

RAPID DETECTION OF *FRANCISELLA TULARENSIS* BY THE IMMUNOAFFINITY ASSAY ABICAP IN ENVIRONMENTAL AND HUMAN SAMPLES

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ABSTRACT

Francisella tularensis is the causative agent of tularemia, a severe zoonotic infectious disease. The natural reservoir of the bacteria is not yet known precisely, but it may persist for over a year in water or mud. It is naturally maintained and spread by various terrestrial and aquatic mammals, and outbreaks in humans are often paralleled by enzootics or epizootics.

The reasons for this seem to be multifactorial, though not well understood. Therefore, for epidemiological and outbreak investigations, a rapid hand-held assay would be helpful.

Here we described a column-based immunofiltration assay called ABICAP, which has several characteristics that permit its application under field conditions. The assay can be performed within 25 min, with a detection limit of 10³ bacteria. Further validation of the assay included the analytical

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range, precision, detection limit and performance for different human, animal and environmental sample matrices, accuracy, recovery, reproducibility and stability.

PRACTICAL APPLICATIONS

The assay is an interesting tool applicable for the rapid detection of *F. tularensis* in various specimens under laboratory and field conditions. It can be used for the identification of reservoirs of *F. tularensis* in epidemiological studies, for the detection of sources of infection in outbreak situations of tularemia and in laboratory investigation of animal and human specimens. The methodological platform can be adapted to the detection of other micro-biological agents and antigens.

INTRODUCTION

Francisella tularensis is an intracellular, nonmotile, nonsporulating, Gram-negative bacterial pathogen that causes tularemia in man and animals. At present, the genus *Francisella* includes two species, *F. philomiragia* and *F. tularensis*. *F. philomiragia* is an opportunistic pathogen often associated with water. Case reports emphasize its role in immunocompromised individuals (Hollis *et al.* 1989).

Four subspecies of *F. tularensis* are described: *F. tularensis* ssp. *tularensis* (also referred to as Jellison biovar A), *F. tularensis* ssp. *holarctica* (Jellison biovar B), *F. tularensis* ssp. *mediasiatica* and *F. tularensis* ssp. *novicida*. These subspecies originate from different regions of the world and differ markedly in their virulence, but are nevertheless closely related phylogenetically (Johansson *et al.* 2004). Biovar A causes mortality rates of up to 30% in untreated patients, and even with appropriate antibiotic therapy, mortality rates can reach 10% (Dennis *et al.* 2001; Tarnvik and Berglund 2003). *F. tularensis holarctica* is also highly infectious and may cause severe diseases, but it rarely causes fatal outcomes in humans.

The natural reservoir of *Francisella* is not yet known precisely, though studies have shown that the organism may persist for over a year in water or mud (Parker *et al.* 1951). It is naturally maintained and spread by various terrestrial and aquatic mammals, and outbreaks in humans are often paralleled by enzootics or epizootics (Ellis *et al.* 2002). In particular, testing rodent feces and contaminated water for *F. tularensis* antigens has proved to be an efficient and practicable method of identifying infection sources and transmission factors in affected households (Grunow and Finke 2002).

In endemic regions, the pathogen causes hard-to-predict outbreaks among animals and humans. There is a real need to control existing and to discover newly emerging endemic regions. In addition, outbreaks of infectious diseases like tularemia require early identifications of the etiological agent and the sources of infection to begin timely and adequate preventive countermeasures.

Affected persons acquire the disease from infected animals by contact, inhalation or ingestion. Other potential sources are contaminated water and bites by vectors such as mosquitoes, flies or ticks.

In addition to its natural occurrence, *F. tularensis* has been classified by the Centers for Disease Control and Prevention as a critical biological agent of high priority (category A) that poses a risk to national security (Centers for Disease Control and Prevention 2000). Such agents require special action for public health preparedness, including enhanced epidemiological capacity to detect and respond to biological attacks and supply diagnostic reagents to state and local public health agencies in the U.S.A. There is still a need to develop and validate diagnostic tests for the detection of *F. tularensis*, not only for a timely onset of therapy and the handling of outbreak investigations, but also for the surveillance of endemic foci.

Serological diagnosis of human tularemia can be performed on different platforms yielding sufficient sensitivity and specificity (Porsch-Ozcurumez *et al.* 2004). The usual incubation period is 3 to 5 days, although it can be as long as 21 days. In most cases, antibodies appear 6 to 10 days after onset of symptoms, i.e., usually about 2 weeks after infection, and reach their peaks at 4 to 7 weeks. Therefore, serodiagnosis is not applicable as a rapid diagnostic approach.

The most sensitive and specific approach for detecting and typing *Francisella* would be standardized molecular techniques. Even though there are a variety of respective reports, none of these assays can be referred to as the gold standard in detecting the pathogen (Eliasson *et al.* 2005). The application of some of these assays is further limited by a lack of data derived from matrix-specific validation protocols.

Immunoassays for the detection of *F. tularensis* encompass immunofluorescence microscopy (Hofer *et al.* 1997; Petersen *et al.* 2004), immunohistochemistry and immunoelectron microscopy (Dennis *et al.* 2001; Zeidner *et al.* 2004). Particularly, a variety of ELISA-based applications and handheld immunochromatographic assays have been described. These methods were applied to detect antigen not only in serum, urine and tissues such as spleen, liver and lungs from infected animals, but also in environmental samples like water and mud (Berdal *et al.* 2000; Grunow *et al.* 2000). Detection of *F. tularensis* in urine from a tularemia patient was reported by Tarnvik *et al.* (1987).

In contrast to these mainly laboratory-based methods, a commercially available hand-held test system, ABICAP (Senova, Jena, Germany), with the capacity of point-of-care testing, has recently been described for its sensitive detection of botulinum neurotoxin types C and D (Gessler *et al.* 2005). The ABICAP system was originally developed for rapid quantification of anaphylatoxins C3a and C5a (Hartmann *et al.* 1993). ABICAP consists of small disposable plastic columns, and all assay components are added in flowthrough. Large sample volumes can be applied, and thereby sensitivity can be increased by immunofiltrating enrichment. The antigen-antibody complex is captured out of the sample matrix, and therefore the system might be unsusceptible to matrix effects, presuming that potentially interfering sample components are small enough to pass through the assay disk.

Based on these findings, a hand-held test system was developed for the rapid and sensitive antigen detection of *F. tularensis*. In this report we present data on the validation of the *Francisella* ABICAP system employing human, animal and environmental samples.

MATERIALS AND METHODS

Preparation of Bacteria

Bacteria were obtained from different sources as indicated in Table 1. Aliquots were stored in Mikrobank tubes (VIVA Diagnostika GmbH, Cologne, Germany) at -70°C . After removal from freezer, *Francisella* strains were plated on cystein-heart agar supplemented with 9% (w/v) sheep blood (CHAB) and cultivated for 72 h at 37°C in humidified atmosphere with 5% CO_2 . Colonies were suspended in phosphate-buffered saline (PBS) (0.15 M, pH 7.4) and adjusted to an optical density of 1.0 at 560 nm ($\text{OD}_{560\text{ nm}} = 1.0$), corresponding to an average of 2.0×10^9 colony-forming units (cfu)/mL.

Analytical specificity of the ABICAP system was tested with a panel of microorganisms (Table 2) that reflect potentially cross-reacting pathogens of the resident and transient flora as well as relevant pathogens in routine microbiological diagnostics or those that cause similar clinical manifestations. Bacteria were grown on appropriate media and adjusted in PBS to the number of cfu as indicated.

Animal Tissues

Randomly collected specific pathogen-free (SPF) inbred and outbred mice were provided by the National Research Center for Environment and Health (GSF, Munich, Germany) and used as negative controls and as sources for tissues used in spiking experiments. Mice were killed routinely in connec-

TABLE 1.
FRANCISELLA STRAINS USED TO ASSESS THE ACCURACY OF THE *FRANCISELLA*
 ABICAP SYSTEM

| Species/Subspecies | Source, Strain No. | n | Absorption (mean, range)* | |
|--|---|---|---------------------------|--------------------------|
| | | | 1 × 10 ⁵ ecfu | 1 × 10 ⁶ ecfu |
| <i>F. tularensis</i> ssp. <i>holarctica</i> | ATCC 29684; IMB F53, F54, F55, F56, F57 | 6 | 2.26, (2.07–2.54) | >3.0, (>3.0) |
| <i>F. tularensis</i> ssp. <i>mediasiatica</i> | FOI FSC 147, FSC 148, FSC 149 | 3 | 2.35, (2.18–2.50) | >3.0, (>3.0) |
| <i>F. tularensis</i> ssp. <i>tularensis</i> | ATCC 6223; FOI FSC 033, FSC 041, FSC 042, FSC 043, FSC 054, FSC 237 | 7 | 1.81, (1.38–2.25) | >3.0, (2.50–>3.0) |
| <i>F. tularensis</i> ssp. <i>novicida</i> | ATCC 15482; FOI FSC 159, FSC 040, FSC 454 | 4 | 0.05, (0.04–0.07) | 0.05, (0.04–0.06) |
| <i>F. philomiragia</i> | ATCC 25015, 25016, 25017, 25018 | 4 | 0.04, (0.03–0.05) | 0.05, (0.04–0.08) |

Bacteria were suspended in phosphate-buffered saline and adjusted to the indicated ecfu.

*Mean values and range (min–max) of absorptions measured are shown as arbitrary units and indicate the specificity of the ABICAP system for the detection of the clinically relevant *F. tularensis* ssp. *tularensis*, *mediasiatica* and *holarctica*.

ATCC, American Type Culture Collection; IMB, Bundeswehr Institute of Microbiology; FOI, Swedish Defence Research Agency.

tion with unusable overproduction in breeding for unrelated experimental purposes. Spleen and liver were excised from a total of 25 uninfected animals. Sets of three organs (0.2–0.4 g each) were pooled and transferred to FastPrep Lysing Matrix A tubes (Qbiogene, Heidelberg, Germany). After addition of 1.0 mL PBS, samples were homogenized and stored frozen (–20°C) until use. Thawed samples were vortexed, and remaining larger tissue particles were allowed to sediment for 5 min. For each determination, 200 µL of the tissue homogenate was used.

Specific pathogen-free female BALB/c mice were purchased from Charles River Laboratories (St. Constant, Quebec, Canada) for experimental infection. Mice were maintained and used in accordance with the recommendations by the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. Their use in the experiments reported here was approved by the animal care committee at the Institute of Biological Sciences. For these experiments, stock cultures of *F. tularensis* LVS (ATCC 29684) were prepared after re-isolation from spleen of a lethally infected mouse. Intradermal inocula (10² or 10⁶ cfu in 50 µL PBS) of these *in vivo*-adapted *F. tularensis* LVS were injected into a skin fold in the shaved mid-belly. The mice were killed 4 days after infection. For subsequent determination of cfu, an area of skin encompassing the injection site and other organs used were excised,

TABLE 2.
PATHOGENS USED TO ASSESS THE ANALYTICAL SPECIFICITY OF THE *FRANCISELLA*
ABICAP SYSTEM

| Species | Source, Strain No. | Absorption (aU) | |
|---|--------------------|----------------------|----------------------|
| | | 1×10^6 ecfu | 1×10^7 ecfu |
| <i>Acinetobacter baumannii</i> | DSMZ 7324 | 0.008 | 0.001 |
| <i>Vibrio cholera</i> | ZIKOB 219512 | 0.015 | 0.018 |
| <i>Enterobacter aerogenes</i> | DSMZ 12058 | 0.018 | 0.002 |
| <i>Enterobacter cloacae</i> | IMB B6 | 0.031 | 0.037 |
| <i>Enterococcus faecalis</i> | DSMZ 2570 | 0.076 | 0.079 |
| <i>Pasteurella multocida</i> | DSMZ 5281 | 0.036 | 0.089 |
| <i>Salmonella typhimurium</i> | ATCC 13311 | 0.054 | 0.056 |
| <i>Shigella flexneri</i> | DSMZ 4782 | 0.050 | 0.045 |
| <i>Staphylococcus aureus</i> | DSMZ 346 | 0.059 | 0.039 |
| <i>Yersinia enterocolitica</i> | ATCC 9610 | 0.035 | 0.032 |
| <i>Yersinia pseudotuberculosis</i> | ATCC 29833 | 0.006 | 0.018 |
| <i>Yersinia pestis</i> | PI EV76 | 0.037 | 0.008 |
| <i>Staphylococcus epidermidis</i> | ZIKOB | 0.018 | 0.035 |
| <i>Streptococcus pneumoniae</i> | DSMZ 20566 | 0.039 | 0.048 |
| <i>Yersinia enterocolitica</i> O:9 | UB S4-223 | 0.024 | 0.038 |
| <i>Salmonella urbana</i> (group N) | HR | 0.025 | 0.021 |
| <i>Achromobacter ruhlandii</i> | DSMZ 653 | 0.070 | 0.053 |
| <i>Achromobacter xylosoxidans</i> ssp. <i>denitrificans</i> | DSM 30026 | 0.039 | 0.069 |
| <i>Aeromonas hydrophila</i> ssp. <i>hydrophila</i> | UR 159 | 0.079 | 0.068 |
| <i>Alcaligenes faecalis</i> ssp. <i>faecalis</i> | DSM 30030 | 0.095 | 0.072 |
| <i>Candida albicans</i> | DSMZ 1386 | 0.099 | 0.067 |
| <i>Citrobacter freundii</i> | DSMZ 30039 | 0.082 | 0.066 |
| <i>Eikenella corrodens</i> | DSMZ 8340 | 0.094 | 0.066 |
| <i>Escherichia coli</i> O157:H | ZIBE E32511 | 0.083 | 0.057 |
| <i>Kingella denitrificans</i> | DSMZ 10202 | 0.052 | 0.052 |
| <i>Klebsiella pneumoniae</i> ssp. <i>Pneumoniae</i> | DSMZ 681 | 0.071 | 0.062 |
| <i>Kingella kingae</i> | DSMZ 7536 | 0.046 | 0.056 |
| <i>Listeria monocytogenes</i> | DSMZ 12464 | 0.057 | 0.044 |
| <i>Branhamella catarrhalis</i> | DSMZ 9143 | 0.053 | 0.052 |
| <i>Morganella morganii</i> | DSMZ 6675 | 0.074 | 0.098 |
| <i>Neisseria meningitidis</i> | DSMZ 10036 | 0.055 | 0.050 |
| <i>Ochrobacterium anthropi</i> | DSMZ 7216 | 0.048 | 0.063 |
| <i>Plesmiomonas shigelloides</i> | DSMZ 8224 | 0.057 | 0.047 |
| <i>Propionibacterium acnes</i> | DSMZ 1897 | 0.085 | 0.051 |
| <i>Proteus mirabilis</i> | ATCC 29906 | 0.056 | 0.046 |
| <i>Proteus vulgaris</i> | DSMZ 30118 | 0.054 | 0.062 |
| <i>Pseudomonas aeruginosa</i> | ZIMUC 48040/E62 | 0.053 | 0.049 |
| <i>Pseudomonas putida</i> | DSMZ 291 | 0.058 | 0.057 |
| <i>Pseudomonas stutzeri</i> | NTCT 10450 | 0.066 | 0.058 |
| <i>Psychrobacter phenylpyruvicus</i> | DSMZ 7000 | 0.093 | 0.057 |
| <i>Rahnella aquatilis</i> | DSMZ 4549 | 0.049 | 0.077 |
| <i>Ralstonia</i> (<i>Burkholderia</i>) <i>pickettii</i> | DSMZ 6297 | 0.044 | 0.054 |
| <i>Serratia proteomaculans</i> | DSMZ4543 | 0.054 | 0.058 |

TABLE 2. CONTINUED

| Species | Source, Strain No. | Absorption (aU) | |
|-------------------------------------|--------------------|---------------------|---------------------|
| | | 1×10^6 cfu | 1×10^7 cfu |
| <i>Sphingomonas paucimobilis</i> | DSMZ 1098 | 0.066 | 0.073 |
| <i>Stenotrophomonas maltophilia</i> | UR 78 | 0.080 | 0.048 |
| <i>Staphylococcus epidermidis</i> | DSMZ 1798 | 0.047 | 0.057 |
| <i>Vibrio parahaemolyticus</i> | UR 26299 | 0.044 | 0.072 |
| <i>Yersinia kristensenii</i> | ATCC 33638 | 0.043 | 0.048 |
| <i>Citrobacter koseri</i> | DSMZ 4595 | 0.098 | 0.049 |
| <i>Acinetobacter calcoaceticus</i> | DSMZ 30006 | 0.091 | 0.063 |
| <i>Klebsiella oxytoca</i> | ZIKIE | 0.041 | 0.063 |
| <i>Serratia marcescens</i> | DSMZ 1636 | 0.071 | 0.058 |
| <i>Staphylococcus haemolyticus</i> | DSMZ 20263 | 0.093 | 0.088 |
| <i>Streptococcus agalactiae</i> | DSMZ 2134 | 0.048 | 0.061 |
| <i>Streptococcus pyogenes</i> | DSMZ 20565 | 0.051 | 0.060 |
| <i>Streptococcus mitis</i> | DSMZ 12643 | 0.048 | 0.058 |
| <i>Yersinia bercovieri</i> | ATCC 43970 | 0.037 | 0.079 |
| <i>Yersinia fredericksonii</i> | UM | 0.004 | 0.094 |

Cells were suspended in PBS and adjusted to 1×10^6 and 1×10^7 cfu as indicated.

aU, arbitrary units; ATCC, American Type Culture Collection; DSMZ, German Resource Center for Biological Material; HR, General Hospital of Regen; NCTC, National Collection of Type Cultures, London; PI, Pettenkofer Institute for Medical Microbiology, Munich; UB, University Hospital of Bonn; UM, University Hospital of Munich; UR, University Hospital of Regensburg; ZIBE, Bundeswehr Central Institute, Berlin; ZIKIE, Bundeswehr Central Institute, Kiel; ZIKOB, Bundeswehr Central Institute, Koblenz; ZIMUC, Bundeswehr Central Institute, Munich.

chopped into small pieces using scissors and homogenized in aerosol-proof homogenizers. The count of cfu was determined on culture plates using appropriate sample dilutions. Subsequently, homogenates were sterilized by heat inactivation (80C for 15 min), confirmed to be sterile by replating on culture plates, then frozen at -80C until use in the ABICAP system.

Environmental Samples

Environmental samples included feces from rabbits and mice as well as water samples from natural sources.

Feces were either collected from specific pathogen-free animals (controls) for spiking experiments or were obtained from outbreak investigations in Kosovo (Reintjes *et al.* 2002). Feces were pooled and aliquoted in portions of approximately 0.3 g. Each portion was transferred to a FastPrep tube and homogenized by adding 1.0 mL PBS and shaking for 2×20 on the FastPrep Instrument (4.0 m/s). Aliquots were stored at -20C until use. Samples were thawed, vortexed and centrifuged at $12,000 \times g$ for 30 s to sediment remaining

large clumps. 200 μL of homogenized samples were used for spiking experiments as indicated below.

Water samples had been collected during a water-borne outbreak investigation in Sweden (Eliasson *et al.* 2002). Samples were stored at 4C until use.

Human Specimens

After written consent was given, EDTA whole blood samples were drawn from three volunteers (coagulation of the blood should be avoided by proper handling). Urine from the same individuals was collected in sterile tubes. All samples were stored at 4C and used within 7 days. Spiking experiments were performed using 200 μL of each sample per determination.

Spiking of Samples

Matrix-specific interferences were evaluated using spiked samples either derived from human or animal specimens, or environmental samples supplemented with defined amounts of *F. tularensis* LVS. Bacteria were diluted with PBS, and 50 μL of the suspension was added to 200 μL of samples negative for *Francisella* that had been processed as indicated above. The resulting absolute amount of spiked bacteria is indicated in Fig. 1. Recovery rates were determined by comparison with PBS samples as reference matrix that were treated identically and measured in parallel.

Sample Preparation

Analytical samples were diluted 1:2 with 250 μL extraction buffer (Abbott, Wiesbaden, Germany) and incubated at 50C for 30 min in order to extract bacterial LPS. Subsequently, samples were further diluted 1:2 with 500 μL sample dilution buffer (Senova, Jena, Germany), vortexed and centrifuged at $12,000 \times g$ for 3 min, or alternatively, filtrated through 0.45 μm pores. Clear supernatants were used for subsequent analysis. The LPS-containing supernatant from defined amounts of bacteria was referred to as *ecfu*.

Immunoaffinity Assay Columns

Hand-held devices used in these investigations are commercially available as ABICAP (Senova). All assay components were provided by Senova, if not indicated otherwise. ABICAP consists of polyethylene (PE) polymer sinter that has a built-in three-dimensional cylindrical filter embedded in disposable plastic columns (54×5 mm, 750 μL sample volume). The columns were prepared in advance as follows: ABICAP PE-sintered filters were washed and degassed in ethanol (96% v/v). Afterwards, filters were washed in ethanol water (50/50) several times, followed by several washing steps in immobili-

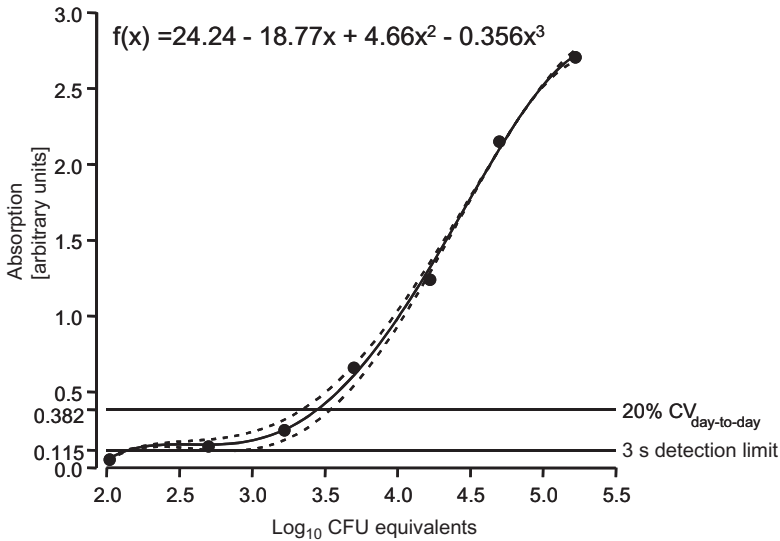


FIG. 1. CALIBRATION CURVE GENERATED WITH THE *FRANCISELLA* ABICAP SYSTEM USING PHOSPHATE-BUFFERED SALINE (PBS) SPIKED WITH INCREASING AMOUNTS OF *F. TULARENSIS* SSP. *HOLARCTICA* LIVE VACCINE STRAIN

Mean values of two determinations performed on two different days are shown. The solid curve was derived from the indicated equation that resulted from third-order polynomial curve fitting. Dashed lines depict day-to-day imprecision at the respective concentration (see Table 3). Functional assay sensitivity (20% coefficient of variance [CV]_{day-to-day}) and 3 s detection limit correspond to results shown for PBS in Table 3.

zation buffer (carbonate buffer, 0.1 M, pH 9.5). *F. tularensis*-specific monoclonal antibody FF/11/6 (Greiser-Wilke *et al.* 1989; Grunow *et al.* 2000) was adsorptively bound on the PE-sintered filters for 1 h using 10 µg of antibody per column in immobilization buffer. Filters were finally blocked and rinsed using 5.5 mg/mL bovine serum albumin (BSA) (Serva, Heidelberg, Germany) in PBS. Dried coated filters were transferred to columns, confectioned and delivered to the laboratory in batches of 2,000 as single-packed blisters together with all reagents required for the ABICAP. For quality assurance, (1) ready-to-use columns were weighted; (2) low, medium and high level inter-assay coefficients of variation were determined in randomly selected columns; and (3) aliquots of retained samples were stored at room temperature and at 4°C over at least 12 months and analyzed after defined intervals.

Protocol of the Immunoaffinity Assay ABICAP

All applied reagents were provided ready-to-use by the manufacturer. 500 µL of clear supernatant of the prepared samples were applied

to the column and incubated at room temperature. After 6 min, 500 μL of the directly poly-horseradish peroxidase (poly-HRP)-labeled monoclonal antibody FF/11/6 (8 $\mu\text{g}/\text{mL}$) was added and incubated for another 6 min. Columns were washed twice with 750 μL PBS containing 0.01% (v/v) Tween and 0.1% (w/v) BSA. Another washing step was performed with 750 μL of substrate buffer containing sodium acetate citrate buffer (0.1 M, pH 4.9) and 0.05% (w/v) 5-bromo-5-nitro-1,3-dioxane as preservative. To prepare the ready-to-use substrate solution, 100 $\mu\text{g}/\text{mL}$ of precipitating 3,3',5,5'-tetramethylbenzidine (TMB) (KPL, Inc., Gaithersburg, MD) were dissolved by the manufacturer in substrate buffer containing 0.04% (w/v) dextran sulphate, 0.006% (v/v) hydrogen peroxide and 0.001% (w/v) dimethylsulfoxide. 500 μL TMB was added and incubated for 6 min. Columns were finally washed once with 750 μL substrate buffer. Absorption was measured immediately in the ABICAP reader system. The ABICAP reader was routinely calibrated with standardized titanium dioxide blank columns.

Capture Enzyme-Linked Immunosorbent Assay

Results obtained by immunoaffinity assay columns were compared to a capture enzyme-linked immunosorbent assay (cELISA) for the detection of *F. tularensis* LPS, which has been described in detail previously and was used here with some modifications (Berdal *et al.* 2000; Grunow *et al.* 2000). Briefly, 96-well microtitre plates (Maxisorp, Nunc, Wiesbaden, Germany) were coated overnight with the *F. tularensis* LPS-specific murine monoclonal antibody FF/11/6. Subsequently, plates were washed and blocked with dry skim milk in PBS-Tween. Samples were prepared as described above. 100 μL was transferred to coated microtiter plates and incubated for 1 h at 37C. After washing, bound antigen was determined by adding 100 μL of horseradish peroxidase-labeled monoclonal antibody FF/11/6 (2 $\mu\text{g}/\text{mL}$ PBS-Tween) and incubated at 37C for 1 h. Optical density was measured at 450/620 nm after washing and adding 100 μL TMB. All tests were run in duplicate.

Statistics

Microsoft Excel was used to analyze data collected for assay validation. Regression analyses and curve fittings were performed employing the Origin 7.5 software package. Methods were compared according to Passing and Bablok (1983).

RESULTS

Analytical Range

Initially, standard curves were generated employing the *F. tularensis* ssp. *holarctica* LVS suspended in PBS as reference matrix (Fig. 2). The range of concentration that is applicable without modification (analytical range) spanned 1.6×10^3 to 1.6×10^5 ecfu corresponding to 0.25–2.7 arbitrary units. A linear correlation was given between absorption and amount of spiked bacteria using linear regression analysis and considering only the five measuring points above 3.5×10^3 ecfu/mL within this range (slope = 1.281 ± 0.094 ; intercept = -3.998 ± 0.401 ; $r = 0.992$; $P < 0.001$).

A plateau was reached at 1×10^6 ecfu without any high-dose hook effect. However, with increasing amount of cells, the columns tended to clog. Eight different matrices were employed for recovery experiments (see below), whereas an overall rate of 2.2% clogged columns (27 out of 1,200) was observed within the analytical range. While no columns were affected when using PBS, in all other matrices clogging appeared in 0.7% to 2.0% of the columns. However, whole blood outperformed significantly, with 10.7% (16 out of 150) affected columns.

Precision

Precision was determined in eight different matrices spiked with four different concentrations of LVS as indicated in Table 3. All spiked matrices yielded comparable results with respect to within-run ($n = 10$) as well as day-to-day precision ($n = 30$). Whole blood samples tended to have higher coefficients of variation, especially at concentrations lower than 1×10^4 ecfu, compared to the other matrices.

Detection Limits

The lowest quantity of ecfu detected in eight different matrices with an alpha error of less than 0.5% was estimated by 10-fold repeated blank measurements. For this purpose, bacteria/PBS suspensions were replaced by sterile PBS, while all other assay components remained unmodified. The limit of detection was defined as the amount of ecfu corresponding to a signal three standard deviations (SD) above the mean absorption of blank measurements ($\xi + 3$ SD). As shown in $\xi + 3$ SD, values ranged from 1.2×10^2 to 1.8×10^3 ecfu according to individual standard curves generated for each matrix. Corresponding absorption values ranged from 0.109 arbitrary units for environmental water samples up to 0.303 arbitrary units for murine feces. Considering the concentration-dependent day-to-day imprecision as shown

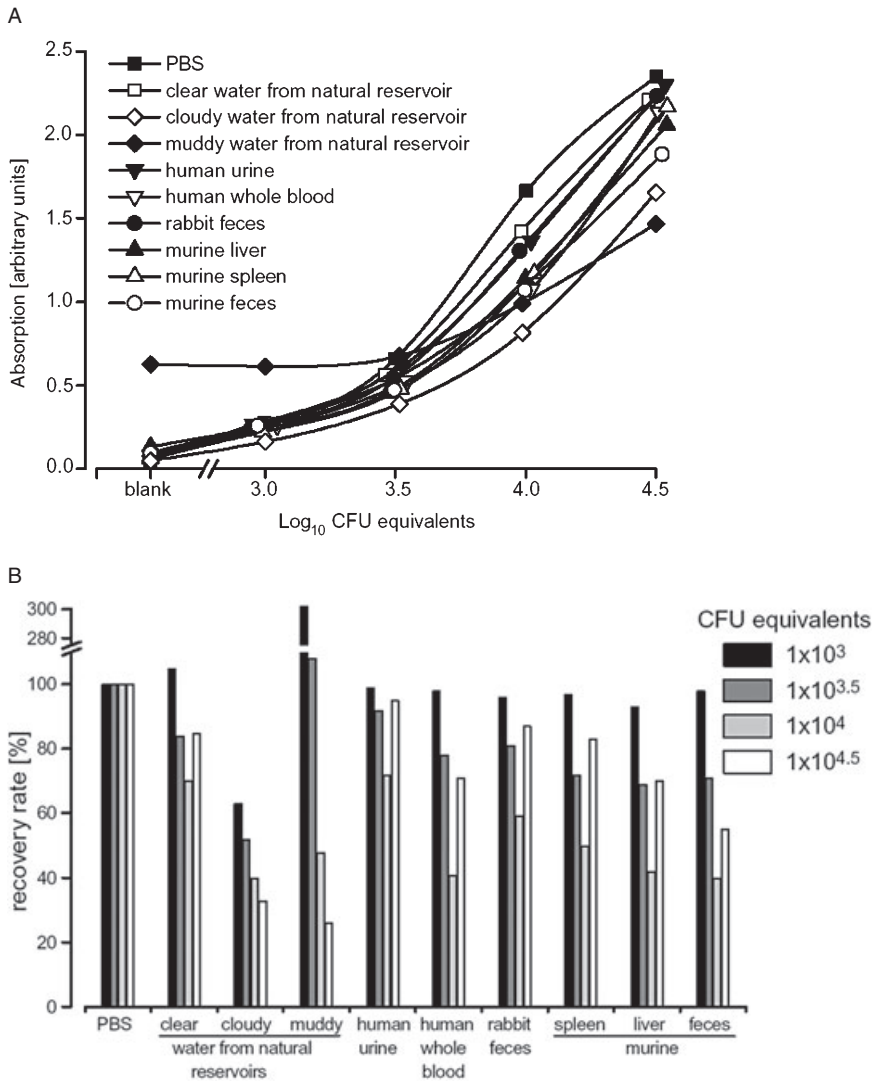


FIG. 2. (A) Absorption signals measured by the *Francisella* ABICAP system using the indicated matrices that were spiked with increasing dilutions of bacterial suspensions. Symbols indicate mean values of triplicate measurements. (B) Recovery rates calculated from results shown in (A). The calculated ecfus were derived from a calibration curve using phosphate-buffered saline (PBS) as reference matrix. Ecfus obtained in the reference matrix were defined as 100%.

TABLE 3.
LIMITS OF DETECTION AND PRECISION OF THE *FRANCISELLA* ABICAP SYSTEM WERE DETERMINED IN THE INDICATED MATRICES

| | ecfu | PBS | Water from natural reservoirs | Human | | Mouse | | Rabbit | |
|--------------------------------------|-----------------|-------|-------------------------------|-------|--------|-------|--------|--------|-------|
| | | | | Urine | Blood | Liver | Spleen | Feces | Feces |
| Detection limit (ecfu)* | | 281 | 115 | 232 | 789 | 1,242 | 290 | 1,871 | 114 |
| Functional assay sensitivity (ecfu)† | | 2,864 | 2,692 | 3,162 | 16,596 | 3,631 | 7,499 | n.a. | n.a. |
| Within-run assay variation (CV%)‡ | 3×10^3 | 13.6 | 17.5 | 15.8 | 27.0 | 17.4 | 14.5 | 11.6 | 15.0 |
| | 1×10^4 | 11.2 | 14.6 | 8.1 | 17.5 | 13.2 | 11.0 | 5.6 | 9.9 |
| | 3×10^4 | 5.7 | 6.2 | 8.1 | 11.1 | 11.9 | 6.3 | 7.8 | 8.2 |
| | 1×10^5 | 3.2 | 4.9 | 5.0 | 10.5 | 7.3 | 4.6 | 5.1 | 6.9 |
| Day-to-day assay variation (CV%)‡ | 3×10^3 | 19.5 | 16.9 | 20.2 | 22.2 | 21.4 | 36.2 | 12.7 | 16.9 |
| | 1×10^4 | 12.3 | 12.7 | 12.3 | 22.0 | 14.1 | 10.5 | 8.6 | 9.7 |
| | 3×10^4 | 7.3 | 8.9 | 9.6 | 12.7 | 11.3 | 10.1 | 7.0 | 8.6 |
| | 1×10^5 | 3.5 | 4.9 | 5.3 | 5.8 | 8.1 | 3.6 | 6.0 | 5.2 |

*Detection limit corresponds to signals three SD above the mean absorption of repeated blank measurements ($\xi + 3$ SD).

†Functional assay sensitivity was defined as the interpolated concentration corresponding to a 20% day-to-day imprecision. In some cases blank measurements did not exceed 20% day-to-day imprecision and thus, assessment of functional assay sensitivity was not applicable.

‡Within-run assay and day-to-day assay variation were based on 10 and 30 repeated measurements, respectively. n.a., not applicable; ecfu, equivalent of colony-forming units; CV, coefficient of variance.

exemplarily for PBS in Fig. 1 (dashed lines), the distribution of signals obtained from blank measurements and those from low amounts of LVS overlapped up to 1×10^3 ecfu. Furthermore, we included the functional assay sensitivity in our validation panel as an important performance criterion, which is defined as the interpolated concentration corresponding to a 20% day-to-day imprecision. Thereby we estimated the amount of ecfu acceptable as a quantitative result with reasonable imprecision (Table 3). This criterion led to a significant increase of the cut-off levels in most of the matrices investigated. The most significant increase was observed in whole blood due to obvious precision problems when using samples spiked with 1×10^4 or less ecfu of LVS. In contrast, day-to-day coefficients of variation for murine and rabbit feces did not exceed the 20% limit. Thus, functional assay sensitivity could not be assessed but was assumed to be identical with the detection limit.

Accuracy and Analytical Specificity

According to the taxonomy of *Francisellaceae* within the genus *Francisella*, there are two species (*F. philomiragia* and *F. tularensis*), and within the latter four subspecies (*F. tularensis* ssp. *tularensis*, *F. tularensis* ssp. *holarctica*, *F. tularensis* ssp. *mediasiatica*, *F. tularensis* ssp. *novicida*) that are known to cause mild to severe symptoms of tularemia in humans. The clinical relevance of other *Francisellaceae* is commonly accepted to be negligible. We therefore tested the accuracy in a set of *Francisella* strains listed in Table 1. All *F. tularensis* ssp. *tularensis*, *holarctica* and *mediasiatica* strains produced a strong positive signal, while all of the *F. tularensis* ssp. *novicida* and *F. philomiragia* strains remained below the detection limit.

Analytical specificity was validated in a set of potential sample contaminants as well as pathogens with relevance in routine microbiological diagnostics (Table 2). Up to 1×10^7 pathogens did not lead to any absorption above the detection limit.

Recovery

Recovery experiments were performed in order to obtain information whether the analyte can be measured in the presence of all the other compounds present in the matrices of authentic samples. Increasing amounts of LVS were suspended in constant volumes of PBS and added to the respective matrices. Figure 2A depicts the resulting curves obtained from four different dilutions of suspended bacteria. Signals spread over proportionally in samples spiked with 1×10^4 ecfu, but converged again at higher levels. Murine specimens and human whole blood led to the most significant loss of signal intensity compared to PBS serving as reference matrix. Figure 2B illustrates the resulting recovery rate based on the respective amount of bacteria using the

calibration curve generated in the reference matrix. Even though murine specimens and human whole blood yielded only poor recovery rates of approximately 40% in samples spiked with 1×10^4 cfu, none of the samples gave false negative results. Recovery rates increased again beyond 1×10^4 cfu due to the saturation effect of the reference curve. The loss of signal intensity was negligible in samples spiked with 1×10^3 cfu, i.e., near the detection limit of the assay.

Water from natural reservoirs varied with respect to the content of soil dissolved in the samples. Therefore, the influence of water quality was assessed in an independent approach. PBS spiked with increasing amounts of LVS was compared with three representative water samples that were either muddy, cloudy or clear, which were spiked in parallel to the reference sample. Muddy water samples led to a significant increase of background signal above the $\xi + 3$ SD cut-off in blanks and also in spiked samples in the range between 3×10^1 and 1×10^4 cfu, and thus led to false positive results (data not shown).

Reproducibility

The *Francisella* ABICAP system was compared with a sandwich ELISA used in routine diagnostics at the German National Consiliar Laboratory for Tularemia and with results obtained from culturing samples on CHAB plates and subsequent counting of cfu.

Initially, PBS samples spiked with varying amounts of LVS were assayed in parallel in the ABICAP system and by ELISA. Signal intensities of both devices showed a linear correlation and random error within an acceptable range (Fig. 3a). In accordance with the higher analytical sensitivity of the ELISA that resulted from the steeper increase of the calibration curve along the reportable range (1×10^3 to 1×10^7 cfu), absorption signals obtained by the ABICAP system were afflicted with a proportional systematic error.

Next, mice were infected artificially with LVS, and their organs were dissected in order to compare the cfu counted after culturing of tissues smeared on CHAB plates with cfu calculated from signal intensities of the ABICAP calibration curve. Figure 3b illustrates the correlation between both approaches over all tissues. Altogether, differences between counted and calculated cfu exceeded 1 log rank in four samples. Particularly, the bacterial load of murine spleen tissues tended to be underestimated by the ABICAP system.

Environmental samples were processed and assayed in parallel using the ABICAP system and by ELISA. Because all available methods are limited regarding sensitivity and specificity, we compared both devices in two independent sample sets: (1) controls that were used to determine cut-off values for authentic samples; and (2) samples from outbreak investigations that included the whole range of potential pre-analytical issues and findings that have to be

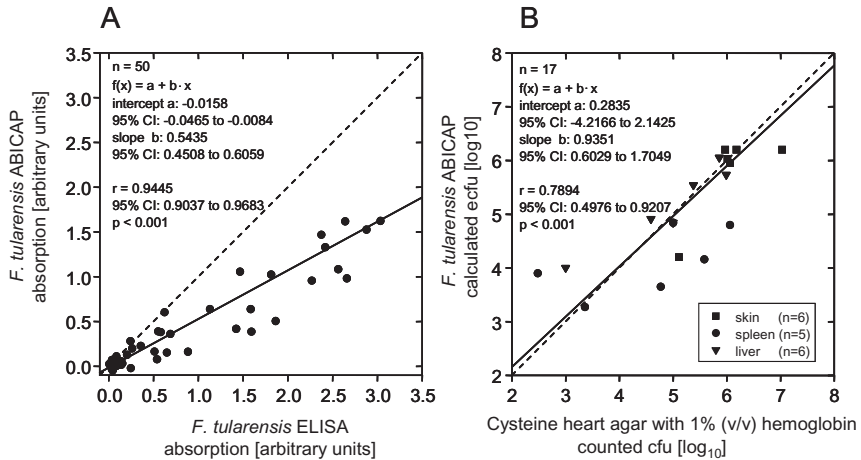


FIG. 3. (A) Method comparison between *Francisella* ABICAP and ELISA using phosphate-buffered saline spiked with LVS. Signal intensities of both assays were compared. (B) Comparison of bacterial loads determined in tissues prepared from mice ($n = 6$) artificially infected by intradermal injection of inocula containing either 1×10^2 or 1×10^6 ecfu of *F. tularensis* LVS. Cfu were either counted after culturing of tissues for 48 h on CHAB plates or calculated from standard curves generated by the *Francisella* ABICAP system. Data of one spleen sample was not included, since no growth of bacteria was observed on CHAB plates; however, absorptions measured by the *Francisella* ABICAP system corresponded to 8×10^4 ecfu equivalents. Scatter plot with linear regression line (solid) and bisecting line (dashed) are indicated. Slope, intercept and 95% confidence intervals (CI) were calculated according to Passing and Bablok (1983).

expected in such settings. Cut-offs were defined as $\xi + 3$ SD and $\xi + 2$ SD of the respective control sets; samples between both values were defined as borderline.

Water samples from environmental sources were collected during outbreak investigations in two different settings in Kosovo (Reintjes *et al.* 2002) and Sweden (Eliasson *et al.* 2002). While water is a suspected reservoir of *F. tularensis* in Sweden, in Kosovo the pathogen is mainly found in rodents. Negative results were obtained for 69 out of 71 water samples from Kosovo by ABICAP and ELISA (97.2% agreement rate; Fig. 4). One sample collected from river water was positive only in the ABICAP assay. However, only massive growth of fungi was seen in the subsequent analysis of the sample. Another sample was most likely false positive by ELISA because only massive bacterial growth was observed during the subsequent investigation. No growth of *Francisella* ssp. was observed in any of the samples. Thus, for both devices, a diagnostic specificity of 98.6% can be assumed using the cut-off values depicted in Fig. 4A and B.

Among the set of water samples ($n = 115$) collected during an outbreak investigation in Sweden, five samples (and another two with borderline results

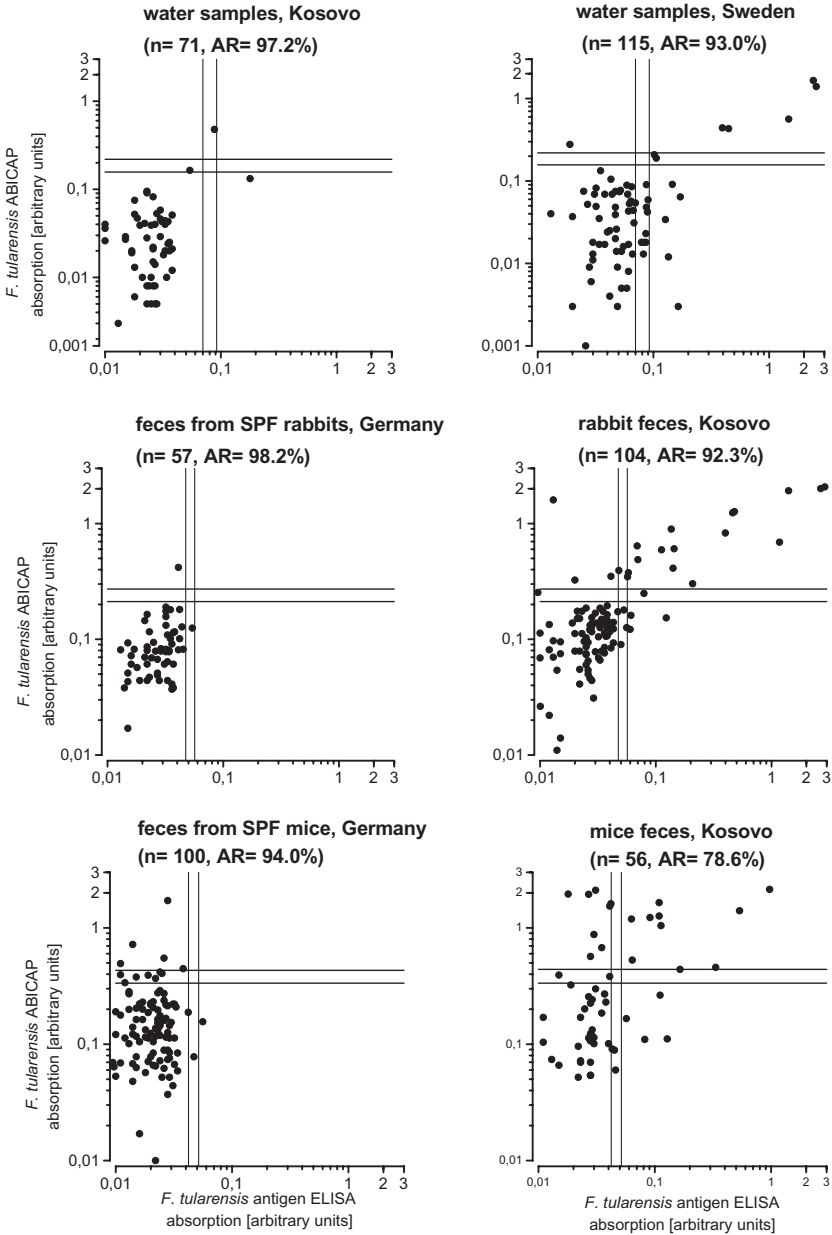


FIG. 4. COMPARISON OF THE *FRANCISELLA* ABICAP AND ELISA USING AUTHENTIC ENVIRONMENTAL SAMPLES

Horizontal and vertical lines indicate cut-offs derived from $\xi + 3$ SD and $\xi + 2$ SD values of the respective control sets (i.e., water samples from Kosovo, SPF rabbits and mice from Germany). Agreement rate (AR) summarizes results that were either positive or negative in both assays in relation to all samples measured.

determined by ABICAP) were positive and 102 samples were negative in both assays, respectively (agreement rate 93.0%). Again, one sample turned out to be positive only by the ABICAP system, while five samples were positive only by ELISA.

Reference intervals for rodent feces were determined both in SPF mice and rabbits. Background levels measured in feces from SPF mice were significantly higher and did not fit into a normal distribution curve compared to data obtained from SPF rabbits. The agreement rate between ELISA and ABICAP was 98.2% and 94.0% when $\xi + 3$ SD cut-off values were chosen as decision criterion.

Feces collected during a tularemia outbreak in Kosovo revealed 15 positive rabbit feces samples out of 104 (14.4%) and an agreement rate with ELISA of 92.3% for rabbit feces. Another nine out of 56 (16.1%) mice feces samples were positive in both ELISA and ABICAP. Since there was only a poor correlation between both assays using feces from mice, agreement rates decreased to 80.4%.

Long-Term Storage

Storage requirements for all reagents and columns were tested over a 12-month period from April 2004 to May 2005. Columns were generally stored at room temperature, whereas the reagents were either stored at 4C or at room temperature in opaque boxes. Three different concentrations of LVS suspended in PBS were aliquoted and assayed periodically. Results were evaluated regarding day-to-day coefficients of variance for the respective concentration of ecfu (Fig. 5). The ABICAP system remained stable over a 6-month period at both temperatures. Storage at 4C extended the time frame until expiry of the columns to at least another 6 months.

DISCUSSION

Infection control practitioners and epidemiologists depend on the fast identification of reservoirs and foci from which a pathogen spreads. Small bench-top analyzers are commonly used for point-of-care testing, but they rely on requirements such as power supply, precautions during transport, ambient conditions, etc. However, vulnerable operator-dependent steps can be automated and, thus, are robust in respect of the technical skill of the personnel. Hand-held single usage devices for microbiological application include dipstick, lateral flow, latex agglutination and direct agglutination. The simplicity and robustness of these assay formats allow their use outside the specialized laboratory as “near-patient” tests and for use in the field. Limitations of these

methods include low analytical sensitivity and imprecision when compared to other immunological techniques such as ELISA or Western blot.

Antibody immuno columns for analytical processes (ABICAP) is an immunoaffinity chromatographic column test that might combine features of hand-held single usage devices with those of established robust formats such as ELISA.

Experience from field investigation reveals the ruggedness and practicality of the ABICAP system (unpublished observation). Advantages of the system are its independence from power supply because flow through the columns only depends on gravity and results could be read off visually. Primitive racks allow processing of 20 or more columns in parallel within a reasonable time frame. The usual assay time is approximately 25 min.

Here, we evaluated the performance of the ABICAP system for the detection of *F. tularensis*.

Tularemia outbreaks among humans are sporadically reported in some areas of the northern hemisphere (Ellis *et al.* 2002). Field investigations are undertaken in order to control the disease or for purposes of surveillance.

We summarized the characteristics and limitations of the *Francisella* ABICAP system when complex matrices were analyzed, such as animal

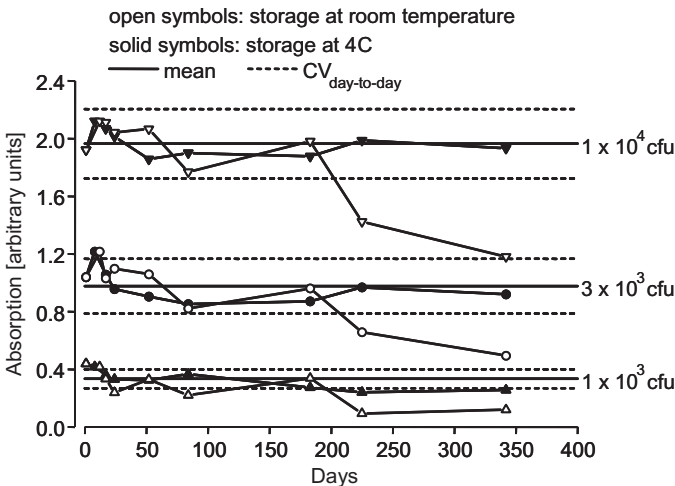


FIG. 5. LONG-TERM STORAGE OF ABICAP COLUMNS OVER 12 MONTHS AT ROOM TEMPERATURE AND 4C

Three different concentrations of LVS suspended in phosphate-buffered saline were aliquoted and periodically assayed. Results were evaluated with respect to day-to-day coefficients of variance (CV) for the respective concentration of ecfu as given in Table 3.

tissues, feces and water samples, as well as human whole blood or urine, which were the only human specimens chosen even though further potential human samples could include biopsies, throat swabs or scrapings from affected tissues depending on the clinical presentation. Appropriate studies have to be undertaken if these specimens are intended for use by the ABICAP system.

Aside from infections through the skin, infections by ingestion and inhalation of a contaminated aerosol could occur. We studied experimentally subcutaneous-infected mice. The clinical outcome of an inhalative or alimentary infection would be the dissemination of the pathogen to target organs like the liver and spleen, where the bacteria could be well detected. Principally, there is no doubt that the assay would also work with lung tissue, which we have proven but have not statistically confirmed. Samples that would be collected during an aerosol-borne outbreak by air collecting systems would be washed out from filters by PBS and subsequently analyzed in this matrix, which is suitable for our assay.

Monoclonal IgG1 antibody (mab) FF/11/6 was employed for the assay design. Mab FF/11/6 was produced by myeloma cells fused with mouse (Balb/C) spleen cells after immunization with *F. tularensis* ssp. *tularensis* ATCC 6223 as formerly described (Greiser-Wilke *et al.* 1989). According to the known properties of the antibody, *F. tularensis* ssp. *novicida* and *F. philomiragia* were not recognized by the *Francisella* ABICAP system. This limitation has, however, only marginal relevance due to the extremely low incidence of tularemia caused by these two species/subspecies (Hollis *et al.* 1989; Wenger *et al.* 1989). More importantly, neither antibody-specific properties nor column-dependent effects led to false positive results, employing a representative panel of relevant pathogens (Table 2).

Based on blank measurements, an absolute number of ecfu between 1.1×10^2 to 1.8×10^3 could be discriminated from negative samples of the respective specimens by the ABICAP system. The determination limit derived from the functional assay sensitivity was reached when samples were spiked with approximately one order of magnitude more ecfu. Thus, the *Francisella* ABICAP seems to be applicable as a sensitive screening device. Noteworthy, sample volumes can be further increased, especially in the case of water samples, in order to further increase the probability of detecting a pathogen (data not shown).

In the case of whole blood, it is still necessary to optimize sample preparation procedures in order to improve the reliability of the system.

The analytical range was restricted at the upper limit to approximately 10^5 ecfu. In contrast, the *Francisella* ELISA was able to discriminate samples loaded with 10^3 to 10^6 ecfu (Grunow *et al.* 2000). Thus, when using the ABICAP device, 10-fold dilutions of the sample might be necessary if a high

bacterial load of the sample is expected and quantitative information is needed. In our view however, this aspect is negligible when using authentic samples from field investigations. In general, only low amounts of the pathogen are present in these samples. Quantification can be achieved by confirmatory assays such as PCR or cELISA under laboratory conditions. In most cases, the signal intensity of the ABICAP system remains strongly positive unless the column is clogged. The latter is visible to the operator because the flow through the column stops, and false negative or false positive results are immediately obvious.

Even more important is the finding that according to spiking experiments, no false negative results are likely to occur due to matrix effects.

All studies undertaken so far to determine the diagnostic sensitivity and specificity of approaches for the detection of *Francisella* are limited regarding the fact that none of them can be referred to as a so-called "gold standard," in particular, if it is claimed that the approach must have employed the respective matrix during validation. Diagnostic animal experiments, in order to enrich and subsequently isolate the pathogen, would most likely fit the requirements applied to a reference method. So far, there are no reports determining the diagnostic sensitivity and specificity of any approach for the direct detection of *Francisella* in authentic samples such as mice and rabbit feces as well as water from natural sources. Considering the low precision of cfu determination, there was an acceptable correlation between signal intensities of the ABICAP system and of a rugged, well-validated *Francisella* ELISA in spiked samples as well as artificially infected animal tissues. However, limited clinical diagnostic data for the *Francisella* ELISA do not allow for assessment of the diagnostic sensitivity and specificity of the *Francisella* ABICAP system based on this assay or other approaches such as PCR or culturing on appropriate growth media as a reference. In one study, a diagnostic sensitivity of PCR of approximately 73% for patients with ulceroglandular tularemia has been shown (Sjostedt *et al.* 1997).

Our studies in outbreak and surveillance settings underline the necessity to determine reference values for every single matrix intended for use, or to include recovery controls by spiking an aliquot of the specimen. Discrepancies in the distribution of signals obtained from negative samples can be expected in completely different matrices such as natural water sources and rabbit feces. Agreement rates between both immunoassays will remain satisfactory if the particularities of the respective matrix are considered. Another important conclusion from our data is that it cannot be assumed that reference values obtained from feces are interchangeable between species, particularly if mice are involved. Reasons for the significant high background levels observed in feces from SPF mice and wild-living mice are not

yet known. At the moment, we cannot explain the difference between the performance of rabbit and murine feces. It can only be speculated that different food components and digested products might be responsible for the observed differences. This phenomenon would mainly impair the specificity of the ABICAP system, and thus increase the number of samples that have to be confirmed by an independent approach. These limitations do not question the ABICAP system's intended use as a screening application in the field. Compared to former findings employing an immunochromatographic hand-held test kit with specific gold-labeled antibodies placed on a nitrocellulose membrane (Grunow *et al.* 2000), the ABICAP system appears to be superior in terms of sensitivity, even though the two devices have not been compared directly. In addition, it seems possible to adapt sample preparation to field conditions. The one-step extraction of LPS of *F. tularensis* works at different temperatures (Bosold 2001) and can be performed after homogenizing solid samples or directly in liquids with subsequent filtration. In the case of problematic matrices, e.g., murine feces, a recovery control at lower concentrations could be included, which could also indicate a high background. Further developments are in progress to combine internal controls using multiple sinters in one column.

In summary, we report performance characteristics of an immunoaffinity chromatographic column test for the specific detection of *F. tularensis* spp. *tularensis*, ssp. *holarctica* and ssp. *mediasiatica*, covering the pathogens responsible for tularemia outbreaks among immunocompetent persons. The assay was proved to have an acceptable precision, recovery rate and a detection limit sufficient for the sensitive detection of *F. tularensis* in a variety of human, animal and environmental samples.

These features, together with the robust assay format, would allow screening for *F. tularensis* by trained operators with minimal technical requirements in the field.

NOMENCLATURE

| | |
|--------|---|
| ABICAP | antibody immuno column for analytical processes |
| LPS | lipopolysaccharide |
| LVS | live vaccine strain |
| ecfu | equivalent of colony-forming units |

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REFERENCES

- BERDAL, B.P., MEHL, R., HAAHEIM, H., LOKSA, M., GRUNOW, R., BURANS, J., MORGAN, C. and MEYER, H. 2000. Field detection of *Francisella tularensis*. *Scand. J. Infect. Dis.* 32, 287–291.
- BOSOLD, A. 2001. *Optimierung und Evaluierung eines Capture-ELISA zum Nachweis von Francisella tularensis und Vergleich mit der PCR.* (Optimization and evaluation of a capture-ELISA for the detection of *Francisella tularensis* and comparison with PCR). MD Thesis, Technical University, Munich.
- CENTERS FOR DISEASE CONTROL AND PREVENTION. 2000. Biological and Chemical Terrorism: Strategic Plan for Preparedness and Response: Recommendations of the CDC Strategic Planning Workgroup; *MMWR* 49(RR04), 1–14.
- DENNIS, D.T., INGLESBY, T.V., HENDERSON, D.A., BARTLETT, J.G., ASCHER, M.S., EITZEN, E., FINE, A.D., FRIEDLANDER, A.M., HAUER, J., LAYTON, M. *ET AL.* 2001. Tularemia as a biological weapon: Medical and public health management. *J.A.M.A.* 285, 2763–2773.
- ELIASSON, H., LINDBACK, J., NUORTI, J.P., ARNEBORN, M., GIESECKE, J. and TEGNELL, A. 2002. The 2000 tularemia outbreak: A case-control study of risk factors in disease-endemic and emergent areas, Sweden. *Emerg. Infect. Dis.* 8, 956–960.
- ELIASSON, H., SJOSTEDT, A. and BACK, E. 2005. Clinical use of a diagnostic PCR for *Francisella tularensis* in patients with suspected ulceroglandular tularaemia. *Scand. J. Infect. Dis.* 37, 833–837.
- ELLIS, J.P., OYSTON, C., GREEN, M. and TITBALL, R.W. 2002. Tularemia. *Clin. Microbiol. Rev.* 15, 631–646.
- GESSLER, F., HAMPE, K. and BOHNEL, H. 2005. Sensitive detection of botulinum neurotoxin types C and D with an immunoaffinity chromatographic column test. *Appl. Environ. Microbiol.* 71, 7897–7903.
- GREISER-WILKE, I., SOINE, C. and MOENNIG, V. 1989. Monoclonal antibodies reacting specifically with *Francisella* sp. *Zentralbl. Veterinarmed. B.* 36, 593–600.
- GRUNOW, R. and FINKE, E.J. 2002. A procedure for differentiating between the intentional release of biological warfare agents and natural outbreaks of disease: Its use in analyzing the tularemia outbreak in Kosovo in 1999 and 2000. *Clin. Microbiol. Infect.* 8, 510–521.
- GRUNOW, R., SPLETTSTOESSER, W.D., MCDONALD, S., OTTERBEIN, C., O'BRIEN, T., MORGAN, C., ALDRICH, J., HOFER, E., FINKE, E.J. and MEYER, H. 2000. Detection of *Francisella tularensis* in bio-

- logical specimens using a capture enzyme-linked immunosorbent assay, an immunochromatographic handheld assay and a PCR. *Clin. Diagn. Lab. Immunol.* 7, 86–90.
- HARTMANN, H.B., LUBBERS, B., CASARETTO, M., BAUTSCH, W., KLOS, A. and KOHL, J. 1993. Rapid quantification of C3a and C5a using a combination of chromatographic and immunoassay procedures. *J. Immunol. Methods* 166, 35–44.
- HOFER, E., SCHILDORFER, H., FLATSCHER, J. and MÜLLER, M. 1997. Zum Nachweis der Tularämie bei Feldhasen (*Lepus europaeus*) in Österreich. *Wien. Tierärztl. Monatsschr.* 81, 301–308.
- HOLLIS, D.G., WEAVER, R.E., STEIGERWALT, A.G., WENGER, J.D., MOSS, C.W. and BRENNER, D.J. 1989. *Francisella philomiragia* comb. nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup *novicida* (formerly *Francisella novicida*) associated with human disease. *J. Clin. Microbiol.* 27, 1601–1608.
- JOHANSSON, A., FARLOW, J., LARSSON, P., DUKERICH, M., CHAMBERS, E., BYSTROM, M., FOX, J., CHU, M., FORSMAN, M., SJOSTEDT, A. ET AL. 2004. Worldwide genetic relationships among *Francisella tularensis* isolates determined by multiple-locus variable-number tandem repeat analysis. *J. Bacteriol.* 186, 5808–5818.
- PARKER, R.R., STEINHAUS, E.A., KOHLS, G.M. and JELLISON, W.L. 1951. Contamination of natural waters and mud with *Pasteurella tularensis* and tularemia in beavers and muskrats in the northwestern United States. *Bull. Natl. Inst. Health* 193, 1–161.
- PASSING, H. and BABLOK, A. 1983. New biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clinical chemistry, Part I. *J. Clin. Chem. Clin. Biochem.* 21, 709–720.
- PETERSEN, J.M., SCHRIEFER, M.E., GAGE, K.L., MONTENIERI, J.A., CARTER, L.G., STANLEY, M. and CHU, M.C. 2004. Methods for enhanced culture recovery of *Francisella tularensis*. *Appl. Environ. Microbiol.* 70, 3733–3735.
- PORSCH-OZCURREMEZ, M., KISCHEL, N., PRIEBE, H., SPLETTSTOSSER, W.D., FINKE, E.J. and GRUNOW, R. 2004. Comparison of enzyme-linked immunosorbent assay, Western blotting, microagglutination, indirect immunofluorescence assay, and flow cytometry for serological diagnosis of tularemia. *Clin. Diagn. Lab. Immunol.* 11, 1008–1015.
- REINTJES, R., DEDUSHAJ, I., GJINI, A., JORGENSEN, T.R., COTTER, B., LIEFTUCHT, A., D'ANCONA, F., DENNIS, D.T., KOSOY, M.A., MULLIQI-OSMANI, G. ET AL. 2002. Tularemia outbreak investigation

- in Kosovo: Case control and environmental studies. *Emerg. Infect. Dis.* 8, 69–73.
- SJOSTEDT, A., ERIKSSON, U., BERGLUND L. and TARNVIK, A. 1997. Detection of *Francisella tularensis* in ulcers of patients with tularemia by PCR. *J. Clin. Microbiol.* 35, 1045–1048.
- TARNVIK, A. and BERGLUND, L. 2003. Tularaemia. *Eur. Respir. J.* 21, 361–373.
- TARNVIK, A., LOFGREN, S., OHLUND, L. and SANDSTROM, G. 1987. Detection of antigen in urine of a patient with tularemia. *Eur. J. Clin. Microbiol.* 6, 318–319.
- WENGER, J.D., HOLLIS, D.G., WEAVER, R.E., BAKER, C.N., BROWN, G.R., BRENNER, D.J. and BROOME, C.V. 1989. Infection caused by *Francisella philomiragia* (formerly *Yersinia philomiragia*). A newly recognized human pathogen. *Ann. Intern. Med.* 110, 888–892.
- ZEIDNER, N.S., CARTER, L.G., MONTENEIRI, J.A., PETERSEN, J.M., SCHRIEFER, M., GAGE, K.L., HALL G. and CHU, M.C. 2004. An outbreak of *Francisella tularensis* in captive prairie dogs: An immunohistochemical analysis. *J. Vet. Diagn. Invest.* 16, 150–152.