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New Method for Plague Surveillance Using Polymerase Chain Reaction To Detect *Yersinia pestis* in Fleas

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***Yersinia pestis*, the plague bacillus, infects a variety of mammals throughout the world and is transmitted by fleas. We developed a polymerase chain reaction (PCR) test using primers designed from the *Y. pestis* plasminogen activator gene to directly detect plague-infected fleas. As few as 10 *Y. pestis* cells were detected, even in the presence of flea tissue, by PCR and then agarose gel electrophoresis and ethidium bromide staining. The feasibility of the assay was demonstrated by using naturally infected *Xenopsylla cheopis* fleas. The detection of *Y. pestis* in fleas by PCR provides a rapid and sensitive way to monitor plague in wild animal populations, allowing public health officials to better assess the potential risk of transmission to humans.**

Yersinia pestis, the bacterial agent of plague, primarily infects small mammals and is cycled from infected to uninfected hosts by fleas. The ecology of plague is complex. Over 200 different species of mammals and at least 80 different species of fleas have been implicated in maintaining *Y. pestis* in enzootic foci throughout the world (13, 14). Plague is well established in certain wild rodent populations in parts of the western United States (13).

Humans can acquire plague through contact with infected mammals or fleas. In the United States, most cases of plague in humans occur during periodic epizootics of plague among wild rodents (13). For this reason, determination of the prevalence and distribution of *Y. pestis* in these animal populations and their fleas is an important part of plague surveillance (14). Fleas are often preferred specimens because they are easier and safer to handle than mammalian tissue (14). The standard public health laboratory methods for plague surveillance have changed little in the 100 years since they were pioneered by Yersin and Kitasato: inoculation of experimental animals with suspect flea or animal tissue and subsequent isolation and identification of *Y. pestis*. These methods are reliable but require weeks for their completion and the use of laboratory animals. Recently, a DNA probe hybridization method was developed to detect *Y. pestis* in fleas (11, 20). However, the DNA probe method only detects fleas infected with 10^5 or more bacteria (11, 20). We describe here a rapid and sensitive alternative method for the direct detection of as few as 10 *Y. pestis* cells in infected fleas using the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Yersinia isolates and cultivation. *Yersinia* cultures were obtained from the Centers for Disease Control, Fort Collins, Colo. The *Y. pestis* strains examined in the present study are listed in Table 1. *Yersinia pseudotuberculosis* serotype 1A and *Yersinia enterocolitica* 70-419-3F were used as negative controls. *Yersinia* cells were grown in brain heart infusion (BHI) broth (Difco, Detroit, Mich.) for 24 h at 27°C and were counted by using a Petroff-Hausser bacterial counting chamber and dark-field microscopy.

Flea samples. *Xenopsylla cheopis* fleas, colonized and maintained as described previously (20), were used in the present study. To infect fleas, 1-week-old white mice were first inoculated intraperitoneally with 0.25 ml of phosphate-buffered saline containing 10^3 cells of the virulent strain *Y. pestis* 195/P. After 48 h, two mice that appeared to be sick were placed in a glass jar containing fleas that had not fed for 7 days. The two flea-infested mice were then transferred immediately to a single clean jar and were held at 27°C and 75% relative humidity to allow the fleas to feed. After 48 h, during which time both mice died, the fleas were recovered. Some of the fleas were killed and stored in 70% ethanol at room temperature; the remainder were killed by freezing and were stored at −20°C. Another set of fleas was fed on uninfected mice in a similar fashion for use as negative control specimens. Fleas were stored for up to 5 months prior to testing.

Fleas were prepared for PCR by a modification of a method described by Webb et al. (21). Individual fleas were placed into separate sterile 0.5-μl polypropylene microcentrifuge tubes. Ethanol-stored fleas were allowed to air dry. By using a micropipet, 50 μl of BHI broth was taken up into a sterile disposable aerosol-barrier pipet tip (1 to 150 μl; PGC Scientifics, Gaithersburg, Md.). Each flea was thoroughly macerated with the end of the pipet tip in a small amount of the BHI broth before adding the rest of the 50 μl of broth to the tube. In some cases, five fleas were pooled in a single tube and triturated in 250 μl of BHI broth (i.e., 50 μl of BHI broth per flea). For experiments used to assess the sensitivity of the method, known numbers of *Y. pestis* 195/P cells in 50 μl of BHI broth were added to uninfected fleas prior to trituration. Tubes containing the flea suspensions were immediately placed at 95°C for 10 min. The tubes were next centrifuged for 10 s at maximum speed (15,600 × *g*) in a microcentrifuge to pellet the flea tissue debris. Sample tubes were then placed on ice and assayed by PCR within 10 min.

PCR. The synthetic oligonucleotide primers used for PCR were based on the nucleotide sequence of the *Y. pestis* plasminogen activator (*pla*) gene (10, 18). PCR primers Yp1 (5′-ATCTTACTTTCCGTGAGAAG-3′) and Yp2 (5′-CTTG GATGTTGAGCTTCCTA-3′) correspond to nucleotides 971 to 990 and 1431 to 1450, respectively, of the *pla* locus sequence reported by Sodeinde and Goguen (18).

The PCR protocol was an adaptation of the "hot start" method of Chou et al. (5). A 2× bottom-layer reagent

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TABLE 1. Geographic origin, identification, and biological source of *Y. pestis* isolates examined by PCR

Geographic area	Identification no.	Source
North America		
United States	NM 77-538	Human
United States	Parrish	Human
United States	NM68FL	Fleas
United States	CALIFST 384	Fleas
South America		
Brazil	P.EXU 17	Human
Bolivia	69-57-1	Human
Ecuador	Ecuador 3	Human
Africa		
South Africa	59V-54	Human
Lesotho	F308/68	Fleas
Zimbabwe	760280	Human
Kenya	Nairobi rattus	Rodent
Madagascar	111	Human
Eurasia		
Yemen	14-47	?
India	195/P	Human
Nepal	JHUPRI	Human
Myanmar (Burma)	14-36	?
Vietnam	13-83	?
Indonesia	JAVA 777	Human

mixture (10 mM Tris [pH 8.3], 3 mM $MgCl_2$, 400 μM [each] the four deoxyribonucleoside triphosphates, 0.6 μM [each] primers Yp1 and Yp2) and an upper-layer dilution buffer (10 mM Tris [pH 8.3], 100 mM KCl) were prepared and were stored at −20°C until they were ready for use. A separate 0.5-μl PCR tube (GeneAmp; Perkin-Elmer, Norwalk, Conn.) was labelled for each flea sample. A total of 50 μl of the 2× bottom-layer reagent mixture was dispensed into each tube, and a single wax bead (AmpliWax PCR Gem 100; Perkin-Elmer) was added. The tubes were put in an 80°C heating block for 5 min to melt the wax. The PCR tubes were then placed at room temperature for 5 min to allow the wax layer to form a solid barrier on top of the bottom-layer reagent. During this time, samples were prepared for PCR by mixing 2 μl of each flea-BHI broth preparation and 43 μl of upper-layer dilution buffer in a separate 0.5-μl microcentrifuge tube. Lastly, 5 μl of AmpliTaq DNA polymerase (5 U/μl; Perkin-Elmer) diluted 1:10 in ice-cold upper-layer dilution buffer and the 45 μl of sample dilution were added in turn to the top of the wax barrier in each PCR tube.

To test different strains of *Y. pestis* directly, samples containing approximately 500 cells in 50 μl of BHI broth were heated at 95°C for 10 min and were then prepared for PCR as described above. Negative control samples contained approximately 10^6 *Y. pseudotuberculosis* or *Y. enterocolitica* cells or 3 ng of *Rickettsia typhi* DNA.

PCR amplifications were conducted in a Perkin-Elmer DNA thermal cycler by using the following program. For cycles 1 and 2, template DNA denaturation was at 95°C for 2 min (this also melted the wax barrier in the tubes, allowing the upper and lower layers to combine), primer annealing was at 51°C for 1 min, and primer extension was at 72°C for 1 min. For cycles 3 to 10, denaturation was at 94°C for 1 min, annealing was at 51°C for 1 min, and extension was at 72°C for 1 min. For cycles 11 to 40, denaturation was at 94°C for 1 min, annealing was at 51°C for 1 min, and extension was at 72°C for 1 min and increased by 5 s per cycle. After the last cycle, primer extension was continued for 10 min at 72°C.

Gel electrophoresis and Southern blot analysis. Following PCR amplification, 30 μl of the 100-μl reaction mixtures was loaded into the wells of a 2% NuSieve GTG–0.5% SeaKem GTG agarose gel (FMC BioProducts, Rockland, Maine), and electrophoresis was done at 2 V/cm in 90 mM Tris (pH 8)–90 mM borate–2 mM EDTA (TBE) buffer containing 0.1 mg of ethidium bromide per ml. For Southern blot analysis, the DNA in the agarose gel was transferred to a nylon membrane (GeneScreen Plus; DuPont NEN, Boston, Mass.) by capillary action (16). The synthetic oligonucleotide probe (5′-ATACTGTGACGGCGGGTCTG-3′) incorporated nucleotides 1181 to 1200 of the *pla* gene and is positioned between the sequences of the two PCR primers (18). The probe was 5′ end-labelled with [γ-^{32}P]ATP and was hybridized with the Southern blot by standard methods (16). Before autoradiography, the membrane was washed twice for 10 min at room temperature in 2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate)–0.1% sodium dodecyl sulfate (SDS)–2 mM EDTA and then twice for 20 min at 37°C in 0.1× SSC–0.1% SDS–2 mM EDTA.

RESULTS AND DISCUSSION

The amplification target for the PCR was a 478-bp segment of the plasminogen activator gene (*pla*) locus of *Y. pestis*. This gene encodes an outer membrane protease that is responsible for two well-known properties of virulent *Y. pestis*, including a plasminogen activator activity, resulting in the lysis of fibrin clots, and weak coagulase activity (1, 17). Cells of the 18 *Y. pestis* strains listed in Table 1 were examined directly by the PCR assay by using the *pla* gene-specific primers. The expected 478-bp PCR product was observed for all of the 18 strains by agarose gel electrophoresis (9).

We evaluated the sensitivity and specificity of the method using uninfected *X. cheopis* flea samples to which known numbers of *Y. pestis* 195/P cells were added. Specificity controls included an uninfected flea to which no bacteria were added, and samples containing *Y. pseudotuberculosis* cells, *Y. enterocolitica* cells, or *R. typhi* DNA. The results are shown in Fig. 1. The predicted 478-bp amplification fragment was seen on the ethidium bromide-stained gel for flea samples containing 10 or more *Y. pestis* cells. No product was evident from the flea samples containing zero or one *Y. pestis* cell, samples containing cells of the two other pathogenic *Yersinia* species, or samples containing *R. typhi* DNA.

Southern blot analysis was performed to confirm that the PCR product was derived from the target *Y. pestis* gene. The DNA in the agarose gel shown in Fig. 1 was blotted and hybridized with an internal *pla* gene probe. The probe hybridized to the 478-bp fragment that was seen on the ethidium bromide-stained gel. This result confirmed that the PCR specifically amplified the expected target. The sensitivity of the method was not markedly improved by the additional step, however; all samples that were positive by Southern blotting were also positive by ethidium bromide staining of the agarose gel (Fig. 1). The probe also hybridized with one or more bands whose apparent sizes were less than 478 bp. These other bands were invisible or were only weakly stained in the agarose gel and most likely resulted from the accumulation of truncated, single-stranded DNA amplification products (5).

These results indicated that the PCR assay of triturated,

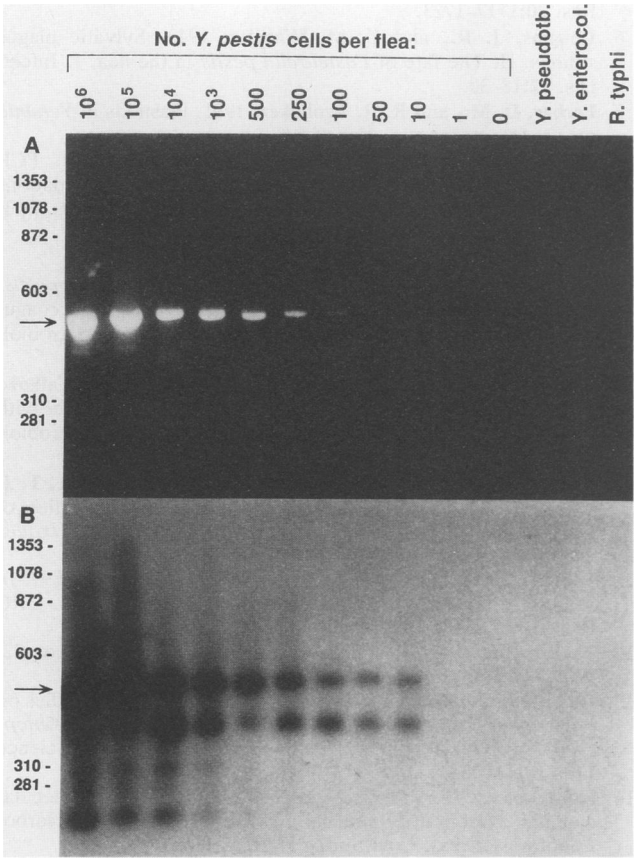

FIG. 1. Sensitivity and specificity of PCR for the detection of *Y. pestis* in fleas. The indicated numbers of *Y. pestis* cells were added to tubes containing an uninfected *X. cheopis* flea and assayed as described in the text. Samples containing 10^6 *Y. pseudotuberculosis* cells, 10^6 *Y. enterocolitica* cells, and 3 ng of *R. typhi* DNA were also tested. PCR amplification products were analyzed by agarose gel electrophoresis (A) and Southern hybridization (B) with a *pla* gene-specific internal probe. The arrows point to the 478-bp PCR product. The migration of the *Hae*III-digested ϕX174 DNA fragments used as size standards (in base pairs) is indicated at the left.

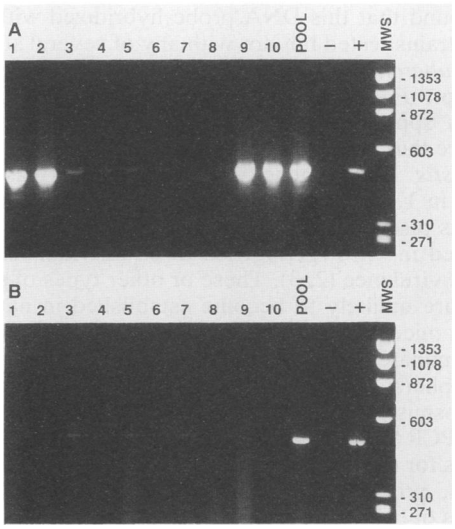

FIG. 2. Detection of *Y. pestis* in infected fleas by PCR. *X. cheopis* fleas that had fed on *Y. pestis*-infected mice and then stored at −20°C (A) or at room temperature in 70% ethanol (B) for 5 months were assayed by PCR. Lanes 1 to 10, results from individual fleas; even-numbered lanes, female flea samples; odd-numbered lanes, male flea samples; pool, samples containing five fleas; lanes −, uninfected flea sample (negative control); lanes +, sample from an uninfected flea to which 500 *Y. pestis* cells were added (positive control); MWS, *Hae*III-digested ϕX174 DNA size standards (in base pairs).

heat-treated flea samples has the potential to specifically detect as few as 10 *Y. pestis* cells in an infected flea. The simple sample preparation method yielded a more sensitive assay than did more elaborate cell lysis procedures that included phenol and chloroform extraction to remove possible PCR inhibitors derived from the fleas or the blood they had ingested (9).

We next tested fleas that had fed on plague-infected mice. Of 20 individual fleas that had been stored at −20°C, 18 (90%) were positive for *Y. pestis* by PCR, as determined by agarose gel electrophoresis. A pool of five fleas that was tested was also positive. Of 20 fleas that had been stored at room temperature in 70% ethanol, 11 (55%) were positive. In addition, three of four separate pools, each containing five ethanol-stored fleas, were positive. A portion of these results is shown in Fig. 2. The method appeared to have somewhat lower sensitivity when ethanol-stored fleas were tested. Ethanol is a recommended preservative for storage of tissue prior to PCR (8), and *Borrelia burgdorferi*, the Lyme disease spirochete, has been detected by PCR in ticks stored for as long as 40 years in 70% ethanol (12). Since ethanol tends to harden flea tissue, a more forceful trituration method with glass beads or a tissue grinder may be required to fully release the bacteria from the proventriculus and midgut of these specimens.

The amount of PCR product varied greatly among individual fleas, as judged by the intensity of ethidium bromide staining. Several variables could account for this. The bacteremia in the two mice at the time of flea feeding was not quantitated, but *Y. pestis* can achieve levels of 10^4 to 10^7 per μl of blood in these animals (6). The stomach capacity of *X. cheopis* is estimated at 0.03 to 0.5 μl and is larger in females than males (6, 15). Fleas of both sexes were tested (Fig. 2), but they were not screened for the presence or the size of their blood meal. Finally, the *Y. pestis*-flea interaction is not well understood and appears to be complex. Some *X. cheopis* fleas that feed on an infected animal do not become infected, probably because of the rapid elimination of the bacteria, whereas other fleas can develop a massive infection (6, 14). These variables underscore the need for a sensitive assay; for instance, important reservoir hosts such as rats and ground squirrels may develop a lower degree of bacteremia than mice (6).

The sensitivity of the method also depends on the conservation of the PCR target in *Y. pestis*. The PCR primers were based on the identical *pla* gene sequence of two different strains (10, 18). These primers successfully amplified *pla* gene DNA from another 18 strains of *Y. pestis* tested that had widely dispersed geographic origins (Table 1). In addition, Campbell et al. (4) reported that the 43 *Y. pestis* strains that they examined were all positive by PCR by using primers derived from a different region of the *pla* gene. Finally, the segment of the *pla* gene that we chose to amplify is contained within a DNA fragment used by McDonough and others (11, 20) as a probe to detect *Y. pestis* in fleas.

They found that this DNA probe hybridized with all 27 *Y. pestis* strains tested but not with any of several strains of *Y. pseudotuberculosis*, *Y. enterocolitica*, nonpathogenic *Yersinia* spp., *Pasteurella multocida*, *Francisella tularensis*, *Brucella* spp., or *Escherichia coli* (11, 20). The *pla* gene sequence thus appears to be unique to and highly conserved in *Y. pestis*. The *pla* gene resides on a 9.5-kb plasmid that is present in *Y. pestis* but not in other *Yersinia* species (2, 7, 17). This plasmid can occasionally be eliminated from cells cultivated in vitro, giving rise to strains that have greatly reduced virulence (2, 3). These or other types of *pla* mutant clones are unlikely to become established in nature, however. In mice, the *pla* gene product is required for *Y. pestis* to disseminate from its initial subcutaneous flea bite site to the peripheral bloodstream, a key event in the pathogenesis and transmission of plague (19).

The PCR method has advantages over other available methods for the detection of *Y. pestis* in fleas. It is simple to perform, produces results within 8 h, and detects small numbers of bacteria without the need for laboratory animals or DNA probe labelling techniques. This method should be useful in plague surveillance studies and could be applied to other types of specimens. It may also prove to be a valuable tool in examining the interaction between *Y. pestis* and its insect host.

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