JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2007, p. 3404–3407 Vol. 45, No. 10
0095-1137/07/$08.00+0 doi:10.1128/JCM.00458-07

Comparison of Hand-Held Test Kits, Immunofluorescence Microscopy, Enzyme-Linked Immunosorbent Assay, and Flow Cytometric Analysis for Rapid Presumptive Identification of *Yersinia pestis*▽

H. Tomaso,[1]* P. Thullier,[2] E. Seibold,[1] V. Guglielmo,[2] A. Buckendahl,[1] L. Rahalison,[3] H. Neubauer,[4] H. C. Scholz,[1] and W. D. Splettstoesser[1]

Bundeswehr Institute of Microbiology, Neuherbergstrasse 11, 80937 Munich, Germany[1]; Groupe de Biotechnologie des Anticorps, Départment de Biologie des Agents Transmissibles, Centre de Recherches du Service de Santé des Armées, 24 Avenue des Maquis du Grésivaudan, P.O. Box 87, 38702 La Tronche, France[2]; World Health Organization Collaborating Center for Plague, Insitut Pasteur de Madagascar, Route de l'Institut Pasteur, Ambatofotsikely-Avaradoha, P.O. Box 1274, Antananarivo 101, Madagascar[3]; and Friedrich Loeffler Institut, Naumburgerstrasse 96a, 07743 Jena, Germany[4]

Received 28 February 2007/Returned for modification 11 April 2007/Accepted 17 July 2007

An in-house immunochromatographic test, Plague BioThreat Alert test strips, ABICAP columns, enzyme-linked immunosorbent assay, flow cytometry, and immunofluorescence microscopy were compared for the detection of the fraction 1 capsular antigen of *Yersinia pestis*, using spiked buffer and clinical specimens. Hand-held test kits proved to be excellent benchtop tools.

Plague, caused by *Yersinia pestis*, is a zoonosis mainly circulating among rodents and their fleas, which can transmit the disease to humans. The most important clinical forms of plague are bubonic, primary septicemic, and pneumonic plague. *Y. pestis* produces a specific fraction 1 capsular antigen (F1) which can be detected as a soluble molecule in clinical specimens early in the course of the disease. Chanteau et al. measured levels of F1 in patients with bubonic plague in the range between 4 ng/ml and 50 μg/ml in serum by using an enzyme-linked immunosorbent assay (ELISA) (1). The median antigen level for the group of patients that survived was 275 ng/ml (25th and 75th percentiles, 59.5 and 521.5 ng/ml), while the concentrations of F1 in sera from seven patients who died were 10,400 to 50,000 ng/ml. In fact, it was only in a single fatal case that the antigenemia was as low as 4 ng/ml of serum. In a second study with 194 patients, also by Chanteau et al. (2), the geometric mean concentrations of F1 in bubo fluid, serum, and urine were 70.8 ng/ml, 42 ng/ml, and 13.5 ng/ml, respectively. Accordingly, Williams et al. used an ELISA to determine F1 antigenemia during acute plague which detected F1 at a concentration of 4 ng/ml with a probability of 95% (11). The two bubonic plague cases in this study had levels of antigenemia of 4 to 8 μg/ml. Recently, hand-held test kits which detect F1 for the purpose of diagnosing plague have been developed and evaluated with clinical samples, but the best evaluated test is not yet commercially available (2, 3). Detection of specific antibodies is not an alternative for diagnosis because of the time lag for seroconversion, and it is limited to retrospective confirmation (10). During bacteremia, the number of *Y. pestis* cells in blood averages 10^6 to 10^8 CFU/ml (7) but can also exceed 10^8 CFU/ml of blood or g of tissue (8), allowing plague diagnosis confirmation by cultivation of *Y. pestis*, which, however, requires at least 24 h and adequate biosafety level 3 laboratory facilities. Isolates are typically identified based on morphology, specific phage lysis, and biochemical tests (8). Due to the severity of plague and the potential use of *Y. pestis* as a biological agent, there is a clear need for rapid identification tools that would at least have presumptive value and would help to shorten the hands-on time under biosafety level 3 conditions (4). One of the purposes of this study was to evaluate, for the first time, the potential of hand-held kits originally designed for soluble F1 detection in clinical samples for this benchtop use.

In order to compare different immunological assay formats and to evaluate commercial products, we compared an in-house immunochromatographic test (ICT) with two rapid commercial assays, namely, Plague BioThreat Alert test strips (BTA; Tetracore, Gaithersburg, MD) and ABICAP columns (Senova, Jena, Germany). To represent ELISA, the standard immunological test, a commercial ELISA for the detection of the F1 capsular antigen of *Y. pestis*, as a soluble molecule and at the surfaces of suspicious bacteria, was chosen (Seramun, Dolgenbrodt, Germany). Additionally, we performed flow cytometry (FC) and immunofluorescence (IF) microscopy with antibodies directed against F1 to identify and directly visualize the bacteria, respectively.

The detection limit for F1 soluble protein was assessed for all assays (Table 1) in triplicate in phosphate-buffered saline (PBS) spiked with clinical samples, using serial dilutions of one batch of purified F1 (from *Y. pestis* strain EV76) purchased from the Russian State Research Centre of Applied Microbiology, Obolensk, Russia. The purity of the protein preparation was confirmed by gel electrophoresis followed by staining with Coomassie blue (6). Regarding detection of F1 at the surfaces of bacteria, it was considered that specificity was at least as important as sensitivity, as this detection is performed after an initial cultivation step. This specificity was evaluated utilizing a representative collection of *Y. pestis* strains, other *Yersinia* sp.

* Corresponding author. Mailing address: Bundeswehr Institute of Microbiology, Neuherbergstrasse 11, 80937 Munich, Germany. Phone: 49-8931683808. Fax: 49-8931683292. E-mail: herberttomaso@bundeswehr.org.

▽ Published ahead of print on 25 July 2007.

TABLE 1. Performance of immunoassays detecting the specific F1 capsular antigen for the identification of *Yersinia pestis*

Method	Producer	Detection limit (ng/ml)[a]				CFU/ml (buffer)	Time (h:min)		Cost per test ($)[b]	Ease of handling[c]
		Buffer (3 replicates)	Sputum (2 specimens)	Serum (20 specimens)	Urine (20 specimens)		Hands-on	Total test procedure		
ICT	In-house	0.75	3.3	3.3	3.3	3×10^3	00:07	00:20	3	Simple
BTA test	Tetracore	35	ND	ND	ND	7×10^3	00:07	00:25	20	Simple
ABICAP test	Senova, Germany	0.25	3.3	30	30	6×10^3	00:20	01:05	10	Simple
ELISA	Seramun, Germany	0.75	3.3	10	10	6×10^3	00:25	02:20	15	Standard
FC	In-house	ND	ND	ND	ND	5×10^3	00:20	00:40	1	Sophisticated
IF microscopy	In-house	ND	ND	ND	ND	10^3	00:30	02:00	3	Standard

[a] ND, not done.
[b] Includes only consumables and may vary depending on the number of tests performed.
[c] Simple, short training is sufficient for a technician; standard, standard microbiological test; sophisticated, special training and experience are necessary.

isolates, and 34 clinically relevant bacterial species grown at 37°C (Table 2) at a fixed concentration. Briefly, material from bacterial colonies was resuspended in PBS and adjusted to an optical density at 600 nm (OD_{600}) of 1.0, corresponding to 5×10^8 bacteria/ml. Inactivation of *Y. pestis* was performed by incubating bacteria in PBS containing 0.1% Tween 20 and 10% formalin for 1 h at room temperature with shaking at 150 rpm. Subsequently, the bacteria were washed twice in PBS containing 0.1% Tween 20 and again adjusted to an OD_{600} of 1.0. The detection limit for bacteria was determined using serial dilutions of *Y. pestis* strain EV76 grown at 37°C without inactivation. The number of CFU was assessed using a standard plating method and confirmed using the *Yersinia pestis* LightMix quantitative real-time PCR assay produced by TIBMOLBIOL (Berlin, Germany). We additionally evaluated ease of handling, costs, and time required to perform the assays (Table 1).

The in-house ICT is a lateral-flow assay, where F1 antigen is sandwiched between the capture anti-F1 rabbit polyclonal antibody immobilized on the strip and the detecting anti-F1 mouse monoclonal antibody conjugated to anti-mouse immunoglobulin G (IgG) colloidal gold beads. The concentration of the purified anti-F1 rabbit polyclonal capture antibody was adjusted to 1 mg/ml in PBS, and the detecting antibody was applied at a concentration of 1 μl/cm of a 1-μg/μl antibody solution in PBS. A conjugate solution was prepared by incubating 2 volumes of gold-conjugated anti-mouse IgG (OD_{400}, 10; BBI) with 1 volume of the anti-F1 monoclonal detection antibody G20 (Squarix, Marl, Germany) at a concentration of 40 μg/ml in gold resuspension buffer (Alchemy Laboratories Ltd., Dundee, United Kingdom). The running buffer contained 0.1 M phosphate buffer (pH 7.0) supplemented with 1% Tween 20. The test devices developed were subjected to stability studies. The strips were kept in sealed tubes with desiccant at 37°C, room temperature, 4°C, or −20°C. The detection limit was not affected after at least 6 months of storage under any of these conditions. The strips were read visually and with a GelDoc XR imaging system (Bio-Rad) using Quantity One 4.6.0 software.

The BTA test strip produced by Tetracore (Gaithersburg, MD) is a lateral-flow assay intended for qualitative analysis of environmental samples in the field. The assay was performed and the results were interpreted according to the manufacturer's instructions. ABICAP columns (Senova, Jena, Germany) contain a filter coated with anti-*Y. pestis* F1 antibodies (G20; Squarix, Marl, Germany). The captured analyte is detected using the same antibody conjugated with PolyHRP40 (SDT, Baesweiler, Germany). The ABICAP system has the theoretical advantage that a large sample volume can be loaded upon the columns, resulting in an enrichment of the soluble analyte (2). Readers for the ABICAP tests (ca. $500) are optional but facilitate the quantification of signals obtained. The test was performed according to the manufacturer's instructions, using a reader. The commercial ELISA applied for the detection of F1 (Seramun, Dolgenbrodt, Germany) provided all necessary reagents and was performed according to the manufacturer's instructions. FC analysis was performed on a Cyflow FL instrument (Partec, Muenster, Germany). Approximately 10^6 bacteria were suspended in 50 μl PBS containing 1% bovine serum albumin and 0.05% sodium azide. The Oregon Green-labeled anti-F1 antibody G20 (Squarix, Marl, Germany) was added at a concentration of 10 μg/ml. For the generic detection of bacteria, propidium iodide was used at a final concentration of 10 μg/ml. After 15 min of incubation in the dark at room temperature, 950 μl of PBS was added and centrifuged for 10 min at 6,000 rpm in a microcentrifuge. The supernatant was discarded, and the pellet was resuspended in 1 ml PBS containing 1% bovine serum albumin and 0.05% sodium azide. Only Oregon Green and propidium iodide double-positive events were counted as *Y. pestis* bacteria. IF microscopy was performed using inactivated bacteria suspended in PBS at a concentration of approximately 10^7 bacteria/ml. Twenty microliters of this suspension was placed on a slide and dried for 1 h at room temperature. The sample was fixed with methanol for 5 min and incubated with 20 μl of the anti-F1 antibody G20 conjugated with Oregon Green (diluted 1:50 in PBS containing 10 μg/ml Evans Blue) for 30 min at 37°C. After three washing steps with PBS, the sample was dried and covered with an antifade reagent containing the DNA-staining dye DAPI (4′,6′-diamidino-2-phenylindole). The sample was analyzed with a standard fluorescence microscope (Axio Star Plus; Zeiss, Germany).

The ABICAP test had the best detection limit in buffer (0.25 ng/ml), but the in-house ICT, which had a detection limit of 0.75 ng/ml in buffer, showed the best results with all clinical samples (sputum, serum, and urine), with a sensitivity of 3.3 ng/ml (Table 1). The ABICAP test sensitivity was 3.3 ng/ml for sputum, but only 30 ng/ml for serum and urine, a value sufficient in most cases for diagnosis with serum but not urine. The

TABLE 2. Specificities of immunoassays targeting F1 capsular antigen of *Yersinia pestis*

Species	Strain[a]	Result[b]					
		ICT	BTA test	ABICAP test	ELISA	FC	IF microscopy
Yersinia pestis	TS (USAMRIID)	+	+	+	+	+	+
	KUMA (Asia)	+	+	+	+	+	+
	Yokohama (Japan)	+	+/−	+	+	+	+
	KIM (Pakistan)	+	+	+	+	+	+
	784 (India)	+	+	+	+	+	+
	785 (Java)	+	+/−	+	+	+	+
	788 (India)	+	+/−	+	+	+	+
	790 (Kenia)	+	+	+	+	+	+
	EV76 (Madagascar)	+	+	+	+	+	+
	"*Y. pestoides*" (Russia)	+	+	+	+	+	+
Yersinia aldovae	ATCC 35236	−	−	−	−	−	−
Yersinia bercovieri	ATCC 43970	−	−	−	−	−	−
Yersinia enterocolitica	DSM 4780	−	−	−	−	−	−
Yersinia frederiksenii	ATCC 33641	−	−	−	−	−	−
Yersinia intermedia	ATCC 29909	−	−	−	−	−	−
Yersinia kristensenii	ATCC 33638	−	−	−	−	−	−
Yersinia mollaretii	ATCC 43969	−	−	−	−	−	−
Yersinia pseudotuberculosis	ATCC 29833	−	−	−	−	−	−
Yersinia rohdei	ATCC 43380	−	−	−	−	−	−
Yersinia ruckeri	DSM 18506	−	−	−	−	−	−
Acinetobacter baumannii	DSM 7324	−	−	−	−	−	−
Aeromonas hydrophila	ATCC 7966	−	−	−	−	−	−
Alcaligenes faecalis	DSM 30030	−	−	−	−	−	−
Brucella abortus	ATCC 23448	−	−	−	−	−	−
Brucella melitensis	NCTC 10094	−	−	−	−	−	−
Burkholderia mallei	ATCC 23344	−	−	−	−	−	−
Burkholderia pseudomallei	ATCC 23343	−	−	−	−	−	−
Candida albicans	DSM 1386	−	−	−	−	−	−
Citrobacter freundii	DSM 30039	−	−	−	−	−	−
Citrobacter koseri	DSM 4595	−	−	−	−	−	−
Enterobacter aerogenes	DSM 12058	−	−	−	−	−	−
Enterobacter cloacae	ATCC 13047	−	−	−	−	−	−
Enterococcus faecalis	DSM 2570	−	−	−	−	−	−
Escherichia coli	ATCC 25922	−	−	−	−	−	−
Francisella tularensis holarctica	ATCC 29684	−	−	−	−	−	−
Francisella tularensis subsp. *tularensis*	ATCC 6223	−	−	−	−	−	−
Haemophilus influenzae	DSM 4690	−	−	−	−	−	−
Klebsiella pneumoniae	DSM 30104	−	−	−	−	−	−
Listeria monocytogenes	DSM 12464	−	−	−	−	−	−
Moraxella catarrhalis	DSM 9143	−	−	−	−	−	−
Morganella morganii	DSM 6675	−	−	−	−	−	−
Neisseria meningitidis	DSM 10036	−	−	−	−	−	−
Ochrobacterium anthropi	DSM 7216	−	−	−	−	−	−
Propionibacterium acnes	DSM 1897	−	−	−	−	−	−
Proteus mirabilis	DSM 4479	−	−	−	−	−	−
Proteus vulgaris	DSM 30118	−	−	−	−	−	−
Pseudomonas aeruginosa	DSM 11810	−	−	−	−	−	−
Salmonella enterica serovar Typhimurium	ATCC 13311	−	−	−	−	−	−
Serratia proteomaculans	DSM 1636	−	−	−	−	−	−
Shigella flexneri	DSM 4782	−	−	−	−	−	−
Staphylococcus aureus	DSM 346	−	−	−	−	−	−
Staphylococcus epidermidis	DSM 1798	−	−	−	−	−	−
Stenotrophomonas maltophilia	DSM 50170	−	−	−	−	−	−
Streptococcus pneumoniae	DSM 20566	−	−	−	−	−	−
Streptococcus pyogenes	DSM 20565	−	−	−	−	−	−

[a] ATCC, American Type Culture Collection; DSM, German Collection of Microorganisms and Cell Cultures; USAMRIID, United States Army Medical Research Institute for Infectious Diseases.
[b] +, positive; −, negative; +/−, faint positive bands present.

third hand-held test evaluated in this study, the commercially available BTA test, had a much lower sensitivity than those of the ICT and the ABICAP test, equal to 35 ng F1/ml in buffer. This sensitivity was not enhanced by using the optional strip reader (ca. $4,000) available from Tetracore (Gaithersburg, MD), and the BTA test strip was not evaluated further. ELISA, with a detection limit of 0.75 ng/ml in buffer, had the same sensitivity with sputum as the in-house ICT and the

ABICAP test (3.3 ng/ml), but its sensitivity with serum and urine (10 ng/ml) was intermediate compared to those of the other two tests. It is notable that the detection limits of previously established immunochromatographic dipsticks are 0.5 ng/ml in buffer (3), and thus equivalent to that of our in-house ICT, and 2 to 4 ng/ml with clinical samples for ELISAs (1, 9, 11).

The order of the evaluated tests regarding their sensitivities for F1 detection at the surfaces of bacteria generally mirrored the order for soluble F1 detection, but differences between test sensitivities were far less marked, as the numbers of bacteria detected ranged from 3×10^3 (ICT) to 7×10^3 (BTA test) (Table 1). In particular, all tests gave a positive response when a colony was resuspended in 500 μl PBS. It is possible that the narrowing of the difference between tests for detecting the multiple copies of F1 at the surfaces of bacteria may be due to an avidity effect that would compensate for the intermediate-level affinity of the antibodies that could be utilized in the test that performed worst on soluble F1. Importantly, the specificity of all tests for *Y. pestis* detection was 100%, as they identified all *Y. pestis* isolates but detected no other bacteria (Table 2).

The ICT, BTA, and ABICAP tests are all simple to perform. The time required to obtain results was less than 25 minutes for the ICT and the BTA test and 45 minutes for the ABICAP test. The ICT was the most economical test, as it was produced in-house and no special equipment was required to perform the test. FC was the most expensive technique due to the equipment cost ($60,000), but reagent costs per assay were relatively low (ca. $1). FC offers the possibility of high-throughput analysis and multiplexing for different targets. The ELISA format allows for the automated, simultaneous assessment of many samples. IF microscopy is useful for small numbers of samples and when visualization of bacteria is desired.

Our results indicate that the in-house ICT is a rapid, reliable, and economical benchtop tool for the detection of F1 for the presumptive identification of *Y. pestis* bacteria. Therefore, this lateral-flow assay should be developed further as a product for diagnostic purposes. The other assays also proved to be specific and offered individual advantages for various applications.

We greatly appreciate the excellent laboratory assistance of M. Goetz, F. Feist, C. Kleinemeier, R. Kuehn, and R. Schneider.

REFERENCES

1. **Chanteau, S., L. Rabarijaona, T. O'Brien, L. Rahalison, J. Hager, P. Boisier, J. Burans, and M. Rasolomaharo.** 1998. F1 antigenaemia in bubonic plague patients, a marker of gravity and efficacy of therapy. Trans. R. Soc. Trop. Med. Hyg. **92:**572–573.
2. **Chanteau, S., L. Rahalison, M. Ratsitorahina, M. Mahafaly, M. Rasolomaharo, P. Boisier, T. O'Brien, J. Aldrich, A. Keleher, C. Morgan, and J. Burans.** 2000. Early diagnosis of bubonic plague using F antigen capture ELISA assay and rapid immunogold dipstick. Int. J. Med. Microbiol. **290:**279–283.
3. **Chanteau, S., L. Rahalison, L. Ralafiarisoa, J. Foulon, M. Ratsitorahina, L. Ratsifasoamanana, E. Carniel, and F. Nato.** 2003. Development and testing of a rapid diagnostic test for bubonic and pneumonic plague. Lancet **361:** 211–216.
4. **Inglesby, T. V., D. T. Dennis, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. D. Fine, A. M. Friedlander, J. Hauer, J. F. Koerner, M. Layton, J. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, M. Schoch-Spana, and K. Tonat.** 2000. Plague as a biological weapon: medical and public health management. JAMA **283:**2281–2290.
5. **Leal-Balbino, T. C., N. C. Leal, C. V. Lopes, and A. M. P. de Almeida.** 2004. Differences in the stability of the plasmids of *Yersinia pestis* cultures in vitro: impact on virulence. Mem. Inst. Oswaldo Cruz **99:**727–732.
6. **Neubauer, H., L. Rahalison, T. J. Brooks, S. Aleksic, S. Chanteau, and W. D. Splettstoesser.** 2000. Serodiagnosis of human plague by an anti-F1 capsular antigen specific IgG/IgM ELISA and immunoblot. Epidemiol. Infect. **125:** 593–595.
7. **Norkina, O. V., A. N. Kulichenko, A. L. Gintsburg, I. V. Tuchkov, A. Y. Popov, M. U. Aksenov, and I. G. Drosdov.** 1994. Development of a diagnostic test for *Yersinia pestis* by the polymerase chain reaction. J. Appl. Bacteriol. **76:**240–245.
8. **Russell, P., M. Nelson, D. Whittington, M. Green, S. M. Eley, and R. W. Titball.** 1997. Laboratory diagnosis of plague. Br. J. Biomed. Sci. **54:**231–236.
9. **Splettstoesser, W. D., L. Rahalison, R. Grunow, H. Neubauer, and S. Chanteau.** 2004. Evaluation of a standardized F1 capsular antigen capture ELISA test kit for the rapid diagnosis of plague. FEMS Immunol. Med. Microbiol. **41:**149–155.
10. **Thullier, P., V. Guglielmo, M. Rajeson, and S. Chanteau.** 2003. Serodiagnosis of plague in humans and rats using a rapid test. Am. J. Trop. Med. Hyg. **69:**450–451.
11. **Williams, J. E., M. K. Gentry, C. A. Braden, F. Leister, and R. H. Yolken.** 1984. Use of an enzyme-linked immunosorbent assay to measure antigenaemia during acute plague. Bull. W. H. O. **62:**463–466.