# Development and Evaluation of a Latex Agglutination Test for the Identification of *Francisella tularensis* Subspecies Pathogenic for Human

WALDEMAR RASTAWICKI[1], KAMILA FORMIŃSKA[2] and ALEKSANDRA A. ZASADA[2*]

[1] Department of Bacteriology, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland
[2] Department of Sera and Vaccines Evaluation, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland

A b s t r a c t

*Francisella tularensis* are highly infectious bacteria causing a zoonotic disease called tularemia. Identification of this bacterium is based on antigen detection or PCR. The paper presents a latex agglutination test (LAT) for rapid identification of clinically relevant *F. tularensis* subspecies. The test can be performed within three minutes with live or inactivated bacteria. The possibility to test the inactivated samples reduces the risk of laboratory acquired infection and allows performing the test under BSL-2 conditions.

K e y   w o r d s: *Francisella tularensis*, *F. tularensis* identification, latex agglutination test, tularemia, zoonosis

Tularemia is a zoonotic, highly infectious disease caused by an intracellular Gram-negative bacterium, *Francisella tularensis*. The disease affects a wide range of hosts including invertebrates, mammals and birds. Humans can become infected by direct contact with an infected animal (through broken skin, scratch or tissue injury), through a bite of haematophagous arthropods (*e.g.* fleas, lice, midges, bedbugs, mosquitoes, ticks), by drinking contaminated water, eating contaminated food, or through inhalation of contaminated dust (Formińska *et al.*, 2015). The clinical presentation in humans depends on the route of infection and varies in symptoms and severity (Eliasson *et al.,* 2006). There are four subspecies of *F. tularensis: tularensis*, *novicida*, *mediasiatica* and *holarctica*. Of these, subsp. *tularensis* and subsp. *holarctica* cause disease in humans, whereas subsp. *mediasiatica* is believed to be of relatively low virulence in humans, and only rare cases of human disease caused by this subspecies are known. *F. tularensis* subsp. *novicida* is non-pathogenic for humans (Celli and Zahrt, 2013).

According to WHO case definition, a confirmation of tularemia case requires recovery of an isolate and identification of the culture as *F. tularensis* by antigen or DNA detection. Commercial biochemical identification systems available in clinical diagnostic laboratories are not suitable for accurate identification of *F. tularensis*.

Alternatively, paired serum specimens with a fourfold difference in titre (tube or microagglutination assay) or significantly (ELISA), with at least one serum positive, are also considered confirmatory (WHO, 2007). However, antibody against *F. tularensis* are detectable in patients' serum 10–20 days post-infection (Koskela and Salminen, 1985). Thus, usefulness of antibody detection tests is limited in severe cases and when a rapid preventive action must be undertaken. On the other hand, a *F. tularensis* antiserum (Becton Dickinson Diagnostic Systems) recommended by WHO for the slide agglutination test for *F. tularensis* culture identification has been withdrawn from the manufacturer's offer and is not available on the market any more. Below we described the latex agglutination test (LAT) for the rapid identification of *F. tularensis* isolates that could be an alternative for the classical slide agglutination test.

For the preparation of sera for coating of latex beads we used pooled serum samples obtained from 25 patients with high level of IgM antibodies to *F. tularensis,* specified as $OD_{450}$ higher than 1,80 by ELISA (Rastawicki and Wolaniuk, 2013; Rastawicki *et al.,* 2015). The gamma globulin fractions of sera were isolated by 40% ammonium sulfate fractionation in cold-bath, and the precipitate was resuspended in phosphate-buffered saline (PBS), pH 7.4. The solution was subsequently dialyzed against PBS for 48 hours until

* Corresponding author: A.A. Zasada, Department of Sera and Vaccines Evaluation, National Institute of Public Health – National Institute of Hygiene, Warsaw, Poland; **e-mail: azasada@pzh.gov.pl**

Table I
Bacterial strains used in the study and results of the latex agglutination test (LAT)
after 1, 3 and 5 minutes of rocking.

| Microorganism | Result of latex agglutination test* | | |
|---|---|---|---|
| | 1 min | 3 min | 5 min |
| *Francisella tularensis* ssp. *holarctica* A 104-15 | + | +++ | +++ |
| *Francisella tularensis* ssp. *holarctica* LVS | + | +++ | +++ |
| *Francisella tularensis* ssp. *tularensis* Ft33 | + | +++ | +++ |
| *Francisella tularensis* ssp. *novicida* Ft26 | – | – | – |
| *Yersinia enterocolitica* ATCC 23715 | – | – | – |
| *Shigella sonnei* WHO SH 16-1 | – | – | – |
| *Serratia marcescens* ATCC 14756 | – | – | – |
| *Pseudomonas aeruginosa* ATCC 27853 | – | – | – |
| *Salmonella* Enteritidis ATCC 13076 | – | – | – |
| *Salmonella* Typhimurium ATCC 14028 | – | – | – |
| *Proteus vulgaris* ATCC 6380 | – | – | – |
| *Escherichia coli* ATCC 25922 | – | – | – |
| *Acinetobacter baumannii* ATCC BAA-1605 | – | – | – |
| *Enterobacter cloacae* ATCC BAA-1143 | – | – | – |
| *Staphylococcus aureus* ATCC 25923 | + | +++ | +++ |
| *Staphylococcus aureus* – clinical strain 1/2017 | – | – | + |
| *Staphylococcus aureus* – clinical strain 2/2017 | – | + | ++ |
| *Staphylococcus aureus* – clinical strain 3/2017 | – | + | ++ |
| *Staphylococcus aureus* – clinical strain 4/2017 | – | – | + |
| *Staphylococcus aureus* – clinical strain 5/2017 | – | – | – |

* – negative; +/++/+++ weak/strong/very strong positive

ammonium sulfate had been removed. Then, 5.0 ml of washed, 1% suspension of 0,81 µm latex particles in glycine-saline buffer (pH 8.2) was added to equal volume of twice-diluted in glycine-saline buffer purified gamma globulins. The mixture was vortexed for 1 min and then allowed to incubate with gentle stirring at 37°C for 6 h. After incubation, sensitized latex particles were recovered by centrifugation, washed twice with glycine-buffered saline and finally diluted to 1% with glycine-buffered saline (pH 8.2) containing 0.1% sodium azide and 0.3% of BSA. For control, the latex reagent particles were sensitized with bovine albumin (Sigma Chemical Co., USA).

The investigated strains of *F. tularensis* (Table I) were cultured on enriched chocolate agar plates. After 48 h of incubation, a small loopful of bacteria from the strain investigated was suspended in 100 µl of PBS. The latex particles sensitized with gamma globulins and the particles sensitized with albumin bovine-control latex reagent, were parallel mixed with the same volume (25 µl) of bacterial suspensions on a black glass plate. The results were read after 1, 3 and 5 minutes of rocking the plate. Agglutination (clumping of cells) was scored as: -negative; +/++/+++ weak/strong/very strong positive. A positive latex agglutination test and a negative

latex control test were confirmatory for *F. tularensis*. To assess potential cross-reactivity of the developed latex test we used the 16 different control bacterial strains as presented in Table I. The procedures of LAT with control strains were the same as for *F. tularensis*.

All manipulations with viable *F. tularensis* strains were done under biosafety level 3 (BSL-3) conditions. Keeping in mind that most diagnostic laboratories work under BSL-2 conditions, to minimize the risk of infection we also evaluated the test using inactivated bacterial suspension. For inactivation the bacterial suspension in PBS was heated at 96°C for 15 min, then cooled and used for the LAT. To verify the effectiveness of inactivation, 50 µl of the suspension were inoculated onto enriched chocolate agar plates and incubated at 37°C for 10 days.

The agglutination reactions with *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* strains after 3 minutes were very strong without any differences between live and inactivated suspensions used. We did not observe positive reactions for *F. tularensis* with the control latex reagent. No positive reactions were observed also by the LAT with *F. tularensis* subsp. *novicida* as well as with the most of control strains. However, a very strong reaction of LAT with *S. aureus*

ATCC 25923 was found after 3 minutes of rocking the plates. For this reason, we decided to investigate the additional five *S. aureus* strains isolated from hospital patients. A weak positive reaction after 3 minutes and a strong reaction after 5 minutes of rocking were observed in two cases.

Antibody coated latex particles are commonly used in diagnostic microbiology for detection, identification or serotyping of many different microbes (Miller *et al.,* 2008; Porter *et al.,* 2008; Sumithra *et al.*, 2013). In the previous work we developed the LAT for detection of antibodies against *F. tularensis* in serum samples (Rastawicki *et al.*, 2015). Here, we present the LAT for identification of *F. tularensis* that could be cultured from all kinds of samples like environmental, food, human and animal tissue samples, *etc*. In accordance with our expectation, the test recognized *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* but not *F. tularensis* subsp. *novicida*. It is because of the unique LPS composition of both subsp. *tularensis* and subsp. *holarctica,* which is different from that of *F. tularensis* subsp. *novicida* (McLendon *et al.*, 2006). The LAT recognises clinically relevant subspecies of *F. tularensis*, opposite to PCR detection of *tul4* gene, which gives positive results for all *Francisella* species and additional PCRs for other targets are necessary to differentiate *Francisella* species and subspecies (WHO, 2007). The lack of cross-reactivity of the LAT with other bacteria, except *S. aureus*, is in accordance with other researchers' results on antibody-based *F. tularensis* identification methods such as cELISA and immunochromatographic assay (Grunow *et al.,* 2000), and reveals that the test is highly specific. The cross-reactivity with some *S. aureus* strains is probably related to the presence of protein A in the cell wall of these *S. aureus* strains. It has been demonstrated that protein A expressed by some *S. aureus* strains has a high ability to bind immunoglobulins (King and Wilkinson, 1981; Romagnani *et al.*, 1981). Our experiments with other latex tests of different commercial companies, for example dedicated to detection of *Salmonella* or *E. coli* strains, also showed cross-reactivity with some *S. aureus* (data not shown). However, it is quite easy to differentiate between *F. tularensis* and *S. aureus* based on commercially available latex agglutination test for *S. aureus* or ability to grow on various microbiological media, the colonial morphology, or Gram staining. *F. tularensis* grows on rich media (enriched chocolate agar – CA, buffered charcoal yeast extract – BCYE, cystine heart agar with 9% chocolatized blood – CHAB, thioglycollate-glucose blood agar – TGBA, GC Agar II with 1% haemoglobin and 1% IsoVitaleX, sheep blood agar – SA) but does not grow on ordinary media; whereas, *S. aureus* easily grows on ordinary media such as nutrient agar (NA) and brain heart infusion agar (BHI). Also, some selective media can be applied.

The LAT developed in our study is inexpensive, simple, rapid and does not need any specialized equipment to be performed. We recommend that the results should be read after 3 minutes of rocking the plate. Thus, the test can be performed much faster compared to PCR or real-time PCR which needs at least one hour, even when the fast polymerases are used (Zasada *et al.,* 2013). Moreover, the test works well with inactivated samples which minimizes the risk of laboratory acquired infection and allows to perform the test under BSL-2 conditions. It is the important characteristic as majority of diagnostic laboratories work under BSL-2 conditions with no access to a BSL-3 laboratory. The LAT reagents shelf life is at least 2 years when stored at 4°C, as it was shown by manufacturers of commercially available latex tests for other microbes as well as our experience with in-house tests. The method is also highly reproducible between different operators (data not shown). The LAT could be an alternative for the slide agglutination test described in WHO guidelines for tularemia (WHO, 2007) when the *Francisella tularensis* antiserum is unavailable. Moreover, the use of latex particles coated with antibodies increases sensitivity of antigen detection significantly when compared to the slide agglutination test with antiserum (Drożdż, 2006).

## Literature

**Eliasson H., T. Broman, M. Forsman and E. Bäck.** 2006. Tularemia: current epidemiology and disease management. *Infect. Dis. Clin. N. Am.* 20: 289–311.

**Formińska K., A.A. Zasada, W. Rastawicki, K. Śmietańska, D. Bander, M. Wawrzynowicz-Syczewska, M. Yanushevych, J. Niścigorska-Olsen and M. Wawszczak.** 2015. Increasing role of arthropod bites in tularaemia transmission in Poland – case reports and diagnostic methods. *Ann. Agric. Environ. Med.* 22: 443–446.

**Celli J. and T.C. Zahrt.** 2013. Mechanisms of *Francisella tularensis* intracellular pathogenesis. *Cold Spring Harb. Perspect. Med.* 3: e010314.

**World Health Organization**. 2007. WHO guidelines on tularemia. WHO Library Cataloguing-in-Publication Data. WHO/CDS/ EPR/2007.7, Geneva, Switzerland.

**Koskela P. and A. Salminen.** 1985. Humoral immunity against *Francisella tularensis* after natural infection. *J. Clin. Microbiol.* 22: 973–979.

**Rastawicki W. and N. Wolaniuk.** 2013. Comparison of usefulness of commercial ELISA Virion/Serion, homemade ELISA and tube agglutination test in serodiagnosis of tularemia. *Med. Dosw. Mikrobiol.* 65: 255–261.

**Rastawicki W., N. Rokosz-Chudziak, A. Chróst and R. Gierczyński.** 2015. Development and evaluation of a latex agglutination

test for the rapid serodiagnosis of tularemia. *J. Microbiol. Method.* 112: 1–2.

**King B.F. and B.J. Wilkinson**. 1981. Binding of human immunoglobulin G to protein A in encapsulated *Staphylococcus aureus*. *Infect. Immun.* 33: 666–672.

**Sumithra T.G., V.K. Chaturvedi, P.K. Gupta, S.C. Sunita, A.K. Rai, M.V.H. Kutty, U. Laxmi and M.S. Murugan.** 2013. Development of a simple and rapid method for the specific identification of organism causing anthrax by slide latex agglutination. *Lett. Appl. Microbiol.* 58: 401–407.

**Miller R.S., L. Speegle, O.A. Oyarzabal and A.J. Lastovica**. 2008. Evaluation of three commercial latex agglutination tests for identification of *Campylobacter* spp. *J. Clin. Microbiol.* 46: 3546–3547.

**Porter B.D., B.D. Ortika and C. Satzke.** 2014. Capsular serotyping of *Streptococcus pneumoniae* by latex agglutination. *J. Vis. Exp.* 91: e51747.

**Zasada A.A., K. Formińska and K. Zacharczuk.** 2013. Fast identification of *Yersinia pestis, Bacillus anthracis* and *Francisella tularensis* based on conventional PCR. *Pol. J. Microbiol.* 62: 453–455.

**Drożdż R.** 2006. The use of microparticles agglutination in clinical practice. *Diagn. Lab.* 42: 211–222.

**McLendon M.K., M.A. Apicella and L.-A.H. Allen.** 2006. Francisella tularensis: taxonomy, genetics, and immunopathogenesis of a potential agent of biowarfare. *Annu. Rev. Microbiol.* 60: 167–185.

**Romagnani S., M.G. Giudizi, R. Biagiotti, F. Almerigogna, E. Maggi, G. Del Prete and M. Ricci.** 1981. Surface immunoglobulins are involved in the interaction of protein A with human B cells and in the triggering of B cell proliferation induced by protein A-containing *Staphylococcus aureus*. *J. Immunol.* 127: 1307–1313.

**Grunow R., W. Splettstoesser, S. McDonald, C. Otterbein, T. O'Brien, C. Morgan, J. Aldrich, E. Hofer, E.-J. Finke and H. Meyer.** 2000. Detection of *Francisella tularensis* in biological specimens using capture enzyme-linked immunosorbent assay, an immunochromatographic handheld assay, and a PCR. *Clin. Diagn. Lab. Immunol.* 7: 86–90.