Diagnosis of Bubonic Plague by PCR in Madagascar under Field Conditions

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The diagnostic value of a PCR assay that amplifies a 501-bp fragment of the *Yersinia pestis* *cafI* gene has been determined in a reference laboratory with 218 bubo aspirates collected from patients with clinically suspected plague managed in a regional hospital in Madagascar. The culture of *Y. pestis* and the detection of the F1 antigen (Ag) by enzyme-linked immunosorbent assay (ELISA) were used as reference diagnostic methods. The sensitivity of PCR was 89% (57 of 64) for the *Y. pestis*-positive patients, and 80.7% (63 of 78) for the F1 Ag-positive patients. The specificity of PCR for the culture-, F1 Ag-, and antibody-negative patients ($n = 105$) was 100%. Because in Madagascar most patients with plague are managed and their clinical samples are collected in remote villages, the usefulness of PCR was evaluated for routine diagnostic use in the operational conditions of the control program. The sensitivity of PCR was 50% (25 of 50) relative to the results of culture and 35.2% (19 of 54) relative to the results of the F1 Ag immunocapture ELISA. The specificity of PCR under these conditions was 96%. In conclusion, the PCR method was found to be very specific but not as sensitive as culture or the F1 Ag detection method. The limitation in sensitivity may have been due to suboptimal field conditions and the small volumes of samples used for DNA extraction. This technique is not recommended as a routine diagnostic test for plague in Madagascar.

Because plague is a fulminating disease and occurs in isolated areas, treatment is based on the patient's clinical findings and the epidemiological context. The biological confirmation by culture of *Yersinia pestis* or by anti-F1 antibody (Ab) detection is retrospective because of the time needed to obtain test results. Although the conventional bacteriological methods are the standard means of diagnosis of plague, they are often unsuitable under the field conditions of many countries with endemic plague due to highly contaminated and badly preserved samples.

The rapid confirmation of plague is of prime importance for patients as well as for the control program since it highly motivates public health officers to seriously and rapidly implement necessary control measures (vector control, administration of prophylaxis to the contact population). The diagnosis of plague during the acute phase of the disease is possible by detection of the F1 antigen (Ag) excreted by *Y. pestis* in bubo or blood by the enzyme-linked immunosorbent assay (ELISA) (3, 13), the immunogold chromatography dipstick assay (S. Chanteau and J. Burans, unpublished data), or the direct immunofluorescence assay (DFA) (9). The PCR method is another rapid, sensitive, and specific alternative test that is able to detect dead bacteria and that requires only a small amount of sample. Several PCR methods for the amplification of *Y. pestis* DNA have been developed, but none of them has been evaluated with clinical samples for their diagnostic value or for their usefulness under the real field conditions of plague control programs (2, 4–8, 12).

We report here the results of a clinical evaluation of the PCR amplification of the *Y. pestis* *cafI* gene compared with the

results of *Y. pestis* culture and F1 Ag detection by ELISA as reference diagnostic tests.

MATERIALS AND METHODS

Patients and clinical samples. (i) **In the regional hospital of Mahajanga.** During the outbreak of bubonic plague in 1997 in the harbor of Mahajanga, Madagascar, a first set of bubo aspirates was collected prior to streptomycin treatment from 218 patients (116 males and 102 females) who had suspected plague and who were admitted to the public hospital (50% inguinal or femoral buboes). The median age of the patients was 13 years (range, 1 to 80 years), and the lethality rate was 3.2% (7 of 218 patients). No past history of plague was known for any of the patients, and self-medication with sulfamide prior to their admission to the hospital was known for 28 of them.

The bubo aspirate (25 to 200 μ l) was collected with a syringe and was diluted in 1 ml of sterile saline. The suspension thus obtained was immediately processed for the isolation of *Y. pestis*. The remaining suspension was kept frozen until it was sent on ice packs to the Plague Central Laboratory, Pasteur Institute, Antananarivo, Madagascar, for the other biological tests. The volume of suspension used was 200 μ l for bacteriology, 100 μ l for F1 Ag ELISA, and 20 μ l for DNA extraction for PCR tests.

Pairs of acute- and convalescent-phase sera for anti-F1 Ab serology were available for 187 patients. Convalescent-phase sera were collected from day 8 to day 30 postinfection.

(ii) **In peripheral dispensaries.** A second set of samples consisted of bubo aspirates collected before streptomycin treatment from 183 patients (104 males and 79 females) with suspected plague who were managed in peripheral dispensaries (that is, dispensaries of remote villages). For 54.6% of the patients, the bubo was located in the inguinal or femoral region. The mean age was 11 years (range, 1 to 59 years), and the lethality rate was 4.3% (8 of 183 patients). No past history of plague was known for any of the patients, and self-medication with sulfamide was known for 23 of them.

The bubo aspirates were absorbed on a sterile swab and were conveyed at room temperature in Cary-Blair agar medium to the Pasteur Institute. Due to the poor means of communication in Madagascar, the delay before receipt of the samples was <1 week for 66 patients, 1 to 2 weeks for 96 patients, and 2 to 4 weeks for 21 patients (median delay, 8 days). Immediately after arrival at the reference laboratory, the samples were extracted from the swabs with 800 μ l of sterile saline and were directly used for the various biological tests.

According to the usual standards of the national control program, sera were not collected from patients from remote villages.

Negative control patients. For ethical reasons, it was not possible to obtain negative control adenitis aspirates from patients without suspected plague. Therefore, the negative patients consisted of patients with clinically suspected plague (105 from Mahajanga Hospital and 102 from peripheral dispensaries) but

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for whom plague could be totally excluded on the basis of negative results by three diagnostic assays (culture and F1 Ag and F1 Ab detection tests).

Diagnostic reference methods. Bacteriologic isolation of *Y. pestis* is considered to be the "gold standard" diagnostic method for the confirmation of plague, as recommended by the World Health Organization (14). To isolate *Y. pestis*, each sample was simultaneously injected into 2 OF1 mice (50 µl plus 1,000 U of penicillin per mouse) and cultivated in pepton broth (50 µl) and on a selective agar plate YCIN (yersinia cefanodin Irgasan novobiocin [YCIN]; 50 µl) (11). Suspected *Y. pestis* colonies were confirmed by the specific phage lysis test and the API 20 biochemical microtest (reference no. 20100; Biomerieux, Marcy l'Etoile, France).

The F1 Ag capture ELISA was the second reference method used. It was kindly provided by the U.S. Naval Medical Research Institute, Bethesda, Md., and was used according to the provider's instructions, as described previously (3). Each sample was tested in duplicate wells (50 µl in each well). The cutoff of the test was 2 ng/ml (100% specificity; S. Chanteau, unpublished data).

ELISA immunoglobulin G anti-F1 Ab detection. A conventional indirect ELISA method (10) was used as a complementary diagnostic test to better discriminate and classify patients and to select the negative control patients. Relative to culture, its specificity was 98.2% and its sensitivity was 91.4% in Madagascar (10).

DNA extraction method. *Y. pestis* DNA was extracted from 20 µl of bubo suspension diluted with 20 µl of TE (Tris-HCl, 10 mM; EDTA, 1 mM [pH 8.0]) in a 5% final concentration of Chelex-100 100/200 mesh resin (reference no. 142-2832; Bio-Rad, Hercules, Calif.). After incubation for 30 min at 56°C and boiling for 10 min, the mixture was centrifuged at 12,000 rpm (5,585 × g) for 2 min. Five microliters of the supernatant, equivalent to 2.5 µl of bubo suspension, was used as the sample for the PCR assay.

PCR assay. The PCR method of Norkina et al. (8) was used, with slight modifications. It amplifies a 501-bp fragment of the gene (*cafI*) in the 110-kb plasmid that encodes the capsular F1 Ag of *Y. pestis*. The sequences of the two primers F1 (5'-CAG TTC CGT TAT CGC CAT TGC-3') and F2 (5'-TAT TGG TTA GAT ACG GTT ACG GT-3'), located at 275 and 776 bp of the gene, respectively, were purchased from Eurogentec (Seraing, Belgium).

The reaction was performed in a microtube containing 5 µl of the sample. A total of 0.5 µl of *Taq* DNA polymerase (5 U/µl; no. NK 9123; United States Biochemical [USB] Amersham, Cleveland, Ohio), 30 µl of mineral oil, and 44.5 µl of a mixture made of 1× buffer (no. 71165; USB Amersham), 2.5 mM MgCl₂ (no. 71167; USB Amersham), 200 µM (each) deoxyribonucleoside triphosphates (Pharmacia Biotech, Orsay, France), and 120 nM (each) primers F1 and F2 were used. The following program was conducted in a HYBAID TRI thermocycler: denaturation at 92°C for 3 min and then 35 cycles of 92°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. The amplified products were detected by electrophoresis on a 1.5% agarose gel stained with ethidium bromide, and their sizes were compared with those of φX174 DNA markers cut with *Hae*III (reference no. 15611-015; Gibco BRL, Paisley, Scotland).

The detection limit of this assay, as determined with negative bubo aspirates spiked with a 10-fold serially diluted suspension of *Y. pestis*, was between 1 and 10 CFU/test. A negative control (distilled water) and a positive control (a negative bubo spiked with 10 CFU of *Y. pestis*) were added in each series.

RESULTS

Evaluation of PCR assay for patients managed in a regional hospital. The specificity of the PCR assay determined with samples from the 105 negative control patients from the regional hospital was 100%.

Of the 218 patients with suspected plague and treated for bubonic plague, 64 patients (29.3%) were *Y. pestis* culture positive, 78 (35.7%) were F1 Ag positive, and 64 (29.3%) were PCR positive.

Relative to culture as the reference test (Table 1), the sensitivity of PCR was 89% (57 of 64 samples), and the rate of agreement between the two tests was 93.6%. Among the seven culture-positive and PCR-negative patients, six were F1 Ag and/or F1 Ab positive (and were thus false negative by PCR given the absolute specificity of the culture method). Among the seven PCR-positive and culture-negative patients, all were Ag F1 and/or Ab F1 positive (and were thus false negative by culture, given the 100% specificity of the Ag detection test). Among the 147 PCR- and culture-negative patients, 105 were true negative (F1 Ag and Ab negative), 22 were Ag F1 and/or Ab F1 positive, and 20 were F1 Ag negative.

When the F1 antigen assay was considered the reference test (Table 2), the sensitivity of PCR was 80.7% (63 of 78 samples).

TABLE 1. Comparative results between PCR and culture under hospital conditions

<i>Y. pestis</i> culture result	No. (%) of specimens with the following PCR result:		
	Positive	Negative	Total
Positive	57 (89)	7 (11) ^a	64 (100)
Negative	7 (4.5) ^b	147 (95.5) ^c	154 (100)
Total	64 (29.3)	154 (70.7)	218 (100)

^a Six of seven specimens were F1 Ag and/or anti-F1 Ab positive.

^b Seven of the seven specimens were F1 Ag and/or anti-F1 Ab positive.

^c A total of 105 of 147 specimens were F1 Ag and anti-F1 Ab negative, 22 of 147 were F1 Ag and/or anti-F1 Ab positive, and 20 of 147 were F1 Ag negative (sera were not available).

Fourteen of the 15 PCR-negative and Ag F1-positive patients were either Ab F1 and/or culture positive (and were thus false negative by PCR). The patient who was found to be PCR positive and F1 Ag negative had a true case of plague, as confirmed by the Ab detection assay. Among the 139 PCR- and culture-negative patients, 105 were true negative (F1 Ab detection assay and culture negative), 14 were Ab F1 or culture positive, and 20 were culture negative.

Evaluation of PCR assay for patients managed in peripheral dispensaries. The specificity determined for 102 culture-negative and F1 Ag-negative patients from peripheral centers was 96% (98 of 102 patients), and thus, 4 patients were PCR positive. Plague cannot be totally excluded for the latter patients since the sensitivities of culture and the F1 Ag assay are not absolute. Furthermore, no Ab detection result was available for them.

Of the 183 patients included in the study, 50 (27.3%) were *Y. pestis* culture positive, 133 (72.6%) were culture negative, and 29 (15.8%) were PCR positive (Table 3). When compared to culture, the sensitivity of PCR was 50% (25 of 50). Sixteen of the 25 PCR-negative and culture-positive patients were F1 Ag positive (and were thus false negative by PCR). The 4 PCR-positive and culture-negative patients were F1 Ag negative. Among the 129 PCR- and culture-negative patients, 19 (14.3%) were F1 Ag-positive (and were thus false negative by PCR).

The F1 Ag status was determined for 170 patients, of whom 54 (31.7%) were F1 Ag positive and 29 (17%) were PCR positive (Table 4). Compared to this reference test, the sensitivity of PCR was only 35.2% (19 of 54 patients).

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TABLE 2. Comparative results between PCR and F1 Ag detection assay under hospital conditions

F1 Ag detection result	No. (%) of specimens with the following PCR result:		
	Positive	Negative	Total
Positive	63 (80.7)	15 (19.3) ^a	78 (100)
Negative	1 (0.7) ^b	139 (99.3) ^c	140 (100)
Total	64 (29.3)	154 (70.7)	218 (100)

^a Four of 15 specimens were F1 Ab positive and/or culture positive, 8 of 15 were anti-F1 Ab positive and culture negative, 2 of 15 were anti-F1 Ab negative and culture positive, and 1 of 15 was F1 Ab negative and culture negative.

^b One specimen was F1 Ab positive.

^c A total of 105 of 139 specimens were anti-F1 Ab negative and culture negative, 1 of 139 was F1 Ab negative and culture positive, 13 of 139 were anti-F1 Ab positive and culture negative, and 20 were culture negative (sera not available).

TABLE 3. Comparative results between PCR and culture for samples collected in the field

<i>Y. pestis</i> culture result	No. (%) of specimens with the following PCR result:		
	Positive	Negative	Total
Positive	25 (50)	25 (50) ^a	50 (100)
Negative	4 (3) ^b	129 (97) ^c	133 (100)
Total	29 (15.8)	154 (84.2)	183 (100)

^a Sixteen of 25 specimens were F1 Ag positive.^b Four of four specimens were F1 Ag negative (sera not available).^c A total of 19 of 129 specimens were F1 Ag positive and 110 of 129 were F1 Ag negative.

before processing of the samples had no significant bearing on the sensitivity.

DISCUSSION

Plague is still a serious public health problem in Madagascar, and the development of a rapid and sensitive diagnostic assay is one of the main objectives of the national plague control program. Such an early detection assay could be used to trigger urgent preventive measures.

The PCR method is usually considered a rapid and sensitive diagnostic tool. Several assays that amplify the *cafI* gene of *Y. pestis* have been evaluated with experimentally infected rodents (7), the blood of infected laboratory mice (8), or *Y. pestis* cultures (12). A PCR test specific for the 16S rRNA gene was not specific enough since it also detects *Y. pseudotuberculosis* bacilli (4). PCRs specific for the plasminogen activator gene were able to identify *Y. pestis* in naturally infected fleas (6) or culture (2, 8). Hinnebusch et al. (5) described a quantitative PCR with primers specific for the *fur* (ferrin ion uptake regulation) gene and demonstrated its usefulness for monitoring of the prevalence of *Y. pestis*-infective fleas.

In laboratory experiments, these PCRs detected between 10 and 80 CFU per test. Yet, none of them has been evaluated with samples from humans with suspected plague. On the basis of the observation that all the *Y. pestis* strains isolated in Madagascar during recent years were F1 Ag positive (10), we evaluated in the reference laboratory of the Pasteur Institute the diagnostic value of a PCR that amplifies the *cafI* gene from samples from patients hospitalized for suspicion of plague. In tests of bubo aspirates, the PCR method could be optimized to detect less than 10 CFU of *Y. pestis* per test. Its specificity was found to be excellent (100%), thus excluding possible laboratory contamination by amplicons. The sensitivity was fair com-

pared to those of culture (89%) and the F1 Ag immunocapture ELISA (80%).

Although a low sensitivity may sometimes be explained by the variability of the DNA sequence of the amplified region from strain to strain or by the presence in some samples of inhibitors of *Taq* polymerase, we believe that the most likely reason is the tiny amount of sample used in one PCR test (2.5 µl) compared to the amount used for culture (200 µl) or the F1 Ag assay (100 µl). So, even though PCR was less sensitive than culture or F1 Ag detection, this technique may be useful when only a very small volume of sample is obtained. Indeed, the bubo size for most of the patients does not exceed 1 cm in diameter and the puncture of buboes is a painful and invasive medical procedure. Thus, in practice, the volume of bubo fluid obtained generally does not exceed 100 µl. The implementation of PCR as a rapid and complementary diagnostic tool as an adjunct to the F1 Ag detection assay may be of help in a hospital laboratory with a well-trained staff.

In Madagascar, the majority of the patients seek medical attention and treatment in remote health care centers that lack laboratory facilities. It is nearly impossible to obtain sterile clinical samples, and a delay of several weeks before receipt of the samples at the reference laboratory is a common situation. Thus, it was relevant to evaluate the PCR method with such samples collected under routine field conditions. The sensitivity was lower for the samples collected in peripheral dispensaries than for the samples collected in the regional hospital of Mahajanga: 50 versus 89% ($P < 5 \times 10^{-6}$) relative to that of culture and 35 versus 80.7% ($P < 1 \times 10^{-7}$) relative to that of the Ag detection assay. This lower sensitivity is likely a result of the destructive activity of DNase due to inappropriate handling of the samples in the field.

Currently, bubonic plague can be diagnosed by the following biological tests: the specific and rapid F1 Ag DFA, ELISA, and the dipstick test, PCR assay, and culture for *Y. pestis* for bubo aspirates obtained during the acute phase and the anti-F1 Ab assay for bubo aspirates obtained during the convalescent phase. As opposed to the DFA, dipstick, and ELISA methods, PCR is an expensive and technically demanding assay. Moreover, the main advantages of the F1-based assays are the copious quantities of F1 Ag excreted at 37°C and its immunogenicity even under tough field conditions (1). Anti-F1 serology is preferred for a retrospective confirmation of the disease or for epidemiological investigations. Culture for *Y. pestis*, although time-consuming and expensive, is an invaluable gold standard diagnostic test that also allows the surveillance of antibiotic resistance and genetic studies of the bacilli.

In conclusion, the PCR method does not meet the necessary performance standards for its use as a routine diagnostic assay in countries such as Madagascar. However, it can be retained as a complementary diagnostic test in reference laboratories or for research purposes.

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TABLE 4. Comparative results between PCR and F1 Ag detection assay for samples collected in the field

F1 Ag detection result	No. (%) of specimens with the following PCR result:		
	Positive	Negative	Total
Positive	19 (35.2)	35 (64.8) ^a	54 (100)
Negative	10 (8.6) ^b	106 (91.3) ^c	116 (100)
Total	29 (17)	141 (83)	170 ^d (100)

^a Twenty-four of 35 specimens were culture positive.^b Six of 10 specimens were culture positive and 4 of 10 were culture negative.^c Ten of 106 specimens were culture positive.^d Thirteen specimens were not tested for F1 Ag.

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