium Escherichia coli (ATCC 9637) was used as negative control.

### 2.2 Preparation of Polyclonal Antibodies

The polyclonal antibodies specific to *F. tularensis* were raised in 10 female BALB/c mice (BioTest, Konárovice, Czech Rep.). Mice were subcutaneously inoculated with 102 CFU of *F. tularensis* in 100 µl of saline solution and after 14 days immunised s.c. with $10^6$ CFU. On 21st day after first inoculation, mice were boosted with 100 µl of antigen solution (prepared as mentioned above) mixed with 100 µl of Freund´s complete adjuvant (FCA) and on 28th day they were inoculated with the same$10^2$ CFU of *F. tularensis* in saline solution, except FCA was used. Non-immune (control) serum was obtained from three donor mice, which were inoculated only with saline solution at the same intervals as used for immunisation. Blood was collected after 35 days by a cut of the ascellary artery. Blood samples were clotted initially for 1 h at 4 °C then again for 1 h at 37 °C, centrifuged first at 95 ×g for 5 min and finally at 375 ×g for 20 min.

### 2.3 Preparation of Monoclonal Antibodies

Monoclonal antibodies against *F. tularensis* were obtained by hybridoma technology using live *F. tularensis* LVS cells. The mouse spleen cells were obtained in the same immunisation protocol as described in the case of polyclonal antibody preparation and were fused with the mouse myeloma cell line Sp2/0-Ag14. After cell fusion using PEG (Sigma, St Louis, MO, USA), the selection in HAT medium (Sigma) was performed. The selective pressure was maintained for 15 days, to guarantee the extinction of all the undesired cells. After this period, all the cells were held in a medium supplemented with HT medium (Sigma). Finally, this medium was removed and the cultures were maintained in DMEM (GIBCO BRL, Grand Island, NY, USA). Hybridomas were expanded and further screened by ELISA using *F. tularensis* antigen. The positive hybridomas were selected for limiting dilution cloning and cryopreserved. From individual clones, isotypes of antibodies were evaluated by mouse monoclonal antibody isotyping kit (Roche, Indianapolis, IN, USA).

### 2.4 Solid-phase Extraction and Protein Amount Determination

The total protein amount of poly- and monoclonal antibodies was determined by Total Protein Kit (Sigma). For determination of immunoglobulin fraction protein amount, solid-phase extraction (SPE) on the column with CBind™ L (Fluka, Buchs, Switzerland) was used according previously used protocol[26]. Column was washed with 20 mM phosphate buffer with 150 mM *NaCl*, p*H* 7.2. glycine buffer (100 mM p*H* 2.2) was used for elution. The column was washed with 20 mM Tris/*HCl* p*H* 7.5 containing 6 M guanidine hydrochloride. After purification, the amount of immunoglobulins was determined by total protein kit.

### 2.5 Indirect Enzyme-linked Immunosorbent Assay

A 96-well microplate (Gama, Ceské Budejovice, Czech Rep.) was coated with 100 µl per well of bacterial cells (*F. tularensis* or *E. coli*) suspended in saline solution for 2 h. Afterwards, the plate was washed with saline solution and blocked with 150 µl of gelatin (Merck, Whitehouse Station, NJ, USA) for 1 h and washed. For blank purposes, only three wells were coated with gelatin. Into each well, 100 µl of 10-time diluted monoclonal or polyclonal antibodies (in triplicate for each sample) was added and plates were incubated at 37 °C for 60 min. After washing with saline solution containing 0.2 per cent of Triton X-100, 100 µl of 1:100 diluted antibodies with specificity against IgM and labeled with HRP (Serotec, Oxford, UK) were added per well. Plates were incubated at 37 °C for 30 min and washed with saline solution containing 0.2 per cent Triton X-100. Finally, 100 µl freshly prepared solution containing 0.5 mg/ml orthophenylendiamine (OPD) and 5 mM $H_2O_2$ was added in dark and allowed to react for 1 min. Reaction was stopped with 100 ml of 2 M $H_2SO_4$. Optical density was measured on ELISA reader MRX (Dynatech Laboratories, Chantilly, VA, USA) with wavelength set to 490 nm.

Titers of monoclonal and polyclonal antibodies were obtained in the same way by ELISA as described above. Bacterial sample in this procedure was replaced by antigen from *F. tularensis* prepared by freeze/thaw cycles. Titers of either IgG or IgM were determined by secondary antibody labeled with HRP (Serotec, Oxford, UK) and specific against mouse IgG or IgM.

## 3. RESULTS AND DISCUSSION

### 3.1 Preparation of Antibody

Two types of antibodies were obtained: polyclonal from mice suffering from tularemic infection combined with consequent immunisation by antigen suspended into Freund´s complete and incomplete adjuvant and monoclonal antibody 3E5B10G4. Monoclonal antibody was determined using isotyping kit as class IgM isotype. Titers obtained by ELISA and amount of protein in antibody solution for polyclonal as well as monoclonal antibody and control (normal) mouse sera are presented in Table 1. It was found that the highest obtained titers were 1:640 for polyclonal antibody and 1:320 for monoclonal antibodies when assayed both for IgM fraction. IgG fractions had minimal titers for both types of antibodies. Amount of immunoglobulin was determined by total protein kit after solid phase extraction and compared with total

**Table 1. Presentation of some basic characteristics of polyclonal and monoclonal antibodies* and control serum obtained from BALB/c mice**

| | Polyclonal antibody | Monoclonal antibody | Control mouse serum |
|---|---|---|---|
| Total protein (mg/ml) | 85.7 | 35.7 | 75.4 |
| Globulins (mg/ml) | 42.5 | 17.5 | 28.0 |
| Titer of IgM | 1:640 | 1:320 | 1:10 |
| Titer of IgG | 1:20 | <1:10 | 1:10 |

* Antibodies were specific against *F. tularensis* and titer was tested by indirect ELISA. Titer under 1:10 is not significant. Globulin content was determined by solid phase extraction and total protein kit.

protein. Monoclonal antibody content was determined to be 17.5 mg/ml and polyclonal antibody of 42.5 mg/ml while the control mouse serum had 28.0 mg/ml.

In this study, no additional steps were used for the concentration or other pretreatment of antibodies in order to reduce costs per analysis. Antibodies, hybridoma culture supernants and mouse immune sera were used in a crude state diluted ten times.

### 3.2 Indirect ELISA for the detection of *F. tularensis*

Dependence of optical density as ELISA output value on concentration of *F. tularensis* as analyte and *E. coli* as negative control, has been expressed for polyclonal antibody in Fig. 1. and for monoclonal one in Fig. 2. Reached limit of detection (LOD) (LOD, S/N=3) was $5.4\times10^6$ CFU/ml for polyclonal antibody and 6.9 × 106 CFU/ml for monoclonal one. Optical density obtained for *F. tularensis* in amount equal to limit of detection was significantly higher from the optical density obtained for *E. coli* adjusted at any concentration.

If the ratio of optical densisty for signals obtained from *F. tularensis* and *E. coli* samples is expressed for both

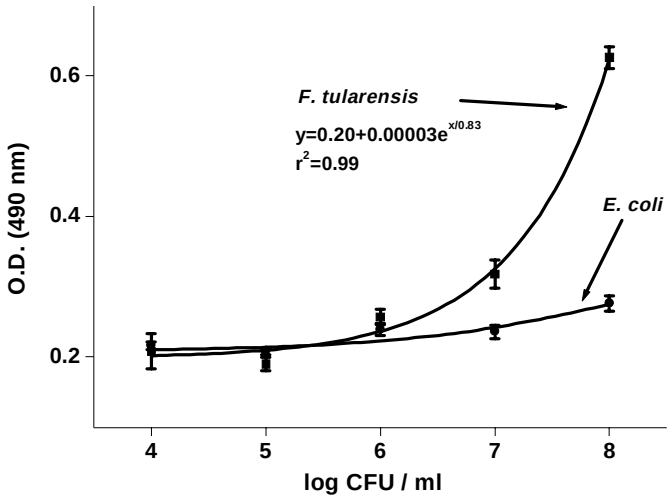


**Figure 1. Indirect ELISA for *F. tularensis* detection using polyclonal antibody. The whole cells were loaded on the microplate and *E. coli* was used as negative control. Output value optical density obtained from ELISA reader was used. The error bars indicate estimated SD (*n* = 3).**

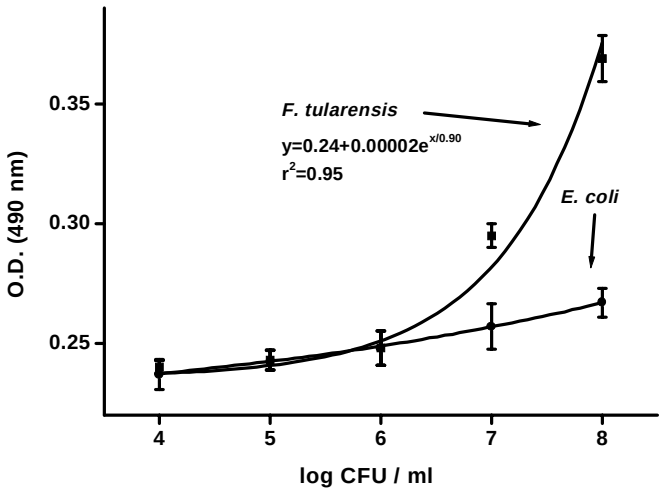


**Figure 2. Indirect ELISA for monoclonal antibody. The whole cells were loaded on the microplate. *F. tularensis* as analyte and *E. coli* as negative control were used. Output value optical density obtained from ELISA reader was used. The error bars indicate estimated SD (*n* = 3).**

of antibodies (Fig. 3), one can see more sensitive signal output for polyclonal antibody in comparison with quite flat output for monoclonal antibody.

Although limit of detection evaluated as ~ $10^7$ is quite above the infection dose for *F. tularensis*; however, on the other side, this limit of detection is quite sufficient when employed after pre-enrichment step. With regard to sensitivity, ELISA is not able to compete with such methods like PCR. On the other side, ELISA is advantageous in the way of number simultaneously analysed samples in combination with low costs per unit analysis. If six wells were used for negative and positive controls, it was possible to contemporary detect as much as 90 samples in one contemporary analysis on one standard microplate. ELISA is convenient

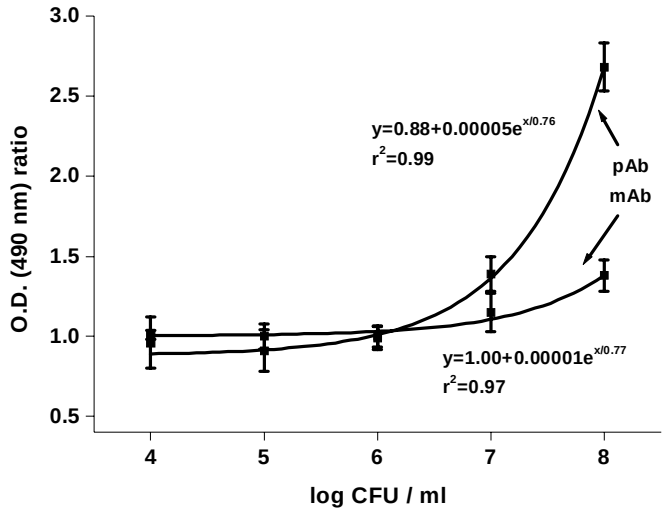


**Figure 3. Plot expressing polyclonal and monoclonal antibodies sensitivity in indirect ELISA. In this figure, value was obtained from the analyte (*F. tularensis*) signal divided by signal received from *E. coli* for each antibody. Data were taken from Fig. 1 and 2. The error bars indicate estimated SD (*n* = 3).**

for characterisation of pre-cultivated microbes (including *F. tularensis*) when combined with classical cultivation tests. Therefore, cultivated cells were used throughout this study to maintain standard test conditions.

One of the main tasks of this work was the comparison of monoclonal and polyclonal antibodies applicability for *F. tularensis* detection. The polyclonal antibody exposed better analytical parameters. Current legislative in Czech Republic and common legislative trends in the world make experiments on animals more difficult, and therefore is simpler to produce monoclonal antibodies from stocked hybridomas. Costs of polyclonal antibody production are not marginally higher than in the case of monoclonal one with regard to all consumables and laboratory equipment needed. Nevertheless, as monoclonal antibody preparation is more elaborative, production of polyclonal antibody remains more readily approachable.

Practical impact of ELISA method for defence purposes is another task of this study. Since facilities for ELISA method are available in local and regional laboratories in many countries including Czech Republic, it predominantly acts as a tool for serological diagnosis in laboratory routine. Any misuse of BW agent, including the one based on *F. tularensis*, should be detectable in a number of laboratories. For this, reason could be ELISA simple approachable due to the presence in clinical labs including the military ones. The present study confirms the applicability of ELISA method for mass processing of bacterial isolates. Even limit of detection (LOD) was approximately on the similar level as in the case of performance of biosensors for *F. tularenis*[27] or *E. coli* [28]. Though the mobile laboratories and reconnaissance teams are typically equipped with portable PCR (such as R.A.P.I.D. or Razor) for its better sensitivity, established non-mobile laboratories should use ELISA method for confirmation of isolates previous or contemporary identification process. Performance of the device was targetedly realised on *F. tularensis*. Accordingly, Centre for Disease Control, Atlanta, enlisted six most important BW agents. One should notice that samples cultivation processes, and ELISA performance were realised just in the same way as one could process real samples.

## 4. CONCLUSIONS

Mouse polyclonal and monoclonal antibodies specific against intracellular pathogen, and one of the most important BW agent *F. tularensis*, were prepared, and consequently the ELISA method for detection of *F. tularensis* was developed. The limit of detection (LOD) was relatively high; on the other side, the high number of contemporary measured samples and low cost per unit analysis is pretty advantageous. From the two tested antibodies, better presumptions to be used in ELISA kit, construction proved the polyclonal one over the monoclonal one.

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