Immunohistochemical Detection of *Yersinia pestis* in Formalin-Fixed, Paraffin-EmbeddedReader Jeannette Guarner, MD,1 Wun-Ju Shieh, MD, PhD,1 Patricia W. Greer, MT,1 Jean-Marc Gabastou, PhD,2 May Chu, PhD,3 Edward Hayes, MD,3 Kurt B. Nolte, MD,4 and Sherif R. Zaki, MD, PhD1

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**Abstract**

*Yersinia pestis* infection usually is limited to lymph nodes (bubo); rarely, if bacteria are aerosolized, pneumonic plague occurs. We developed an immunohistochemical assay using a monoclonal anti–fraction 1 *Y pestis* antibody for formalin-fixed tissues. We studied 6 cases using this technique. Respiratory symptoms were prominent in 2 cases; histologically, one showed intra-alveolar inflammation, and the other had alveolar hemorrhage and edema. By using the immunohistochemical assay, we found intact *Yersinia* and granular bacterial antigen staining in alveoli, bronchi, and blood vessels. Of the remaining cases, 2 had septicemia and 2 had a bubo. Pathologic changes included lymphocyte depletion, necrosis, edema, and foamy macrophages in lymph nodes; multiple abscesses in the spleen; fibrin thrombi in glomeruli; and unremarkable lungs. By using the immunohistochemical assay, we identified intact bacteria inside monocytes and granular antigen staining in blood vessels. The immunohistochemical assay provided a fast, nonhazardous method for diagnosing plague. The immunohistochemical assay localizes bacteria, retaining tissue morphologic features, and can help define transmission mechanisms.

Plague, a zoonotic disease caused by *Yersinia pestis*, usually is acquired through the bite of an infected flea or a skin abrasion when handling an infected mammal and results in a skin pustule and lymphadenitis (bubo).1-4 Through lymphohematogenous spread, *Y pestis* can cause septicemia and secondary pneumonia. Primary pneumonia occasionally occurs when an individual inhales infected airborne particles coughed by a patient or animal with plague. Transmission through inhalation has been speculated to be the cause of great mortality during 4 world pandemics and has made *Y pestis* a potential agent for bioterrorism.5 Airborne transmission was suspected during an outbreak of plague in Ecuador in 1998.6 This outbreak started when members of a small Andean community attended the funeral of a girl who had been exposed to sick guinea pigs that were being prepared for consumption. There were 12 deaths, including at least 2 people with probable primary pneumonic plague.

Several laboratory methods have been used to detect plague infection: culture, direct immunofluorescence assay, serology, and polymerase chain reaction (PCR). Culture is the standard method for confirming a diagnosis of plague. Both culture and direct immunofluorescence assay require sputum, blood, or aspires from lymph nodes, which often are difficult to obtain and are hazardous to handle.7 Serologic tests that detect the fraction 1 (F1) antigen of *Y pestis* in a passive hemagglutination assay require acute- and convalescent-phase serum samples showing a 4-fold rise in specific antibody titer.8,9 PCR has been used for diagnosis and surveillance.9

We developed an immunohistochemical assay to detect *Y pestis* in formalin-fixed tissues and applied it to a series of plague cases in an effort to maintain morphologic features and to minimize handling of hazardous specimens. We discuss...
transmission and pathogenesis issues that transpired from the study of these cases and are important for diagnostic purposes.

Materials and Methods

We studied formalin-fixed, paraffin-embedded autopsy tissues from 3 patients from Ecuador, 2 of whom were associated with the 1998 outbreak and 1 who was a sporadic suspect case, and 3 sporadic cases from the southwestern United States. Demographic and clinical information was obtained when available. In all cases, \textit{Y. pestis} was cultured from the blood or recognized by PCR.

We placed 4-µm sections of the tissues on Fisher Plus slides (Fisher Scientific, Pittsburgh, PA). The sections were deparaffinized and rehydrated in graded alcohol washes and placed in water. All tissue sections were digested in 0.1 mg/mL Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) in a 0.6-mol/L concentration of tris(hydroxymethyl)aminomethane (Tris), pH 7.5/0.1% calcium chloride for 15 minutes, washed in Tris saline and polysorbate 20, and later blocked with normal swine serum. A mouse monoclonal antibody against F1 of \textit{Y. pestis} (Naval Research Institute, Bethesda, MD) was applied to the tissue sections and allowed to incubate for 60 minutes. This step was followed by sequential application of biotinylated swine antimouse antibody, avidin-alkaline phosphatase complex, and naphthol/fast red substrate (DAKO, Carpinteria, CA). Sections then were counterstained in Mayer hematoxylin (Fisher Scientific) and mounted with aqueous mounting medium (DAKO). Negative controls consisted of each case’s tissue sections incubated with normal mouse serum instead of the antibody against \textit{Y. pestis}.

Validation of the immunohistochemical assay was performed by applying the technique to smears of \textit{Y. pestis} cultures. The plague antibody did not cross-react with paraffin blocks of cases known to have tularemia, cat-scratch disease, legionnaires disease, syphilis, Rocky Mountain spotted fever, or leptospirosis.

Results

Table I lists the origins of the specimens, available demographic and clinical information, primary clinical manifestation, and major histopathologic diagnosis on each case. Irrespective of initial symptomatology, septicemic disease eventually developed in all patients. All patients were male, and their ages ranged from 8 to 54 years; 4 were 13 years or younger. A history of exposure to wild animals was documented for only 1 of the sporadic cases from the United States (case 4).

The specimens studied were derived from a variety of procedures, including full autopsies (cases 2, 4-6), postmortem fine-needle lung and liver biopsies (case 1), and a postmortem finger amputation (case 3). Tissues included lung (4 cases); liver (4 cases); kidney (3 cases); lymph nodes (3 cases); spleen (2 cases); thyroid (2 cases); and skin, bone marrow, central nervous system, gastrointestinal tract, adrenal gland, pancreas, prostate, and testis (1 case each).

The lung, lymph nodes, kidney, and spleen showed histopathologic abnormalities, while other tissues studied appeared unremarkable. The lung tissue of the patients from the outbreak in Ecuador showed acute pneumonia consisting of intra-alveolar infiltration by neutrophils and mononuclear cells (case 1) and intra-alveolar hemorrhage and edema (case 2). In both cases, abundant bacteria could be identified with H&E stains. The lung sections from the sporadic cases (cases 4 and 6) were unremarkable or had focal intra-alveolar edema; acute pneumonia was not observed in either of them. The lymph nodes examined (cases 4-6) showed various degrees of lymphoid depletion (all cases), necrosis (1 case), and foamy macrophages in the sinusoids (2 cases). The spectrum of histologic findings could be present in the same patient when lymph nodes from different locations were sampled. The spleen was examined in 2 cases: multiple abscesses were found in case 4, while case 6 showed increased numbers of macrophages in the sinusoids. The kidney from case 6 showed fibrin thrombi in the glomeruli, suggesting disseminated intravascular coagulation.

Table I

<table>
<thead>
<tr>
<th>Case No./Sex/ Age (y)</th>
<th>Origin</th>
<th>Available Clinical Information</th>
<th>Primary Manifestation</th>
<th>Major Histopathologic Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/37</td>
<td>Ecuador</td>
<td>Fever, headache, cough, outbreak case</td>
<td>Respiratory</td>
<td>Acute pneumonia</td>
</tr>
<tr>
<td>2/M/54</td>
<td>Ecuador</td>
<td>Father of index case</td>
<td>Respiratory</td>
<td>Intra-alveolar hemorrhage and edema</td>
</tr>
<tr>
<td>3/M/13</td>
<td>Ecuador</td>
<td>Fever, headache</td>
<td>Systemic</td>
<td>Unremarkable</td>
</tr>
<tr>
<td>4/M/8</td>
<td>United States</td>
<td>Fever, shock, animal exposure</td>
<td>Lymphadenopathy</td>
<td>Lymphoid depletion</td>
</tr>
<tr>
<td>5/M/10</td>
<td>United States</td>
<td>Fever, groin pain</td>
<td>Lymphadenopathy</td>
<td>Lymphoid depletion</td>
</tr>
<tr>
<td>6/M/13</td>
<td>United States</td>
<td>Pain in legs, diarrhea, vomiting</td>
<td>Systemic</td>
<td>Focal intra-alveolar edema</td>
</tr>
</tbody>
</table>

* The clinical data and primary manifestations for the Ecuadorian patients were obtained indirectly and postmortem from family members and neighbors.
By using the immunohistochemical assay, we were able to
detect intact bacteria inside macrophages and extracellularly
in the blood vessels of all tissues studied. Granular antigen staining
(fine stippling) usually accompanied the intact bacteria.

Abundant extracellular bacteria were present in the alve-
olar spaces in the lung specimens from cases 1 and 2 Image 1. Case 1 had intact bacteria in the larger bronchi Image 2. Granular antigen staining was found extracellularly, coating the alveolar walls and the blood vessels. In the lung specimens from cases 4 and 6, we found intact bacteria and gran-
ular staining in the interstitial vessels and connective tissues Image 3; bacteria were not observed in the alveolar spaces or in the lumen of the bronchi.

In the liver (cases 1, 2, 4, and 6), intact bacteria were
present in the Kupffer cells; granular staining accompanied in
various degrees the intact bacteria in the liver sinusoids. In the kidney specimens from cases 4 and 6, abundant granular antigen staining and bacteria were present in the blood vessels and delineated the glomerular structures Image 4. However, the kidney specimen from case 2 showed intact bacteria focally inside mononuclear cells in the interstitium. The amputated finger specimen (case 3) showed abundant granular antigen staining and intact bacteria in the blood vessels of the dermis Image 5, subcutaneous tissues, and bone marrow. The spleen of case 4 showed intact bacteria and granular staining in the abscesses. Two patterns were seen in the lymph nodes: those that showed lymphoid depletion had bacteria present in the blood vessels only, but those from patients who had necrosis demonstrated intact intracellular

bacteria in macrophages and abundant granular staining in the subcapsular sinusoids. In the brain (case 5), intact bacteria and granular antigen staining were present predominantly in the meninges, although they also were seen in the parenchymal blood vessels in smaller amounts. The gastroin-
testinal tract (case 6) demonstrated bacteria in the blood vessels and focally in the lamina propria of the mucosa.
Discussion

We used an immunohistochemical assay to identify *Y. pestis* in formalin-fixed, paraffin-embedded tissues, a technique that has been used previously to study animal models of plague.10,11 Because this assay can be applied to formalin-fixed tissues, it can decrease the exposure of laboratory personnel to potentially hazardous microorganisms found in fresh tissues, and it can provide a useful new tool to evaluate postmortem needle-biopsy samples. We demonstrated *Y. pestis* antigens and bacteria in skin vasculature, indicating that plague potentially can be diagnosed in skin biopsy specimens. A similar diagnostic procedure has proven useful during outbreaks of Ebola hemorrhagic fever, in cases of Rocky Mountain spotted fever, and in anthrax cases related to the recent bioterrorist attacks.12-14 In addition, the immunohistochemical assay can be used to retrospectively review autopsy material in certain epidemiologic situations, and it can provide a diagnosis when the only tissues available are paraffin embedded. This technique also allows visualization of bacteria, their antigens, and the surrounding inflammatory reaction in various tissue structures, thus increasing knowledge of the pathogenetic mechanisms of this infection.

Two forms of plague pneumonia have been described: primary and secondary. Primary pneumonia refers to infection acquired through airborne particles, while secondary pneumonia is a consequence of lung seeding from septicemic plague. Classic pathologic descriptions suggest that it is possible to distinguish primary and secondary pneumatic plague since in the former, bacteria are found mostly in the alveoli, while in the latter, they will be found in the interstitium.2,3,15,16 However, this distinction could be difficult to establish when there is autolysis and bacterial overgrowth because the autopsy was not performed in a timely fashion or when the pneumonic plague has advanced, spreading into other tissue compartments. In the cases described herein, the immunohistochemical assay was useful because we could distinctly identify *Y. pestis* and its antigens in the different compartments: air spaces (bronchi, alveoli) and interstitium. The patients with intact bacteria in the air spaces probably inhaled the microorganisms and had primary pneumonic plague. The cases with bacteria only in the interstitium indicate septicemia and correspond to secondary pneumonia. Being able to distinguish primary from secondary pneumonic plague has important implications for epidemiologic investigations, including events of possible bioterrorism in which airborne release of *Y. pestis* would produce primary pneumonic plague. The immunohistochemical assay for *Y. pestis* can be a helpful tool not only to document the mode of transmission but also to specifically diagnose plague.

Sections from the lungs in this series of cases showed that several histopathologic patterns can be observed, including acute pneumonia, intra-alveolar hemorrhage and edema, a combination of the previous two, and even an unremarkable lung parenchyma. Previous descriptions of either primary or secondary pneumonic plague indicate that the inflammatory reaction to the infection is usually minimal.2,3 *Y. pestis* secretes a series of proteins (Yop H, Yop E, and Yop...
M) that block the host inflammatory response, a process that explains the lack of inflammation in infected tissues.\textsuperscript{5} However, focal or diffuse intra-alveolar inflammation associated with manifestations of pneumonia, similar to what we saw in one of our cases, have been reported occasionally in animal models and human cases.\textsuperscript{4,16,17} We speculate that host factors can produce different inflammatory responses resulting in varied histopathologic patterns of disease; alternatively, the tissue samples obtained may represent different stages as the disease progresses.

Septicemic plague can occur after either bubonic or pneumonic plague. Septicemia was present in all of the cases we studied, since bacteria were present in blood vessels of the kidney, central nervous system, bone marrow, skin of finger, and other distal locations. From our immunohistochemical results, it is evident that the bacteria are found in the blood vessels both inside monocytes and as free organisms. Intact bacteria also seed the interstitium of organs such as kidney and spleen. Granular antigen staining usually was present in the same locations in which intact bacteria were seen and probably represents breakdown products of the bacteria. The presence of \textit{Yersinia} antigens in lymphoid tissues may indicate an attempt by the host to mount a response to the infection.

One of our cases showed fibrin thrombi in the glomeruli, representing disseminated intravascular coagulation. Disseminated intravascular coagulation has been reported to occur in up to 13% of monkeys with experimentally induced pneumonic plague.\textsuperscript{10} Another monkey animal model reported the presence of fibrin thrombi in the glomerular capillaries of 80% of the fatalities; the thrombi were not observed in other tissues.\textsuperscript{18} In our patient, the thrombi were associated with intense granular antigen staining in the glomeruli. We speculate that the presence of \textit{Yersinia} antigens in the glomeruli of this patient may have been the stimulus that initiated the coagulation cascade.

The immunohistochemical assay can provide a fast, nonhazardous method for diagnosing plague when cultures are not obtained and only formalin-fixed tissues are available. Further study of skin biopsy specimens is necessary to determine whether this tissue can be used to diagnose septicemic plague. The immunohistochemical assay also can be a valuable research tool since it localizes bacteria in the context of tissue morphologic features. Pathologists should be aware that deaths due to plague may show varied histopathologic manifestations.

\textbf{References}