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Identification of a *Yersinia pestis*-Specific DNA Probe with Potential for Use in Plague Surveillance

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A 900-base-pair DNA fragment derived from a 9.5-kilobase plasmid in *Yersinia pestis* hybridized specifically with *Y. pestis* DNA. We demonstrated the feasibility of using this DNA fragment to detect plague bacilli directly in fleas, suggesting that this *Y. pestis*-specific DNA probe may be useful for plague surveillance in the field. Additional applications for this DNA probe may include plague diagnosis and pathogenesis research.

Yersinia pestis, the etiologic agent of plague, infects wild rodent populations in various regions of Asia, Africa, and the Americas, including the western United States (22, 34). Periodically, this microorganism is transmitted from these animals to humans through a flea intermediate (2). Despite the susceptibility of *Y. pestis* to available antimicrobial therapy, the mortality associated with these outbreaks is high (37). Timely diagnosis is particularly germane to plague epidemiology, since untreated bubonic plague can rapidly progress to septicemic and pneumonic states. Pneumonic plague is transmissible from human to human by droplet infection and is almost uniformly fatal unless effective treatment is begun promptly (26). Because of this continuing epidemic potential, it is essential to maintain surveillance of known plague foci and to investigate the source of all human plague cases. When the threat to humans is judged to be high, local authorities are advised to take appropriate control measures to reduce the risk of a major plague outbreak (2).

Surveillance involves screening fleas and animal carcasses collected throughout areas in which plague is endemic for the presence of *Y. pestis* (2). The laboratory identification of *Y. pestis* from such sources usually requires animal passage to obtain a pure culture. Organisms are recovered from necropsied liver and spleen tissue and identified by using standard microbiological techniques, phage sensitivity typing, and immunofluorescent antibodies directed against fraction I, a major capsular antigen (20, 26, 39, 40). This screening procedure is costly and time consuming; confirmation of plague carriage may take 2 weeks. A rapid diagnostic technique capable of detecting *Y. pestis* directly in fleas or infected animal tissue would be an asset to any plague surveillance program. It would also provide a tool with which plague transmission and the factors affecting it could be studied. Previous attempts to develop such a technique by using immunofluorescent antibodies have proved unreliable (15). Our interest in *Yersinia* pathogenesis (23) has led us to develop a *Y. pestis*-specific DNA probe that is potentially useful for plague surveillance. We assume that such a DNA probe could also be used for plague diagnosis in a clinical setting.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and their sources are listed in Table 1. Unless otherwise stated, bacteria were grown as follows. All *Y. pestis* strains except EV76-6 (24) and TRU were grown at 28°C in either heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) or brain heart infusion broth (Difco Laboratories, Detroit, Mich.). *Brucella* strains were grown in *Brucella* media (BBL) at 37°C. *Pasteurella multocida* strains were grown at 37°C in brain heart infusion broth. All other strains were grown at 28°C in either heart infusion broth or L broth (GIBCO Diagnostics, Madison, Wis.). Strains were stored lyophilized or at −20°C in 50% glycerol in L broth.

DNA purification, digestion, and electrophoresis. Plasmid DNA was isolated from *Y. pestis* EV76-6 by using the method of Birnboim and Doly (7) and purified on a CsCl gradient. DNA was digested according to the recommendations of the manufacturers by using restriction endonucleases purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Horizontal agarose gel electrophoresis was carried out in TAE (0.04 M Tris–0.02 M sodium acetate–0.001 M EDTA, pH 8.0) buffer in a field strength of 1 V/cm for approximately 12 to 18 h. Lambda phage DNA cut with *Hin*dIII or with *Pst*I was used for size markers. DNA bands were visualized with UV light after being stained with ethidium bromide. The agarose (Bethesda Research Laboratories) concentration used ranged from 0.7 to 1.7%.

Plasmid mapping. *Bam*HI, *Hin*dIII and *Pst*I restriction endonuclease cleavage sites in plasmid DNA were mapped with respect to each other by using double and triple enzyme combinations. Additional enzymes (*Eco*RI, *Cla*I, and *Bgl*I) were added to the map by determining their positions relative to an enzyme which cleaved the plasmid at a single site and then relative to each other.

Probe purification and labeling. DNA fragments were prepared by digesting isolated plasmid DNA with the appropriate enzymes. The fragments were electrophoresed into 0.7% low-melting-point agarose (Bethesda Research Laboratories), and bands containing the desired fragments were cut from the gel. DNA was recovered by melting the agarose plug at 65°C and extracting several times with phenol saturated with 0.01 M Tris chloride–0.001 M EDTA, pH 8.0. Phenol was removed by running the sample through a spin column (Bio-Gel P10; 200/400 mesh; Bio-Rad Laboratories, Richmond, Calif.) prepared in an Eppendorf tube. Purified

TABLE 1. Strains used and their reactivities with probe fragments

Species (no. of strains tested)	Reactivity[a] with fragment:			Source or reference
	A	B	C	
Y. pestis (27)	+	+	+	T. Quan[b], 24
Y. pseudotuberculosis (33)	−	+/−	+/−	T. Quan[b]
Y. enterocolitica (26)	−	ND[c]	ND	W. Hill[d] and J. Farmer III[e]
Yersinia spp.[f] (5)	−	ND	ND	W. Hill[d] and J. Farmer III[e]
P. multocida (27)	−	ND	ND	T. Quan[b]
F. tularensis (2)	−	ND	ND	T. Quan[b]
Brucella spp.[g] (3)	−	ND	ND	J. Douglas[h]
E. coli (3)	−	ND	ND	23, 33

[a] +, All strains reacted with probe; −, no strains reacted with probe; +/−, a subset of strains reacted with probe.
[b] Plague Branch, Division of Vector Borne Viral Diseases, Centers for Disease Control, Fort Collins, Colo.
[c] ND, Not done.
[d] Division of Microbiology HFF-234, Food and Drug Administration, Washington, D.C.
[e] Enteric Identification Laboratory, Centers for Disease Control, Atlanta, Ga.
[f] Includes *Y. aldovae*, *Y. intermedia*, and *Y. frederiksenii*.
[g] Includes *B. abortus* and *B. suis*.
[h] Department of Microbiology, University of Hawaii, Honolulu.

DNA was labeled by nick translation by using [^{32}P]dCTP (Amersham Corp.) to a specific activity of 2×10^8 cpm/μg. Labeled fragments were separated from unincorporated nucleotides by again running the sample on a Bio-Gel P10 spin column.

Colony blot hybridization conditions. Samples (10 μl) of bacterial cultures were spotted onto nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.). The number of organisms per spot ranged from 10^5 to 10^7, depending on the experiment. Filters were then treated as described by Maniatis et al. (18) with some modifications. Briefly, filters underwent successive treatments of 5 min each on blotting pads (Schleicher & Schuell) soaked with (i) 10% sodium dodecyl sulfate (SDS); (ii) 1.5 M NaCl and 0.5 M NaOH; and (iii) 1.5 M NaCl–0.5 M Tris chloride (pH 8.0) before being air dried and baked for 15 min at 80°C in a vacuum. After being baked, filters were wet in a solution of 6× SSPE (1× SSPE is 0.15 M NaCl–0.01 M NaH_2PO_4–0.001 M EDTA, pH 7.4) and washed in 50 mM Tris chloride (pH 8.0)–1 M NaCl–1 mM EDTA–0.1% SDS for 1.5 to 2 h at 42°C with shaking. After filters were sealed in plastic bags, prehybridization was performed in 50% formamide–5× Denhardt solution (1× Denhardt solution is 0.2% Ficoll [Pharmacia Fine Chemicals, Piscataway, N.J.]–0.2% polyvinylpyrrolidone–0.2% bovine serum albumin [Pentax fraction V])–5× SSPE–0.1% SDS–100 μg of denatured salmon sperm DNA per ml for 2 to 4 h at 42°C before the addition of 10^6 cpm of ^{32}P-labeled probe per ml of hybridization buffer. After hybridizing overnight at 42°C, the filters were washed three times for 10 min each in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)–0.1% SDS at room temperature with shaking and then washed twice for 1.5 h each in 1× SSC–0.1% SDS at 68°C with shaking. After being air dried, filters were exposed to X-ray film (XAR; Eastman Kodak Co., Rochester, N.Y.) for various amounts of time.

Flea infection. Two white mice (RML outbred-NIH; Rocky Mountain Laboratories, Hamilton, Mont.) were inoculated intraperitoneally with 0.1 ml of an overnight brain heart infusion broth (Difco) culture of a fully virulent strain of *Y. pestis* (NM77-538). The mice were restrained 12 h postinoculation, and 25 fleas (*Diamanus montanus*) were placed with each mouse in a 1-gal (3.785-liter) jar. An additional 25 fleas were placed on each mouse 12 h later. The 100 fleas were collected from both jars 50 h postinoculation, incubated at 27°C with high humidity for 2 additional days, and then frozen overnight. The next morning, 25 fleas were thawed and positioned on a nitrocellulose membrane, covered with a sheet of cellophane wrap, and blotted onto the filter by rolling a curved spatula over each flea in a head-to-tail direction. Another group of 25 fleas that had fed on a noninfected mouse served as negative controls.

RESULTS

Previous work has shown that virulence in *Yersinia* species is mediated in part by a 70-kilobase-pair plasmid common to all three pathogenic *Yersinia* species (25). *Y. pestis* also carries a 9.5-kilobase-pair pesticin plasmid (5, 11) that has been uniquely associated with its pathogenicity (9). We thought that this latter plasmid might serve as a source for *Y. pestis*-specific DNA. Plasmid DNA was purified from *Y. pestis* EV76-6 (24) and used to generate a restriction map from which potential probe fragments could be identified. The plasmid DNA was digested with 10 enzymes. Two restriction endonucleases, *Pvu*II and *Bcl*I, failed to cleave the DNA, while three others, *Bam*HI, *Hin*dIII, and *Pst*I, appeared to cut at single unique sites. The remaining five enzymes, *Alu*I, *Bgl*I, *Cla*I, *Eco*RI, and *Hae*II, each cut the plasmid two or more times. A partial restriction map of the 9.5-kilobase pesticin plasmid is shown in Fig. 1.

By using this restriction map as a guide, several fragments (labeled A, B, and C in Fig. 1) were selected for use as possible probes. Because *Yersinia pseudotuberculosis*, *Francisella tularensis*, *P. multocida*, and *Brucella* spp. can cause epizootics and rodent die-offs similar to those observed with plague (28), we screened each fragment for cross-reactivity with these organisms. *Rickettsia typhi* was included because it is carried by some of the same flea vectors. *Y. pseudotuberculosis* was of particular interest to us because it shares greater than 90% DNA homology with *Y. pestis* (6, 19) and is capable of surviving in fleas (8). (*F. tularensis* has also been isolated from fleas [14], but its DNA homology with *Y. pestis* is minimal [29].) Of the fragments tested, only fragment A, a 900-base-pair (bp) *Bam*HI-*Hin*dIII fragment, hybridized specifically with *Y. pestis* but not with *Y. pseudotuberculosis* (Fig. 2), *Y. enterocolitica* or other nonpathogenic *Yersinia* species, *P. multocida*, *R. typhi*, *F. tularensis*, and *Brucella* spp. (Table 1). All 27 *Y. pestis* strains tested reacted with this probe except the avirulent strain TRU, which lacks the pesticin plasmid. Under our conditions, a minimum of 10^5 organisms were required for detection by this DNA probe (not shown).

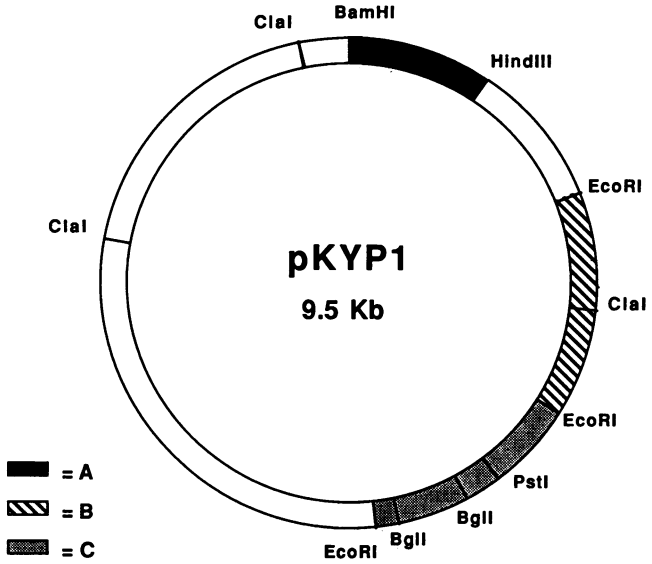

FIG. 1. Restriction map of plasmid pKYP1. Regions A, B and C indicate fragments used as DNA probes. Kb, Kilobase.

An additional test of the usefulness of a probe for plague surveillance is its level of background reactivity with biological materials, including fleas and their natural flora. To measure this for fragment A, we performed a reconstitution experiment, the results of which are shown in Fig. 3. Ground fleas (*Thrassis bacchi*) were suspended in either phosphate-buffered saline (PBS) or PBS containing *Y. pestis* at a concentration of 10^8 organisms per ml. Samples of these suspensions containing the equivalent of one flea or 10^6 plague bacilli or both were spotted onto nitrocellulose filters and probed with fragment A. Only those samples containing *Y. pestis* organisms produced a signal, and the presence of flea material did not interfere with this detection. Negative results were also obtained when samples of mouse and human fecal material, representing a diverse mixture of naturally occurring microorganisms, were probed with fragment A (Fig. 3).

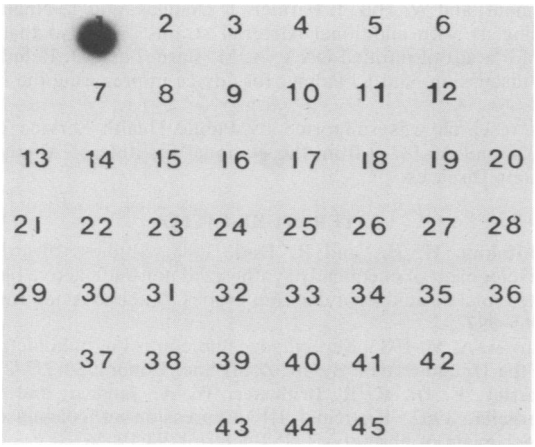

FIG. 2. Colony blot probed with fragment A. Numbers 1 to 6 correspond to *Y. pestis* EV76-6, *E. coli* LE392(pYV019::Tn*5*) and LE392, *Y. enterocolitica* YE8081, and *P. multocida* PM107 and PM108, respectively. Numbers 7 to 45 represent an assortment of *Y. pseudotuberculosis* isolates.

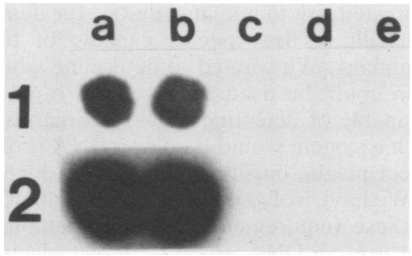

FIG. 3. Panel 1 is an autoradiograph of a nitrocellulose filter spotted with EV76-6 (lane a), EV76-6 and ground flea suspension (lane b), ground flea suspension (lane c), human feces (lane d), or mouse feces (lane e) and probed with ^{32}P-labeled fragment A. Panel 1 was slightly underexposed to show that the presence of ground fleas in lane b did not quench the strength of the hybridization signal compared with lane a. Panel 2 is the same autoradiograph overexposed to demonstrate the lack of reactivity with flea and fecal debris in lanes c to e.

To test the feasibility of using a DNA probe to detect *Y. pestis* organisms directly in fleas, we blotted individual plague bacillus-fed fleas onto nitrocellulose and processed these filters as if they were colony blots (Fig. 4). The "infected" panel contained 25 fleas that were allowed to feed on plague-infected mice. Arrows point to nine spots that correspond to fleas that reacted with the probe. No flea-associated hybridization signals were detected in the control panel, which contained the contents of 25 fleas that fed on uninfected mice.

DISCUSSION

Implementation of plague control programs is costly and depends on the use of rodenticides and insecticides which are themselves health and safety risks (22). Targeted rodent and flea populations may also develop resistance to the chemicals being used against them, foiling future control efforts (13). Clearly, such programs should not be instituted unless there is strong evidence for the presence of plague

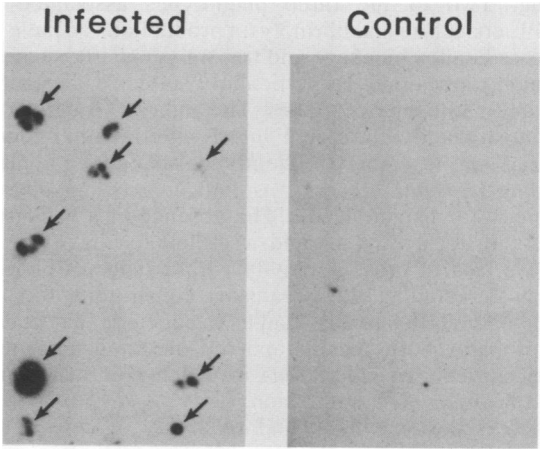

FIG. 4. DNA probe hybridization for the detection of *Y. pestis* in squashed whole fleas. Laboratory-reared fleas, *D. montanus*, were fed on either an experimentally infected laboratory mouse (left) or a noninfected control mouse (right); there were 25 fleas in each group. Arrows indicate hybridization signals corresponding to blotted fleas. Signals without arrows do not correspond to squashed fleas and represent nonspecific reactions.

with an associated risk to humans, that is, the demonstration of plague bacilli in flea species capable of transmitting disease to humans. We wanted to determine whether DNA hybridization could be used to develop a rapid diagnostic technique capable of detecting *Y. pestis* organisms directly in fleas. Such a system would require a DNA probe that can reliably detect plague bacilli in fleas with a high degree of specificity. We have isolated a *Y. pestis*-specific DNA probe that meets these requirements and have demonstrated the feasibility of using a DNA probe to detect plague bacilli in fleas without prior culturing. Other applications may include rapid confirmation of human plague cases, particularly the pneumonic form, when it may be necessary to identify and treat or quarantine patient contacts.

The restriction map we obtained for pKYP1, the plasmid from which our probes were isolated, is similar to that previously reported for pYP1 by Goncharov et al. (12). The major differences observed lie in our placement of two *Bgl*I sites (Fig. 1). Results with *Bam*HI, *Hin*dIII, *Eco*RI, *Pst*I, and *Pvu*II did not differ significantly. Presumably, pKYP1 is also identical to the 6-megadalton pesticin plasmid, termed pEL100 by Ferber and Brubaker (11).

A measure of the predictive value of any detection system must take into account the levels of both false-negative and false-positive results. We refer to these properties as reliability and specificity, respectively. The reliability of fragment A as a probe for detecting plague organisms was determined by screening isolates of *Y. pestis* collected from both human plague cases and routine rodent and flea surveillance from a variety of locations over a period of several years. All strains tested hybridized with the probe except the avirulent strain TRU, which did not react. This result is expected because TRU lacks the pesticin plasmid. The association of this plasmid with virulence (9) may explain its high rate of carriage in wild plague isolates (4, 38) and argues for the usefulness of pKYP1-derived DNA fragments as probes for plague surveillance. In order to transmit plague bacilli to a new host, a flea must become "blocked" with a solid mass of bacteria and blood products. By interfering with the normal function of a sphincter valve in the foregut of the flea, this blockage allows an infectious flea to regurgitate its stomach contents (including plague bacilli) upon feeding. Two of the three phenotypes associated with pKYP1, coagulase and fibrinolysin production (3), have been implicated in flea blockage and transmission (10), suggesting that such probes may be particularly suited for flea surveillance and pathogenesis studies. The ability of fragment A to distinguish readily between *Y. pestis* and the other zoonotic bacterial species most frequently coisolated with plague, including *Y. pseudotuberculosis*, indicates its high degree of specificity for plague bacilli. The absence of a background signal with fecal samples and with flea specimens demonstrates a lack of cross-reactivity with a range of biological materials, including the organisms constituting the flea's normal flora. These results lead us to conclude that fragment A is probably both specific and reliable enough for use in plague surveillance operations in which it is necessary to screen fleas for plague infection.

Whereas the specificity and reliability of detection are inherent properties of a DNA probe, its sensitivity is more a function of the system in which the probe is used. In these studies a minimum of 10^5 organisms were required for detection by a colony blot type of analysis. Our preliminary studies suggest that this level of sensitivity may be sufficient for direct detection of plague-infected fleas without additional culturing. We cannot be sure that we can reliably directly detect 100% of the infected fleas with our method at this time, but a high degree of detection would not be inconsistent with the findings of other investigators, who report experimental flea infection levels of 30 to 50% (32, 35, 36) and that the average number of organisms in a blocked flea is between 10^5 and 10^6 (27). In these studies, the probe would not have been able to detect the smaller number of organisms in unblocked fleas. However, greater sensitivity could be achieved by incubating filters spotted with samples overnight on nutrient agar to increase the bacterial count before probing (21). Alternatively, a DNA amplification technique such as the polymerase chain reaction (31) could be employed to increase the hybridization signal. By using this technique, probe sequences in the sample could be specifically increased manyfold in less than 2 h, allowing detection of a single bacterium (30). Our results demonstrate the feasibility of using DNA hybridization techniques to screen fleas directly for plague infection. An additional use for this method may be the detection of plague bacilli in tissue samples, such as animal carcasses sent in from the field during plague surveillance. Presumably, human specimens, including bubo or lung aspirates and blood samples, would be amenable to this type of diagnostic approach as well. Using a nonradioactive label such as biotin (1, 16, 17) in place of ^{32}P to visualize the probe might make this technique adaptable for on-site use in the field, since the required equipment and reagents are generally portable and nonperishable.

We have reported a restriction map for the 9.5-kilobase-pair pesticin plasmid of *Y. pestis* EV76-6 and identified a portion of it that is uniquely *Y. pestis* specific by colony blot analysis. This DNA fragment appears to encode part of the coagulase gene (K. A. McDonough and S. Falkow, unpublished results). We have also demonstrated the feasibility of using DNA probe technology to detect *Y. pestis* organisms directly in fleas by using a modified colony blot approach. The *Y. pestis*-specific probe and the flea blot method described here may have future uses in plague surveillance, diagnosis, and pathogenesis studies.

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