LcrV Capture Enzyme-Linked Immunosorbent Assay for Detection of *Yersinia pestis* from Human Samples

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In the United States, there is currently a major gap in the diagnostic capabilities with regard to plague. To address this, we developed an antigen capture assay using an essential virulence factor secreted by *Yersinia* spp., LcrV, as the target antigen. We generated anti-LcrV monoclonal antibodies (MAbs) and screened them for the ability to bind bacterially secreted native *Yersinia pestis* LcrV. Anti-LcrV MAb 19.31 was used as a capture antibody, and biotinylated MAb 40.1 was used for detection. The detection limit of this highly sensitive *Yersinia* LcrV capture enzyme-linked immunosorbent assay is 0.1 ng/ml. The assay detected LcrV from human sputum and blood samples treated with concentrations as low as 0.5 ng/ml of bacterially secreted native *Y. pestis* LcrV. This assay could be used as a tool to help confirm the diagnosis of plague in patients presenting with pneumonitis.

There is currently a major gap in our diagnostic capabilities with regard to plague. Direct visualization of the organism in clinical specimens is insensitive and nonspecific, and culture is too slow (3). A sensitive and specific antigen capture assay that detects the F1 capsular antigen has been developed (4). However, F1 is not required for virulence (9) and, although rare, F1-negative *Yersinia pestis* strains have been isolated from a number of different host species and from a human infection (1, 3a, 22). To overcome the F1 assay, a fully virulent *Y. pestis* strain lacking the F1 capsular antigen could be used to evade detection. Therefore, an F1 assay cannot be depended on in case of a biological attack.

We selected the LcrV antigen as a target for antigen capture. LcrV is a 37-kDa protein secreted by the type III secretion system (20) that, like F1, is expressed at body temperature and, unlike F1, is a key virulence factor (7) (14). In addition, LcrV is expressed on the *Y. pestis* cell surface before the establishment of bacteria target cell contact (14).

DNA sequencing of the LcrV antigen revealed that two evolutionarily distinct types of V antigen exist in *Yersinia* spp. One type is expressed by *Y. enterocolitica* serotype O:8 (designated LcrV-YenO8 or V-O:8), and the other type is expressed by *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* serotypes O:3, O:9, and O:5,27 (LcrV-YenO8 or V-O:8), and the other type is expressed with concentrations as low as 0.5 ng/ml of bacterially secreted native *Y. pestis* LcrV. This assay could be used as a tool to help confirm the diagnosis of plague in patients presenting with pneumonitis.

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**MATERIALS AND METHODS**

**Cloning.** (i) YPIII(pCD1). YPIII(pCD1) was constructed using P1 phage transduction to introduce pCD1 marked with Tn5 into YPIII as described previously (23).

(ii) pET28a-LcrV. The LcrV gene was PCR amplified from *Y. pseudotuberculosis* YPIII(pBI1) with a 5′ NdeI primer (GGATCCCATATGATTAGAGCC TACGAACAAAACC) and a 3′ EcoRI primer (GAATTCATTTACCAGACG TGTCATCTAGC) and was cloned into the Ndel-EcoRI sites of pET28a-His vector. *Escherichia coli* DH5α was transformed with the plasmid, the antibiotic-resistant colonies were isolated in TBY medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 1 g of glucose per liter) supplemented with 30 μg of kanamycin/ml, and purified DNA was characterized via restriction pattern analysis before being sequenced (Stony Brook University, Stony Brook, N.Y.).

**LcrV protein purification.** *E. coli* strain BL21(DE3) was transformed with the plasmid encoding the target gene and grown in 1,000 ml of TBY medium (5 g of NaCl, 10 g of tryptone, 5 g of yeast extract, 25 μg of chloramphenicol, and 30 μg of kanamycin/liter) at 37°C in a shaking incubator. When the optical density at 600 nm (OD600) reached 0.4 to 0.6, protein expression was induced by adding IPTG (isopropyl-β-d-thiogalactospyranoside) to a final concentration of 1.0 mM, and the cells were grown for another 3 h. The cultures were harvested by centrifugation in 160-ml volumes at 5,000 × g for 5 min at 4°C. The cell pellets were stored at −20°C until use. Once thawed, the cell pellets were incubated at room temperature with 3.2 ml of CellLyte B-II (Sigma) and DNase I (5.0 μg/ml) for 15 min. Cellular debris was pelleted by centrifugation at 25,000 × g for 15 min at 4°C, and the combined supernatant “crude extract” was used for further purification of the recombinant protein. The crude extract was applied directly to a chromatography column containing 75 ml of His-Select HC nickel affinity gel (Sigma) previously equilibrated with buffer (50 mM NaPO4 [pH 8.0] and 0.3 M NaCl) at a flow rate of about 1.0 ml/min. The column was then washed with...
buffer (50 mM NaPO4 [pH 8.0], 0.3 M NaCl, and 250 mM imidazole) and then recombinant protein was eluted off the column in approximately 80 ml of elution from the column was stable and near that of the equilibration buffer. The

**P. aeruginosa**

**Y. enterocolitica**

**Y. pestis**

**YPIII(pIB1)**

**Y. pseudotuberculosis**

additional volumes of equilibration buffer until the A$_{280}$ of the material eluting from the column was stable and near that of the equilibration buffer. The recombinant protein was eluted off the column in approximately 80 ml of elution buffer (50 mM NaPO4 [pH 8.0], 0.3 M NaCl, and 250 mM imidazole) and then concentrated to 10 ml in a stirred ultrafiltration cell using a molecular size cutoff of 30 kDa (Amicon). The concentration was determined by Bradford protein assay (Bio-Rad), and the protein stored at $-70^\circ$C.

**Generation of MAbs.** Monoclonal antibodies (MAbs) were generated against rLcrV by using a protocol adapted from Leah et al. (11), Rathjen and Geczy (16), and Sugasawara et al. (17).

**Purification of MAbs.** Purification of MAbs was accomplished by affinity chromatography on 1-ml columns of protein G-agarose (Sigma) in Econocolumns (1 by 10 cm; Bio-Rad) as described previously (6). Typically, 500 ml of serum-containing supernatant was diluted with 1/10 volume 1 M Tris–Cl (pH 8.0). The sample was run through the column at a flow rate of 0.8 ml/min controlled by Peristaltic Pump P-1 (Pharmacia LKB), followed by washes of 10 column volumes with 0.1 M Tris (pH 8.0) and 10 column volumes of 10 mM Tris (pH 8.0) at a flow rate of 0.8 ml/min. Antibody was eluted with 0.5-ml fractions of 50 mM glycine (pH 3.0) into a 1/10 volume of 1 M Tris (pH 8.0). Antibody-containing fractions were identified by determining the OD$_{280}$, confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and then pooled and dialyzed against phosphate-buffered saline (PBS) with two changes of buffer overnight at 4°C. The protein yield was determined by a Bradford protein assay (Bio-Rad) and typically ranged from 2 to 10 mg per 500-ml culture.

**Biotinylation of MAbs.** Anti-LcrV MAb 40.1 was biotinylated by using an EZ-Link Sulfo-NHS-Biotinylation kit (Pierce), according to the manufacturer’s instructions. Briefly, 2.5 mg of MAb 40.1 in PBS was diluted into BupH PBS (Pierce), and 0.17 mg Sulfo-NHS-biotin was added. The reaction was incubated at room temperature for 30 min. A 1-ml desalting column was used to pre-equilibrated with 30 ml of PBS. The sample was then applied to the column. Eluted fractions of 0.5 ml were collected after application of 0.5-ml aliquots of PBS. Eluted protein was detected by determining the OD$_{280}$ and the presence of antibody was confirmed by SDS-PAGE. Antibody-containing fractions were combined and stored at 4°C.

**LcrV treatment of human sputum and blood.** Purified rLcrV standards were prepared by serial dilution of the stock to 2,000, 200, 20, 2, 0.5, or 0.1 ng/ml and added to fresh human sputum and blood. The volume of extraction buffer PBS-TS (PBS plus 4% Tween 20 for sputum) or PBS-TB (PBS plus 1.25% Tween 20 for blood) was added, and these components were mixed until there was complete dissolution of the sample. Sputum samples were vortexed vigorously after the addition of the extraction buffer. In addition, we tested YPIII(pCD1) culture supernatant containing Y. pestis–secreted native LcrV (sLcrV). A fresh YPIII(pCD1) culture was induced for LcrV secretion under low-calcium conditions (Luria-Bertani medium containing 0.02 M sodium oxalate and 0.02 M MgCl$_2$) for 3 h at 37°C. Induced culture was mixed with human sample at 1:2 and diluted to 1:100, 1:200, 1:400, 1:800, 1:1,600, and 1:3,200. Untreated human samples were used as negative controls. To determine the approximate concentration of secreted LcrV in culture, the induced YPIII(pCD1) culture was compared to a bovine serum albumin (BSA) standard by SDS-PAGE and stained with Coomassie blue. Then, 10 ml of YPIII(pCD1) culture was induced for secretion of LcrV under low-calcium conditions as described above and centrifuged, and 2 ml of the supernatant was subjected to SDS-PAGE against a BSA standard composed of 1,000, 200, or 100 ng of protein.

**Determination of the minimum number of cells necessary to produce a positive result on LcrV capture ELISA.** YPIII(pCD1)-induced culture was diluted in sputum at 1:2, 1:100, 1:200, 1:400, 1:800, 1:1,600, and 1:3,200 and incubated at 37°C for 3 h to allow induction of the type III secretion system, and then 1/10 of the sample was plated in Luria-Bertani plates. Plates were incubated at 37°C, and colonies were counted the following afternoon. In parallel, 4% Tween 20 was

**TABLE 1. Bacterial strains used in this study**

| Strain        | Description                                           | Source or reference          |
|---------------|-------------------------------------------------------|------------------------------|
| YPIII(pCD1)   | *Y. pseudotuberculosis* expressing *Y. pestis* LcrV serotype O:3 | J. B. Bliska (this study)    |
| YPIII(pIB1)   | *Y. pseudotuberculosis*                                | 2                            |
| Y. pestis KIM10+ | *Y. pestis* lacking LcrV                               | 13                           |
| Y. enterocolitica 8081 | *Y. enterocolitica*, LcrV serotype O:8              | 15                           |
| P. aeruginosa  |                                                       | ATCC 27853*                  |
| B. cerasus     |                                                       | Stony Brook University*      |
| F. tularensis LVS |                                                        | ATCC 29684**                 |

* *, strains kindly provided by George Tortora, Stony Brook Hospital, Stony Brook, N.Y. **, strain generously provided by K. Elkins, Center for Biologies Evaluation and Research, Food and Drug Administration, Rockville, Md.

**FIG. 1.** Analysis of the MAbs used to design the LcrV capture ELISA. (A) MAbs obtained against LcrV were analyzed by Western blotting or dot blotting. For Western blotting, approximately 2 μg of rLcrV and 10 ng of native LcrV (sLcrV, secreted from YPIII(pCD1)) were subjected to SDS-PAGE. For dot blotting, approximately 10 μg of rLcrV and 30 ng of sLcrV from YPIII(pCD1) culture were placed on a polyvinylidene difluoride membrane. Hybridoma supernatant was used as the primary antibody, and the secondary antibody was alkaline phosphatase-labeled anti-mouse IgG. (B) Design of the LcrV capture ELISA.
added to the rest of the sample, which was then vigorously mixed and incubated at 37°C for 15 min; then, 100 μl of each sample was tested in quadruplicate against a rLcrV standard curve. Two independent experiments were performed.

**LcrV capture ELISA.** Hybridoma supernatants diluted 1:2 or purified MAb (MAb19.31) diluted at a concentration of 2 μg/ml in filter-sterilized ELISA coating buffer (15 mM Na2CO3, 35 mM NaHCO3 [pH 9.6]) was applied to a 96-well Maxisorp U-bottom immunoplate (Nunc) at 0.1 ml per well and then incubated for 1 h at 37°C. The coated plate was blocked with PBS containing 0.05% Tween 20 and 1% BSA (Fraction V; Sigma), referred to here as the "sample diluent," at 0.2 ml per well for 1 h at room temperature. Recombinant or native secreted LcrV was added to the plate at 0.1 ml/well in sample diluent, followed by incubation for 1 h at 37°C. The plate was washed twice with filter-sterilized PBS containing 0.05% Tween 20 (PBST) and five times with distilled H2O. Biotinylated anti-LcrV MAb40.1 was added at a concentration of 2.5 to 5 μg/ml in sample diluent at 0.1 ml/well, incubated for 1 h at 37°C, and washed as described above. Neutravidin-alkaline phosphatase conjugate (Pierce) was diluted to 1:1,000 in sample diluent, added at 0.1 ml/well, incubated at 37°C for 30 min, and washed as described above. The assay was developed with 50 μl of PNPP substrate (Sigma) added to each well. The plate was read at OD405 in a Spectramax microplate reader (Dynatech). A positive value was counted as one, with an OD value superior to three standard deviations above the average of the negative control. For determination of the detection limit of the assay and specificity, albumin was used as the negative control. To further validate the sensitivity of the assay the relative percent value (X) was calculated as follows: (sample OD – blank OD)/(positive control OD – negative control OD) × 100. We considered positive values to be those that deviated plus or minus 50 points from 100.

**Statistical analysis.** The standard deviation for each datum point was calculated and plotted through plus and minus error bars. Three or more replicates were performed for each sample, and experiments were repeated at least twice.

**Protein extracts.** *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Francisella tularensis* cultures were extracted by using 200 μl of CelLytic B-II (Sigma) according to the manufacturer’s instructions.

**Bacterial strains.** The strains used in this study are listed in Table 1.

**RESULTS**

MAbs and design of the LcrV capture ELISA. *Y. pseudotuberculosis* LcrV was expressed as a His-tagged fusion protein and purified on a nickel affinity column. Protein purity was checked by SDS-PAGE, and the reactivity of rLcrV was checked by Western blotting against rabbit anti-LcrV polyclonal antibody. Several MAbs were identified against purified rLcrV (Fig. 1A). Ten 96-well plates were seeded, and about every well had a clone. Of these, 72 hybridomas with a strong immunoglobulin G (IgG) response to rLcrV were transferred; 29 of these were expanded. At this point, *Y. pestis* LcrV native protein secreted by the YPIII(pCD1) strain (sLcrV) was used...
to screen MAbs. Only hybridomas positive against *Y. pestis* sLcrV were selected for expansion.

To develop the LcrV capture ELISA, two MAbs were required, each with different epitope specificities. First, anti-LcrV MAb 40.1 was identified by its ability to strongly detect LcrV in ELISA by using sLcrV as the coated antigen. This MAb was concentrated, biotinylated, and used to identify an MAb to be used to capture the antigen.

We screened for capture MAbs by diluting hybridoma supernatants 1:2 in coating buffer and then coating the antibodies onto an ELISA microtiter plate. sLcrV was then applied for capture, and captured antigen was detected by using biotinylated MAb 40.1, followed by Neutravidin AP. By this technique, we identified MAb 19.31 as having the ability to capture sLcrV and yet it did not interfere with the binding by MAb 40.1, suggesting that these two MAbs recognize different epitopes on the sLcrV protein (Fig. 1B).

**Determination of the detection limit of the assay.** We examined the detection limit of the LcrV capture ELISA by coating a microtiter plate with MAb 19.31 hybridoma supernatant di-

![Graph](image)

**FIG. 3.** Detection of rLcrV from human sputum (A) and blood (B) treated samples by capture ELISA. Two independent experiments (experiment I in red and experiment II in black) and the respective rLcrV standards for each test are represented. The rLcrV standard was prepared with the same concentrations of protein used previously to treat the samples. The negative control was a human sample not treated. The averages of three OD₄₀₅ values are represented, and the standard deviations were determined.
TABLE 2. Relative percent values of detection of rLcrV from treated human samples

| rLcrV concn (ng/ml) | Sputum | Blood |
|---------------------|--------|-------|
| 2                   | 101 < X < 106 | 62 < X < 88 |
| 1                   | 86 < X < 116 | 89 < X < 98 |
| 0.5                 | 86 < X < 133 | 64 < X < 80 |
| 0.1                 | 956 < X < 197 | 69 < X < 414 |
| 0                   | X ≤ 100        | X ≤ 100        |

*The relative percent value (X) was calculated as described in the text for the OD readings plotted in Fig. 3A (sputum) and B (blood) to further validate the sensitivity of the assay. We considered positive values to be those that deviated plus or minus 50 points from 100.

Sensitivity of assay for detection of LcrV from human samples. A specimen pretreatment protocol was tested to verify its effectiveness in liberating *Y. pestis* LcrV from human sputum and blood. Human sample datum points were plotted against the rLcrV standard obtained for that ELISA plate. Three replicates were performed for each sample. Experiments have been repeated at least twice. We detected rLcrV from human sputum (Fig. 3A) and blood (Fig. 3B) with concentrations as low as 0.5 ng of purified protein/ml. Furthermore, 0.5 ng/ml was the lowest detectable concentration of rLcrV for which a positive relative percent value (X) could be obtained for both sputum and blood (Table 2).

To verify the approximate concentration of sLcrV in YPIII (pCD1) culture, 2 ml of the induced culture was subjected to TCA precipitation, and total protein was subjected to SDS-PAGE side by side with a BSA standard of 1,000, 200, or 100 ng. The gel was stained with Coomassie blue (Fig. 4). The sLcrV concentration observed was about 50 ng (~25 ng/ml), which is equivalent to the value determined by Fields et al. (7).

In addition, we tested human sputum and blood treated with induced YPIII(pCD1) culture supernatant containing *Y. pestis* LcrV (Fig. 5). For determination of sLcrV concentration in the same plate, a curve of the rLcrV standard was run on the same plate. These data are represented in the inset graph. The minimum amount of sLcrV detected from treated human blood and sputum was observed at 1:800, which is equivalent to 0.5 ng/ml on the standard curve. As expected, this is the same concentration detected from human samples treated with rLcrV. Extrapolating from the rLcrV standard curve, we estimate the sLcrV concentration per milliliter of induced YPIII (pCD1) culture to be ~400 ng/ml (if rLcrV OD~0.5 = ~1 ng/ml and sLcrV OD~0.5 = ~1:400, then 1 × 400 = 400 ng/ml).

Determination of the minimum number of cells necessary to produce a positive result on LcrV capture ELISA. The LcrV capture assay detected a positive signal from YPIII(pCD1)-induced culture diluted in sputum at 1:800. The number of *Yersinia* cells counted on the 1:800 plate was 5 × 10^5 cells/ml. A similar number was obtained when we plated an induced *Yersinia* culture diluted 1:800 in PBS (~10^6 cells/ml). We conclude that there are no inhibitory substances in sputum that block or diminish detection of this antigen by LcrV capture ELISA.

Determination of specificity. Specificity was tested with culture medium from an LcrV-negative strain of *Y. pestis* (strain KIM10+) and culture medium from *Y. enterocolitica* secreted LcrV (strain 8081, serotype O:8). To check for cross-reactivity with PcrV, a protein extract from *P. aeruginosa* was also tested. In addition, since the clinical presentation of pneumonic plague can be mistaken as tularemia or anthrax pneumonic infection, a differential diagnosis with these two pathogens is required. A protein extract of the vaccine strain of *F. tularensis* (LVS) and a protein extract and a solution of spores of *B. cereus* were tested. We tested *B. cereus* instead of *B. anthracis* because tight regulations make it virtually impossible to obtain even a protein extract of *B. anthracis*. We chose *B. cereus* as the test organism since *B. anthracis*, *B. thuringiensis*, and *B. cereus* belong to the *B. cereus* group. Helgason et al. proposed that they are, in fact, one species based on genetic evidence (10). Because they all share common antigens, this is a good test of specificity.

The results are presented in Fig. 6. The LcrV capture ELISA developed detects LcrV strongly in protein extract and culture medium of *Y. pseudotuberculosis* expressing *Y. pestis* LcrV (positive control), less strongly in protein extract and culture medium of *Y. enterocolitica* (control for cross-reactivity with other *Yersinia* species), and not at all in protein extract or culture medium of *Y. pestis* KIM10+ (does not express LcrV), in protein extract or spores of *B. cereus*, and in protein extract of *F. tularensis* LVS. In addition, the LcrV capture ELISA does not cross-react with PerV, a closely related protein secreted by *P. aeruginosa*.

**DISCUSSION**

We developed an LcrV capture ELISA with a detection limit of 0.1 ng/ml of purified recombinant protein. LcrV has a molecular size of 37 kDa (37,000 g/mol), so 0.1 ng/ml is ~2 pM, which is probably close to the theoretical limit of sensitivity. The sensitivity of the assay for detection of LcrV from human...
samples was 0.5 ng/ml. The difference between the detection limit and the concentration detected from human samples suggests that there is some room for improvement of the sensitivity.

The most likely mode of attack with plague, anthrax, or tularemia would be by aerosol. If *Y. pestis* is used, a key in controlling the number of casualties is rapid identification of the organism, so that appropriate quarantine measures can be taken. Signs and symptoms of pneumonic plague can begin 1 day after infection (3a), and victims become vectors (8). Currently, the definitive laboratory diagnosis of plague is made by the culture of *Y. pestis*, along with appropriate microbiological
studies, such as microscopy of stained preparations, characteristics of growth in liquid and solid media, biochemical characterization, and lysis of cultures by specific bacteriophages. Additional methods include the detection of Y. pestis antigens with specific antibodies in direct fluorescence and rapid dipstick tests, or the detection of Y. pestis-specific DNA sequences by real-time PCR (1). Molecular detection assays can effectively identify Y. pestis and may be the ideal method to use. However, these methods are not as widely available as ELISA technology.

An excellent Y. pestis antigen capture assay was developed by the Institute Pasteur in Madagascar and established the utility of antigen capture in the diagnosis of plague. This assay is based on the detection of the F1 capsular antigen (4). When the assay was first developed, the F1 antigen was believed to be an important virulence factor. It is now clear that this is not the case. Fully virulent F1-negative strains of Y. pestis that can produce non-Y. pestis LcrV antigen exist (1), and lethal human pneumonic plague secondary to an F1-deficient isolate has been reported (9). F1-negative and F1-positive Y. pestis strains are equally deadly, opening up the possibility that an enemy could utilize a virulent F1-deficient strain of Y. pestis to evade detection by the existing F1 capture assay.

In the United States most hospital laboratories, including even small community hospitals, are able to perform ELISA. To help fill an important gap in the laboratory diagnosis of Y. pestis infection, we developed an LcrV capture ELISA with MAbs to both capture and detect this important virulence factor. This assay is designed as a tool to be used to help confirm the diagnosis of plague in patients presenting with clinical symptoms of pneumonia.

We chose LcrV as a capture target because it is an essential virulence factor (7), it is secreted (20), and it is expressed on the surface of Y. pestis at 37°C (14). This LcrV capture ELISA detects all three pathogenic Yersinia spp.: Y. pestis, Y. enterocolitica, and Y. pseudotuberculosis. The antibodies used in our assay are cross-reactive with both major LcrV serotypes (V-O:3 and V-O:8). Thus, rather than being a deterrent, the fact that the assay is not specific for Y. pestis is an advantage because it is more likely that it could detect modified organisms. The specificity of the assay comes from the clinical presentation of the patient. Y. pestis is the only Yersinia spp. that causes pneumonia. Thus, in cases of pneumonia a positive result from a sputum sample would indicate that the patient had been infected with Y. pestis (plague).

The LcrV concentration in human extracellular fluid has not been determined, but the concentration of secreted LcrV has been estimated to be about 20 ng/ml in vitro (7). In the present study, we estimate by using the LcrV capture assay the secreted LcrV concentration in induced Y. pestis infected human samples as low as 0.5 ng/ml. We developed a highly sensitive LcrV capture ELISA with a detection limit threshold of 0.1 ng/ml. It detected LcrV from human samples treated with concentrations as low as 0.5 ng/ml of bacterially secreted native Y. pestis LcrV. This assay fills an important void in our ability to detect Y. pestis in sputum samples. We plan to adapt this assay to a rapid lateral flow format.

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