

Field Evaluation of an Immunoglobulin G Anti-F1 Enzyme-Linked Immunosorbent Assay for Serodiagnosis of Human Plague in Madagascar

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Bacteriological isolation of *Yersinia pestis* is the reference test for confirming plague infection, but recovery of the pathogen from human samples is usually very poor. When the etiology of the disease cannot be bacteriologically confirmed, it may be useful to possess alternative tests such as detection of specific circulating antibodies to help guide the diagnosis. In the present study, the immunoglobulin G (IgG) anti-F1 enzyme-linked immunosorbent assay (ELISA) has been applied to various human sera to evaluate its large-scale applicability in the high-endemicity plague foci of Madagascar. The sensitivity of the test was found to be 91.4%, and its specificity was 98.5%. The positive and negative predictive values were 96 and 96.6%, respectively. Seroconversion was observed on day 7 after onset of the disease. Patients with a positive ELISA result could be separated into high (82%) and low (18%) IgG anti-F1 responders. Cross-reactions with eight other infectious diseases prevalent in Madagascar were scarce and were found in 1 of 27 *Mycobacterium tuberculosis*-, 3 of 34 *Schistosoma haematobium*-, and 1 of 41 *Salmonella*-infected patients. Finally, the efficiency of the IgG anti-F1 ELISA was evaluated during the Mahajanga, Madagascar, plague outbreak of 1995. When the number of ELISA-positive patients was added to the number of bacteriologically confirmed and probable cases, the number of positive patients was increased by 35%. In conclusion, although it does not replace bacteriology, IgG anti-F1 ELISA is a useful and powerful tool for retrospective diagnosis and epidemiological surveillance of plague outbreaks.

Plague is one of the most threatening diseases which still have active foci in different parts of the world. During the past 15 years, 24 countries in Africa, Asia, and the Americas reported 18,739 human plague cases (25). The situation worsened recently. More human cases have been reported to the World Health Organization since the beginning of the 1990s (1990 to -94) than during the entire 1980s. Furthermore, plague recently reappeared in areas where it had been absent for 15 (Malawi and Mozambique), 30 (India), or 62 (Mahajanga, Madagascar) years. For these reasons, plague is now considered a reemerging disease.

It is difficult to have an accurate estimate of plague incidence since several countries do not properly report their cases or have the laboratory skills for diagnosis. The only means of confirming the diagnosis of plague is to isolate the microorganism from the clinical specimen (bubo aspirate, expectoration, or organ). Since isolation of *Yersinia pestis* is not easily performed by field laboratories, direct examinations under a microscope of a stained bubo or sputum smear may help orient diagnosis. However, because of the acuteness and severity of the disease, clinically suspected cases are often treated without taking of biological samples for bacteriological investigations. Under these conditions, the only way to retrospectively con-

firm the diagnosis is via the search for *Y. pestis*-specific antibodies.

The detection of antibodies against F1 purified antigen by the passive hemagglutination test (PHA) has been the serodiagnostic tool most widely used. This method has recently been employed during the 1994 plague outbreak in India (10). PHA has been recommended as a diagnostic test (4, 9, 11), a marker of vaccine efficacy (16, 22), and a tool for the detection of enzootic plague among animal populations (5, 19, 20). Although PHA is simple, rapid, and cheap, its sensitivity and specificity are not always satisfactory (23). The lack of specificity of this method may be attributed partly to the fact that PHA detects all kinds of antibody isotypes, including immunoglobulin M (IgM), which have been shown to be of poor specificity (8, 20, 24). An enzyme-linked immunosorbent assay (ELISA) developed in 1979 to detect F1-specific circulating antibodies in humans and animals (6) was found to be more reliable than PHA, especially when detecting IgG at the convalescent phase of the disease (24). Despite its potential advantages, the ELISA has not been widely used in field situations.

In the present study, evaluation and optimization of an IgG anti-F1 ELISA for human plague serodiagnosis in Madagascar were performed. This test was then applied during the 1995 outbreak in Mahajanga to the sera of patients suspected of having the plague.

MATERIALS AND METHODS

Nomenclature and definitions. A confirmed case of plague corresponds to a patient from whom a *Y. pestis* strain has been isolated. A probable case means

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that the strain was not isolated but that direct examination of the stained sample smear was suggestive of plague. Probable and confirmed cases are considered bacteriology positive and are reported to the World Health Organization.

The sensitivity of the ELISA is the percentage of positive results obtained with the sera of confirmed patients. The specificity is the percentage of negative results obtained with the sera of healthy controls. The Receiver Operating Characteristic (ROC) curve represents variation in sensitivity and specificity depending on different cutoff values (15).

IgG anti-F1 ELISA. F1 antigen, a 15.5-kDa capsular protein, was purified from *Y. pestis* culture (strain A1122) at the Institut Pasteur in Paris, France (1). The ELISA procedure was as follows. F1 antigen at 0.1 µg/well in carbonate buffer, pH 9.6, was used to coat 96-well ELISA plates by overnight incubation at 4°C. Plates were washed three times in phosphate-buffered saline (pH 7.2)–0.1% Tween 20 (PBST) and saturated with PBST–5% skim milk. The human serum samples were diluted 1/100 and incubated at 37°C for 1 h. After three washings with PBST, anti-human IgG horseradish peroxidase conjugate diluted 1/3,000 (Bioss) was incubated at 37°C for 1 h, before *o*-phenylenediamine was added as indicator reagent. Results were determined by reading absorbance at 492 nm (A_{492}) on an automatic microplate reader (LabSystem Multiskan MS). As controls, one negative and two positive sera (high and low titers) were included in each series of experiments.

Sensitivity, specificity, and predictive values of the ELISA. The sensitivity of the test was assessed by testing one serum each from 35 Malagasy patients with confirmed bubonic plague. The sera were collected between day 10 and day 180 after onset of clinical symptoms. The specificity was determined with 94 sera from healthy persons (66 Malagasy and 28 European subjects) living in Madagascar, who reported having no history of plague or other infectious disease endemic in this country (typhoid fever, shigellosis, tuberculosis, malaria, bilharziasis, toxoplasmosis, and cysticercosis).

Positive and negative predictive values (PPV and NPV) were calculated, based on the number of confirmed and probable plague cases determined from (i) 2,153 samples of clinically suspected plague patients collected between 1993 and 1995 at the central reference laboratory and (ii) 342 samples of suspected patients collected at the Mahajanga Hospital during the 1995 outbreak.

Cross-reactivity with other infectious diseases. Cross-reactions with other bacterial or parasitological diseases prevalent in Madagascar were evaluated with sera of 150 Malagasy patients who had antibodies against *Salmonella typhi* and *Salmonella paratyphi* ($n = 41$), *Toxoplasma gondii* ($n = 9$), *Plasmodium falciparum* ($n = 11$), *Taenia solium* (larval stage, $n = 12$; positive bacteriological culture or parasitological exam for *Shigella flexneri*, $n = 11$), *Mycobacterium tuberculosis* ($n = 27$), *Schistosoma mansoni* ($n = 5$), and *Schistosoma haematobium* ($n = 34$).

Cross-reactions with other yersinioses were determined with 6 *Y. enterocolitica* and 13 *Y. pseudotuberculosis* agglutination-positive sera from patients living in France (kindly provided by the Centre de Biologie Médicale Spécialisée, Institut Pasteur, Paris).

Kinetics of anti-F1 IgG. The evolution of anti-F1 IgG antibodies during the first month of infection was studied in 84 bubonic plague patients with positive bacteriology. The results are represented by the "box and whiskers" plotting method (12). The long-term antibody persistence was assessed on paired sera from 11 confirmed plague patients (the first serum was collected between days 8 and 15 of the disease, and the second serum was collected between month 9 and year 6 postinfection).

RESULTS

Characteristics of the IgG anti-F1 ELISA. The high value (good sensitivity and specificity) of the ELISA could be demonstrated by its ROC curve (Fig. 1). The chosen threshold was $A_{492} = 0.35$, corresponding to the optimum sensitivity and to the high specificity needed, 98.5%. Accordingly, the sensitivity was 91.4%. The threshold value of 0.35 was chosen for all subsequent experiments.

Plague prevalence for the samples received at the central laboratory was 28.4% (612 probable and confirmed cases among 2,153 suspected patients). Thus, the PPV of the IgG anti-F1 ELISA was 96%, and its NPV was 96.6%.

In the city of Mahajanga, plague prevalence during the outbreak of 1995 was 29.5% (101 probable and confirmed cases among 342 suspected patients). The PPV and NPV of the test were then 95.8 and 96.3%, respectively.

Cross-reactivity with other infectious diseases. Among the sera of 150 patients having a positive serological, bacteriological, or parasitological diagnosis for one of the most frequent endemic infections in Madagascar, cross-reactions with F1 antigen were observed in *M. tuberculosis* (1 of 27)-, *Schistosoma haematobium* (3 of 34)-, and *Salmonella typhi* and *Salmonella*

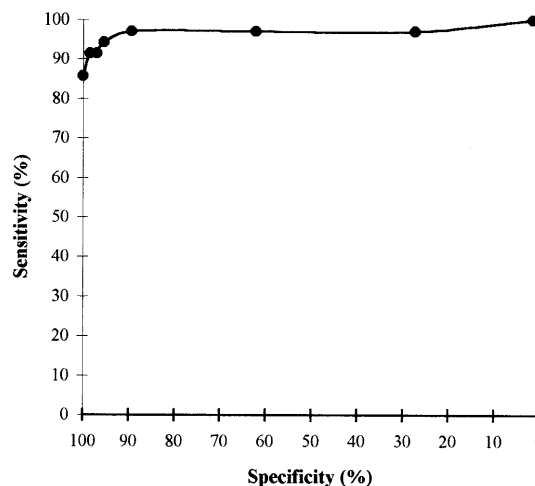


FIG. 1. ROC curve of the IgG anti-F1 ELISA (variation in sensitivity and specificity in relation to variation in the A_{492} threshold [15]).

paratyphi (1 of 41)-infected patients. Cross-reactivities were also observed for 2 of 13 patients with a positive agglutination test for *Y. pseudotuberculosis*. No cross-reaction was observed with other infections such as shigellosis, toxoplasmosis, malaria, or taeniasis.

Follow-up of the IgG anti-F1 antibody response during and after plague infection. The gradual change in the amount of IgG anti-F1 antibodies during the first month of infection was studied with 331 sera taken from 84 confirmed or probable plague patients. The results are shown in Fig. 2. Seroconversion occurred on day 7 following onset of disease, and the plateau was reached by day 15.

Two groups could be distinguished among those patients. The major group, 69 of 84 individuals (82%), corresponds to high-antibody-responder patients ($A_{492} > 1$), and the minor group, 15 of 84 individuals (18%), corresponds to low-responder patients ($A_{492} < 1$). Figure 3 shows the kinetics of mean absorbance in the groups of very high and very low responders.

Long-term antibody persistence was studied with paired sera from 11 confirmed patients (9 high responders and 2 low responders). As expected, the sera taken 9 months or 3 years postinfection from the two low-responder patients were negative. Among the nine high-responder patients, five were seronegative 7 months to 5 years later, and four were still seropositive 4 to 6 years postinfection.

Reproducibility of the ELISA. The reproducibility of the test was assessed with the three control sera included in 17 independent series of experiments. The mean A_{492} and its standard deviation (SD) were 0.083 and 0.022, respectively, for the negative control, 0.793 and 0.106 for the low-positive control, and 1.868 and 0.116 for the high-positive control. The coefficients of variation of the test (SD/mean A_{492}) for these controls were 26.5, 13.4, and 6.2%, respectively.

Application of the test to the 1995 plague outbreak in Mahajanga. The epidemiological features of this outbreak have been described previously (3). Five hundred sixty patients were identified as plague-suspected cases between March and December 1995. During the epidemic, bacteriological analysis of bubo aspirates was performed on 342 patients. Fifty-five of them were culture positive, and 46 were only smear positive. Anti-F1 serology was applied to the sera of 339 patients collected at least 6 days after onset of disease. The serological

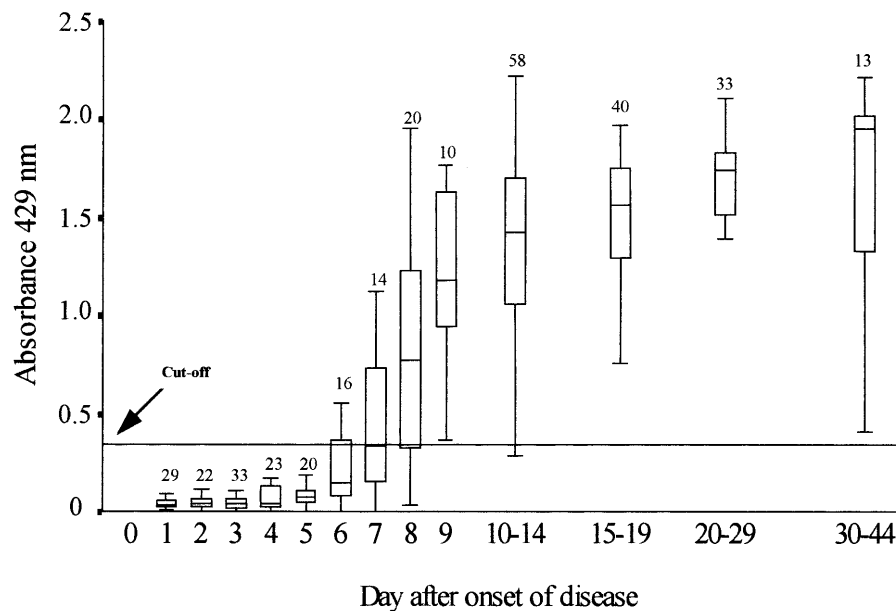


FIG. 2. Kinetics of IgG anti-F1 antibody titers during human *Y. pestis* infection plotted by the box and whiskers method (12). Upper and lower horizontal bars correspond to maximum absorbance and minimum absorbance, respectively. Boxes represent values between the 25th and 75th percentiles; the bars inside these boxes represent the median values. The numbers of samples are indicated.

results for patients with different bacteriological results were as follows. Of the 39 confirmed cases, 32 (82.1%) were ELISA positive; of the 27 probable cases, 6 (22.2%) were ELISA positive; of the 154 negative cases, 15 (9.7%) were ELISA positive; and of the 119 patients for whom no sample was available for bacteriological analysis, 20 (16.8%) were ELISA positive. The 73 ELISA-positive patients accounted for 21.5% of the 339 tested.

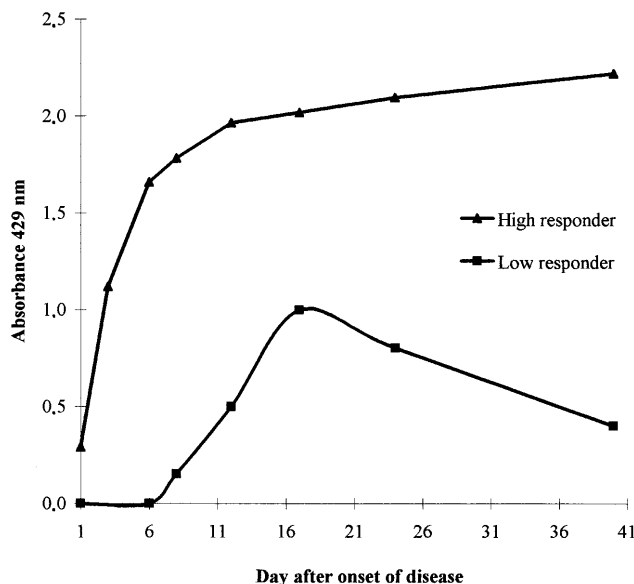


FIG. 3. Evolution of IgG anti-F1 antibodies over time in very high and very low responders among confirmed plague patients. The data are group means. Very high responders are those with absorbance above the mean absorbance + SD for all the patients, and very low responders are those with absorbance below this value.

DISCUSSION

In Madagascar, the poor sanitary and economic situation renders difficult the control and surveillance of plague. Every year, approximately 1,000 patients have clinical symptoms of plague, and about 200 of them are bacteriologically confirmed or probable cases (7, 25). Laboratory techniques including microscopy, bacteriological culture, and mouse infection are available at the central reference laboratory in Antananarivo, Madagascar, but at the district level, only microscopy identification is implemented. Since most of the suspected cases of plague are detected in remote small villages, the clinical samples, if taken, do not reach the central laboratory for several days, weeks, or even months. This long conveying period accounts at least partly for the poor recovery of the bacteria from the clinical specimens. Indeed, *Y. pestis* is isolated from only 15% of the samples forwarded to the central laboratory for bacteriological confirmation. Thus, plague incidence is certainly underestimated in Madagascar. Implementation of a serodiagnostic test would help a more accurate evaluation of the real incidence of plague in Madagascar.

The suitability of an IgG anti-F1 ELISA for retrospective plague serodiagnosis in field conditions and in the sanitary context of Madagascar has been investigated. After optimization of the parameters of the reaction (amount of F1 per well, dilutions of sera and conjugate, cutoff value, etc.), a sensitivity and specificity of 91.4 and 98.5%, respectively, were obtained. Cross-reactivities with other pathogens were scarce and involved *M. tuberculosis*, *Salmonella*, *Schistosoma haematobium*, and *Y. pseudotuberculosis*. The possibility that these patients had past exposure to *Y. pestis* cannot be ruled out. The cross-reaction observed with *Schistosoma haematobium* should not interfere with the interpretation of the ELISA since, except for the coastal city of Mahajanga, plague and schistosomiasis foci do not overlap. Similarly, cross-reactions with *Y. pseudotuberculosis* should not be taken into account since enteropathogenic *Yersinia* has never been isolated in Madagascar (13).

In order to evaluate its practical value, the ELISA was tested during the 1995 plague outbreak in Mahajanga. Plague was imported into the city in 1902, during the third pandemic, and persisted in this area until 1928. After 62 years of silence, the disease reappeared, first as a small outbreak in 1991 and then as a more important epidemic in 1995 (3). From March to December 1995, pairs of acute- and convalescent-phase sera from 339 suspected plague patients were collected and analyzed by ELISA. Seventy-three of the patients had IgG anti-F1 antibodies in their convalescent-phase sera. However, only 82.1% of the bacteriologically confirmed patients were seropositive compared to the 91.4% sensitivity found with positive-control sera. Several explanations could be proposed to explain this difference and why few patients are seronegative. Firstly, the *Y. pestis* strains isolated from these patients could be non-F1 producers. However, a retrospective analysis of the corresponding isolates demonstrated that they all synthesized F1 antigen in vitro. A more extensive study of 453 *Y. pestis* strains isolated in 1994 and 1995 also indicated that all isolates studied were F1 producers (8). Secondly, it is known that during extremely severe and acute infections, the immune system may be paralyzed. This may have been the case for the seronegative patients. However, it should be noted that all these patients survived plague infection. Thirdly, the sera may have been taken at too early a stage of the infection, before the appearance of specific antibodies. The control sera used to determine the sensitivity of the test were collected between days 10 and 180 after the onset of the disease. In Mahajanga, theoretically only sera taken 6 or more days after onset of disease were analyzed. However, the exact onset of symptoms may have been inaccurately estimated by the patient or registered at the hospital, due to the disorganization resulting from the outbreak. Fourthly, the immune system in some individuals may have a gap in the anti-F1 response. In previous studies, it has been reported that 8 to 12% of the vaccinees did not produce detectable F1 antibodies in response to several booster inoculations (14). The hypothesis that persons with type O blood (21) may be refractory because of the similarity between H antigen and F1 antigen could not be validated (14). For our culture-positive but ELISA-negative patients, it may be worth testing other *Yersinia* antigens such as the pYV-encoded Yop antigens which have been shown effective for the serodiagnosis of *Y. enterocolitica* and *Y. pseudotuberculosis* infections (2).

On the basis of bacteriology results alone, 101 of the 342 suspected cases analyzed were positive (55 by culture and 46 by microscopy only). Although a positive serological test cannot prove the diagnosis of plague, the very good sensitivity and specificity of the IgG anti-F1 ELISA should allow the diagnostician to classify a clinically suspected case with a positive serology as a probable case and therefore to officially report it. On this basis, 35 additional clinically suspected plague cases which were not identified because the bubo pus was either not obtained or bacteriologically negative could be added to the number of plague cases reported during the Mahajanga outbreak. Taking into account these seropositive patients would have resulted in a 35% increase in the number of reported plague cases in Mahajanga.

Given that anti-F1 antibodies are likely to persist for several years in a large majority of patients, the results of the ELISA may be difficult to interpret in the highlands of Madagascar, where plague has been endemic for a long time. However, a seroconversion, or an increase in the antibody titer, observed with two serum samples taken several days apart from a clinically suspected patient is highly suggestive of an acute plague infection. Anyhow, antibody persistence and the fact that

100% of the *Y. pestis* strains isolated in Madagascar are F1 positive (8) indicate that the ELISA could be a valuable tool for estimating the incidence of *Y. pestis* infection among the highland population and for undertaking epidemiological surveillance. Mass seroepidemiological studies, including young children, can be performed easily with blood collected by finger pricking on calibrated filter paper disks (8).

In conclusion, the IgG anti-F1 ELISA, once evaluated and standardized for a given country, is a simple and useful tool for either plague retrospective diagnosis or epidemiological surveillance. However, it has to be used in conjunction with culture.

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