Development and testing of a rapid diagnostic test for bubonic and pneumonic plague

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Summary

Background Plague is often fatal without prompt and appropriate treatment. It affects mainly poor and remote populations. Late diagnosis is one of the major causes of human death and spread of the disease, since it limits the effectiveness of control measures. We aimed to develop and assess a rapid diagnostic test (RDT) for plague.

Methods We developed a test that used monoclonal antibodies to the F1 antigen of Yersinia pestis. Sensitivity and specificity were assessed with a range of bacterial cultures and clinical samples, and compared with findings from available ELISA and bacteriological tests for plague. Samples from patients thought to have plague were tested with the RDT in the laboratory and by health workers in 26 pilot sites in Madagascar.

Findings The RDT detected concentrations of F1 antigen as low as 0.5 ng/mL in up to 15 min, and had a shelf life of 21 days at 60°C. Its sensitivity and specificity were both 100%. RDT detected 41.6% and 31% more positive clinical specimens than did bacteriological methods and ELISA, respectively. The agreement rate between tests done at remote centres and in the laboratory was 89-8%. With the combination of bacteriological methods and F1 ELISA as reference standard, the positive and negative predictive values of the RDT were 90-6% and 86-7%, respectively.

Interpretation Our RDT is a specific, sensitive, and reliable test that can easily be done by health workers at the patient's bedside, for the rapid diagnosis of pneumonic and bubonic plague. This test will be of key importance for the control of plague in endemic countries.

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Methods

Development of plague RDT

To develop monoclonal antibodies (Mab) against F1 antigen, we immunised mice (Biozzi BP/2 strain) with F1 antigen that was purified as Chen and Meyer described from Y pestis strain 1855S/97 (Madagascar). The fusion and screening methods used have been described. 67 hybridomas with strong IgG responses against F1 were obtained, 16 of which were cloned and characterised. For development of the plague RDT, two monoclonal antibodies (IgG1 and κ chain isotype) that bound to two different epitopes of the F1 antigen were selected on the basis of their high affinity, and were purified by ammonium sulphate precipitation and fast protein liquid chromatography (Pharmacia, Boîn d’Arcy, France) according to the column manufacturer’s instructions. Mab B18–1 (kD <5×10^13 mol) was conjugated to gold particles as the mobile phase, and Mab G6–18 (kD=1×10^10 mol) was deposited on nitrocellulose as the capture antibody. The hybridomas B18–1 and G6–18 were stored at the Collection National des Microorganismes (Paris, France).

Our RDT was derived from a prototype developed by the Naval Medical Research Institute (Bethesda, Maryland, USA) and assessed in Madagascar. For this new test, we used a combination of B18–1 and G6–18 produced at the Institut Pasteur, whereas the original RDT combined F1–04-A-G1 Mab with a polyclonal rabbit antiserum, and was based on one-step, vertical-flow immunochromatography. The colloidal gold particles (40 nm diameter) were conjugated to anti-F1 Mab B18–1 (British Biocell International Cardiff, UK) and lyophilised (A nm=3) onto polyester release pads (Filfit, Ambérieu en Bugey, France). An automatic thin layer chromatography sampler (CAMAG, Muttenz, Switzerland) was used to spray the anti-F1 capture Mab G6–18 in a line, at a concentration of 2 µg/cm, onto nitrocellulose membrane (Immunopore FP, Whittman International, Chateau Giron, France); together with the control capture line, which was affinity-purified goat anti-mouse IgG (ICN Biomedicals, Aurora, Ohio, USA), sprayed as a line higher up the strip at a concentration of 0·8 µg/cm. Cellulose filter paper was used for the wicking pad and sample pads (Schull and Schleicher, Ecquevilly, France).

The immunostrips were trimmed to a width of 5 mm and stored in a waterproof bag at 4°C, or in 5 mL disposable plastic tubes at room temperature at non-central sites. The test was done in the plastic tube with a sample volume of about 200 µL. After 10–15 min, a positive result appeared as two pink lines (upper control line and lower F1-positive line), and a negative result as a single upper pink control line. Semiquantitative analysis was possible, with the intensity of the test line scored from 0·5 to 4 (indicating the concentration of F1 antigen in the test sample). The cutoff and the range of F1 concentration that could be detected was measured with tenfold dilutions (50 µg/mL to 0·05 ng/mL) of purified F1 antigen. The reliability of the RDT was assessed with two concentrations of purified F1 antigen (0·5 ng/mL and 1 µg/mL).

The shelf life of the strips in the laboratory was assessed by testing twice per week for 3 weeks after storage at 60°C, 4°C, −20°C, and −80°C. The reliability of the RDT was assessed by repeating the test ten times on the same sample and in ten different series of tests.

Reference tests and samples

We used two reference standard tests for the diagnosis of plague. The first was gram staining and isolation of Y pestis, either directly from the patients’ samples or after mouse inoculation. A specimen was judged to be confirmed as being plague when the culture was positive, presumptive when culture was negative but microscopy was positive, and negative when both tests were negative. Confirmed and presumptive cases were notified to WHO. The second method was the immunocapture ELISA for detection of F1 antigen with F1–04-A–G1 anti-F1 Mab. The lowest concentration of antigen detectable with this test was 2 ng/mL.

To assess the sensitivity of the plague RDT we used 85 strains of Y pestis that had been cultured in brain-heart broth at 37°C for 72 h. 55 of these were isolated in Madagascar from 1996 to 2000 (35 from patients with bubonic plague, four from those with pneumonic plague, seven from rats [Rattus norvegicus], three from fleas [Xenopsylla cheopis], seven from shrews [Suncus murinus], and one from a hedgehog [Setifer setosus]). 30 strains came from 14 other countries (collection of the WHO Collaborating Centre for Yersinia, Institut Pasteur, Paris, France): five of biotype Antiqua (four from Kenya, one former USSR), five of biotype Medievalis (Kurdistan [four], Turkey [one]), and 20 of biotype Orientalis (Germany [two], Morocco [one], Turkey [one], India [one], Namibia [two], Vietnam [seven], Brazil [two], Argentina [one], Senegal [one], South Africa [one], Congo [one]). Additionally, we used 198 clinical specimens from plague patients that had tested positive by both bacteriology and F1 ELISA: 17 sputum samples, 35 serum samples, 43 urine samples, and 103 bubo aspirates.

To assess the specificity of the test we used 189 cultures of yersinia and other enterobacteria grown in brain-heart broth, at 37°C for 72 h. These samples were: 71 cultures of enteropathogenic yersinia, consisting of Y pseudotuberculosis (44 strains, serotypes I–V from 17 different animal species and environmental samples isolated in 22 different countries), and Y enterocolitica (27 strains isolated in seven countries and belonging to six biotypes [1A–5] and to various serotypes); 52 non-pathogenic yersinia of various serotypes belonging to eight species and isolated in various countries [Y kristensenii (nine), Y intermedius (ten), Y mollaretii (ten), Y frederiksenii (ten), Y bercovertii (ten), Y rhodei (one), Y ruckeri (one), and Y aldovae (one); and 66 enterobacteria belonging to 19 genera and 55 species (Escherichia spp [eight], Enterobacter spp [ten], Erwinia sp [one], Hafnia sp [one], Serratia spp [ten], Klebsiella spp [ten], Salmonella spp [four], Shigella spp [four], Letonia sp [one], Citrobacter spp [two], Edwardsiella spp [two], Proteus spp [two], Providencia spp [two], Morganella spp [one], Kuevorea spp [two], Bvadicia sp [one], Cedeeia spp [two], Lentinorella spp [two], and Oesumbacterium sp [one]). Additionally, we used 137 clinical specimens that were negative for plague: 47 sputum samples from suspected tuberculosis patients (14 positive for Mycobacterium tuberculosis), 66 normal serum samples diluted 1/10, and 24 undiluted normal urine samples. For ethical reasons, bubo aspirates were not taken from patients who were not thought to have plague.

The negative and positive predictive values (NPV and PPV, respectively) of the F1 dipstick were calculated by Bayes’ theorem, and 95% CI by Fleiss’ quadratic approximation method (Sp=sensitivity, Se=sensitivity):

\[
NPV = \frac{Sp \times (1-Se)}{(1-NPV \times (1-SP))}
\]

\[
PPV = \frac{Se \times (1-SP)}{(1-St Spe \times (1-Se))}
\]

The agreement between RDT and reference tests results (bacteriology or F1 ELISA, and bacteriology combined
We undertook a prospective study to assess the effectiveness of the F1 dipstick during the 2000–01 plague season in Madagascar, as part of the national plague control programme. We obtained a biological sample (bubo aspirate, sputum, or post-mortem organ puncture, as appropriate) from patients with suspected plague, and the patients were treated with streptomycin. Samples were sent on a swab in Cary Blair agar, at room temperature, to the Central Laboratory at the Institut Pasteur de Madagascar. On arrival, the specimen was washed out of the swab by incubation in 1 mL phosphate-buffered saline, and was tested with the two reference methods and with RDT. The mean transport times of the positive and negative samples to the Central Laboratory were compared with the Kruskal-Wallis non-parametric test.

To test the RDT in remote areas, a network of 26 pilot sites was set up from Dec 1, 2000, to Jan 25, 2001, consisting of six district hospitals and 20 health care centres in remote areas (figure). After the efficiency of the plague RDT had been assessed at the Central Laboratory, kits—each composed of one syringe, one needle, PBS, one plague RDT in a disposable test tube with silica gel, one Cary Blair tube, one swab, a user’s manual, and a questionnaire—were distributed to these field sites. The robustness of the test under field conditions was assessed, based on several criteria: reliability of the results obtained by non-biologist staff, presentation of the kit, ease of manipulation and interpretation, shelf life under real field conditions, length and content of the training programme, and intelligibility of the illustrated manual.

The RDTs were stored at room temperature during the study (20–30°C). 29 medical doctors, 19 nurses, and nine health workers participated in the assessment. They were trained onsite for 3 h as to how to obtain clinical samples and to use, read, and archive the dipsticks. An illustrated instruction guidebook, in French and Malagasy, was given to each centre. The plague RDT was done at the patient’s bedside. The trained personnel were then asked to send a sample of the specimen to the Central Laboratory for a dipstick test with the same batch of strips, bacteriological identification, and FI ELISA assay. The technicians who did the tests at the Central Laboratory were not aware of the results obtained in the pilot centres.

From May to June, 2001 (end of the plague season), the 26 sites were visited again, RDT results were compared, and the registers were checked. The health personnel were asked to fill in an individual user’s questionnaire, to provide criticisms, comments, and observations about the plague RDT and the instruction book.

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

**Role of the funding source**

The lower detection threshold of the newly developed plague RDT was 0·5 ng/mL, and the range of F1 concentrations extended from 0·5 ng/mL to 50 μg/mL (5 log), without any hook effect (in which no signal is detected for high concentrations). Results obtained were much the same after storage of RDT for 21 days at 60°C, 4°C, –20°C, and –80°C.

The RDT had a sensitivity of 100% on freshly isolated Y pestis strains and positive control clinical samples: all 55 bacterial cultures and all 198 positive specimens tested were positive with the RDT. Positive RDT results were obtained for 28 of 30 Y pestis strains from various continents. PCR
assays done on the two RDT-negative strains with primers internal to the calp gene yielded no amplification product, indicating that this gene (or the entire pFra plasmid) was deleted in these two strains. Comparison of the plasmid restriction profiles of these strains with that of wild-type strain 6/69 from Madagascar confirmed the absence of the restriction fragments corresponding to pFra (data not shown). The specificity of the plague RDT was also 100% for all bacterial cultures and all negative control clinical specimens. Of the 283 bubo aspirates from suspected plague patients that tested negative in bacteriology and ELISA, 256 (90.5%) also tested negative by RDT, but 27 (9.5%) gave weak positive results with this test (score of 0·5).

From Dec 1, 2000, to May 30, 2001, 671 cases of suspected plague were declared to the national surveillance system: 598 (89%) survived, 61 (9%) died, 12 (2%) had an unknown outcome. 642 (96%) patients had bubonic plague, 12 (3%) had pneumonic plague, and nine (1%) had an unknown clinical status. In total, 691 clinical specimens were sent to the Central Laboratory in Cary Blair agar: 643 (93%) were bubo aspirates, 13 (12%) sputa, and 35 (5%) postmortem lung or liver puncture samples. The median transport time to the Central Laboratory of the 691 samples was 8 days, ranging from 0 to 66 days (25th percentile 5 days, 75th percentile 14 days). The mean transport time was 10·6 days (SD 9·6) for bacteriologically confirmed specimens and 12·1 days (11·9) for negative specimens (non-significant difference, Kruskall-Wallis non-parametric test).

After recovery of the samples from Cary Blair media, \textit{Y. pestis} was cultured from 182 (26%) samples (classified as confirmed specimens), whereas 15 (2%) were negative by culture but positive by microscopy (classified as presumptive specimens; table 1). Thus, the total number of plague patients identified by bacteriology was 197 of 691 suspected. With RDT, 279 (40%) specimens were positive for plague, 116 (42%) of which tested negative both by microscopy and culture. The concordance between the results of bacteriological and plague RDT results was 78·3% (κ=0·47, 95% CI 0·40–0·54; moderate agreement). Therefore, use of plague RDT alone led to identification of 41·6% more cases than when only bacteriology was used (279 vs 197 positive tests). However, 31 (17%) samples tested positive with the plague RDT were positive by bacteriology.

When F1 ELISA was used as the reference method, results obtained with this technique and with RDT were highly concordant (concordance 90·3%), results of the two tests agreed for 624 of 691 specimens tested) and strongly correlated (Kendall's correlation coefficient \( r = 0·834, p < 0·001 \)). \( \kappa = 0·79 \) (95% CI 0·77–0·81; good agreement). All ELISA-positive samples also tested positive on RDT. Moreover, RDT detected 67 weakly positive samples (score of 0·5) among the 479 samples that tested negative by ELISA (table 1). Therefore, use of RDT resulted in 31% (67 of 212) more positive results than were obtained with F1 ELISA alone. When we used a combination of bacteriological tests and F1 ELISA as the reference standard (table 1), 266 of the 691 specimens tested positive, whereas with RDT alone, 279 tested positive (\( \kappa = 0·75, 95\% \text{ CI } 0·73–0·77 \)).

At the 26 remote sites, 128 patients (122 survivors and six patients who died) were suspected, on clinical grounds, to have plague (123 bubonic and five pneumonic forms) and were treated. Results of RDTs done by health workers at the patient's bedside, and by skilled technicians at the Central Laboratory, on the same specimens (table 2) were concordant in 115 of 128 patients (90%). Four tests done at remote sites (3%) were not valid since the control line was absent (table 3). With bacteriology and F1 ELISA as reference standards, 58 samples were identified as positive among the 128 tested, whereas RDTs done at remote sites and at the Central Laboratory identified 53 and 55 positive samples, respectively. Agreement between results from RDTs done at the field sites and bacteriology was moderate (\( \kappa = 0·62, 95\% \text{ CI } 0·57–0·66 \)), but agreement was very good when RDT in the field was compared with F1 ELISA (0·83, 0·78–0·87). The false negatives with RDT corresponded with samples that tested positive in culture after amplification in two mice (low bacterial load) but negative with F1 ELISA. Five patients who were identified at remote sites as plague-infected were negative for plague with the combination of reference tests.

On the assumption that the combination of the two reference tests is the gold standard (which is not the case), the calculated PPV and NPV would be 90·6% (95% CI 78·6–96·5) and 86·7% (76·4–93·1), respectively, at the pilot sites, and 92·7% (81·6–97·6) and 90·4% (80·7–95·7), respectively, at the Central Laboratory. There was 85% concordance between the results of the combined reference methods and those for the plague RDT in the field (\( \kappa = 0·76, 95\% \text{ CI } 0·71–0·80 \)), and 91% concordance between the combined and RDT methods at the Central Laboratory (0·82, 0·77–0·86).

The 57 individual user's questionnaires gave a mean overall satisfaction score of 17·6 of 20 [range 14–20]. Most (51 of 57, 90%) of the health staff thought that the RDT would help to reduce plague morbidity and mortality in Madagascar.

Sputum obtained from 16 patients with suspected pneumonic plague were tested with the plague RDT as well as with the two reference tests for comparison (table 4): 13 sputa were tested only at the Central Laboratory; the remaining three sputum samples were also tested at the field sites. The eight patients for whom the diagnosis of plague was confirmed by bacteriology also tested positive by RDT (one tested negative by ELISA). However, two patients who tested negative by bacteriological methods gave a weak positive result with the plague RDT, one of whom also tested positive by ELISA.

### Table 1: Comparison between plague RDT, bacteriology, and F1 ELISA for specimens tested at Central Laboratory

<table>
<thead>
<tr>
<th>Number (%) samples</th>
<th>Bacteriology</th>
<th>F1 ELISA</th>
<th>Combination of reference tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Confirmed</td>
<td>Presumptive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>279 (40%)</td>
<td>151 (63%)</td>
<td>12 (80%)</td>
</tr>
<tr>
<td>Negative</td>
<td>412 (60%)</td>
<td>31 (17%)</td>
<td>3 (20%)</td>
</tr>
<tr>
<td>Total</td>
<td>691</td>
<td>182</td>
<td>15</td>
</tr>
</tbody>
</table>

The median transport time to the Central Laboratory of the 691 samples was 8 days, ranging from 0 to 66 days (25th percentile 5 days, 75th percentile 14 days). The mean transport time was 10·6 days (SD 9·6) for bacteriologically confirmed specimens and 12·1 days (11·9) for negative specimens (non-significant difference, Kruskall-Wallis non-parametric test).

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### Table 2: Comparison between plague RDTs done at remote sites and at Central Laboratory on the same specimens

<table>
<thead>
<tr>
<th>RDT, remote sites</th>
<th>Total patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive (n=55)</td>
<td>49 (92·5%)</td>
</tr>
<tr>
<td>Negative (n=73)</td>
<td>4 (7·5%)</td>
</tr>
<tr>
<td>Total patients (%)</td>
<td>53</td>
</tr>
</tbody>
</table>
Discussion

We have shown that our RDT is as specific as, and at least as sensitive as, the two available standard methods. The excellent specificity of the RDT, its low detection threshold, and the higher number of positive specimens detected among samples from patients with suspected plague, suggest a greater sensitivity than bacteriology and ELISA. Thus, in the operational conditions of the plague control programme at the Central Laboratory, we recommend a combination of bacteriological tests and RDT for diagnosis and surveillance of plague in Madagascar.

Our new test detected F1 antigen at a wider range of concentrations than other tests, in 10–15 min, without prozone effect. Absence of prozone phenomenon is essential to avoid false-negative results, especially in post-mortem samples or sputum samples that contain very high concentrations of F1 antigen. The highest F1 concentration ever noted in clinical samples was 30 μg/mL (S Chanteau, unpublished).

The existing reference tests, which can only be done in the laboratory—the isolation of Y pestis and the detection of F1 antigen by ELISA—are specific, but lack sensitivity in all countries in which plague is endemic. The failure of these standard assays is due to the deterioration, contamination, or both, of specimens during their long transport time to the laboratory, antibiotic treatment of patients before sampling, and the diffusion of F1 antigen in the Cary Blair transport medium. The difficulty could be solved by a second training session focusing on interpretation of the test results. Furthermore, other investigators have shown that some patients who were originally diagnosed as negative with the reference tests (and positive with the initial plague dipstick) subsequently underwent F1 antibody seroconversion.16

Conversely, 116 samples that tested positive by plague RDT gave negative results in bacteriological tests, probably for the reasons given above. These reasons explain the fair concordance between RDT and bacteriology. Similarly, 67 samples that tested negative with F1 ELISA gave weak positive results with RDT, probably because the detection threshold of RDT was lower than that of ELISA. Finally, 27 of the 283 clinical specimens classified as negative with the combined bacteriological and ELISA tests tested positive by RDT. Since the RDT was highly specific and had a low detection threshold, we suggest that these 27 specimens were true plague-positive specimens, and that the plague RDT is thus more sensitive than the two reference assays.

Of the 30 Y pestis strains of the three biotypes and from different continents, most tested positive by RDT, indicating that this test is potentially applicable to plague foci worldwide. The two strains that tested negative proved to have total or part deletions of the cfa1 gene; this deficiency seems to be due to the loss of the 110 kb plasmid, probably during the long storage time of the strains in the laboratory.

The invalid tests in the conditions of the remote sites (no control line in 3% of the specimens) were due to moistening of the dipsticks, caused by the humidity of the air. This drawback can be overcome by improvement of the RDT packaging, with waterproof bags instead of a disposable test tube. We expect the shelf-life of the RDT in such conditions to be about 2 years at room temperature, on the basis of stability after 21 days at 60°C.21

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The test kit should therefore tolerate storage at room temperature for long periods in plague endemic zones. The other discordant results were due either to weak positive bands that were not seen in the field, or to a misinterpretation of the control and positive lines. This difficulty could be solved by a second training session focusing on interpretation of the test results.

During non-central evaluation of the RDT, this test was applied to the diagnosis of pneumonic plague as well as bubonic plague. Although sputum contains high concentrations of F1 antigen, we thought that its density and viscosity might inhibit antigen-antibody interactions and obstruct the flow of immune complexes through the dipstick. This difficulty was overcome by diluting sputum samples with saline or PBS (1 in 2 to 1 in 10 dilution). Since the sensitivity and specificity of the test with samples from patients with confirmed and suspected pneumonic plague was high, the RDT seems to be a useful method for rapid, easy, and non-invasive confirmation of this disease. The availability of a reliable onsite diagnostic test should result in rapid treatment of patients and efficient administration of chemoprophylaxis to the contact population; these measures should, in turn, help to prevent the spread of pneumonic plague in endemic countries or in cases of bioterrorist use.

Table 4: Diagnosis of pneumonic plague in sputum with plague RDT, F1 ELISA, and bacteriology
Assessment of the RDT results and of the users’ questionnaires showed that the technical transfer of the test to remote pilot sites was successful and allowed the reliable diagnosis of bubonic and pneumonic plague. These findings led to a general request from health staff working in plague foci for this test to be made available at their centres. Bacteriological and ELISA tests are between five and 50 times more expensive than the RDT, and they cannot be used in remote locations.

Overall, we have shown that with our test, the rapid and cost-effective diagnosis of bubonic and pneumonic plague could be easily achieved by health workers in remote sites. Use of the test could help to reduce mortality (through rapid and efficient treatment of patients), morbidity (by rapid implementation of preventive measures), and insecticide resistance of fleas (through rational use of expensive insecticides). This kit will be distributed to all the districts and health care centres of the endemic plague areas of Madagascar in 2002. The next step will be, with the assistance of international organisations, to make the plague RDT available to other countries with endemic plague worldwide.

Contributors
S Chanteau designed the study, developed anti-F1 Mabs and RDT, planned the user’s guide book, trained health staff in the field, analysed data, and wrote the manuscript. L Rahalison supervised assays at Central Laboratory, trained health staff in the field, and made the user’s guide book (French and Malagasy languages). L Raisifasoana produced RDT kits, trained health staff in the field, and did RDT and ELISA tests at the Central Laboratory. J Foulon assessed RDT with bacterial cultures and did PCR. L Raisifasoamanana coordinated the plague national control programme and the network of health centres. É Carniel designed the study, supervised the assessment of RDT with bacterial cultures, and wrote the manuscript. F Nato coordinated the Institut Pasteur RDT project, supervised the development and production of the anti-F1 Mabs, and wrote the manuscript.

Conflict of interest statement
None declared.

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