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RESEARCH ARTICLE

Application of chromosomal DNA and protein targeting for the identification of *Yersinia pestis*

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Purpose: A comprehensive strategy was developed and validated for the identification of pathogens from closely related near neighbors using both chromosomal and protein biomarkers, with emphasis on distinguishing *Yersinia pestis* from the ancestral bacterium *Yersinia pseudotuberculosis*.

Experimental design: Computational analysis was used to discover chromosomal targets unique to *Y. pestis*. Locus identifier YPO1670 was selected for further validation and PCR was used to confirm that this biomarker was exclusively present in *Y. pestis* strains, while absent in other *Yersinia* species. RT-PCR and Western blot analyses were utilized to evaluate YPO1670 expression and MRM MS was performed to identify the YPO1670 protein within cell lysates.

Results: The described study validated that YPO1670 was exclusive to *Y. pestis*. PCR confirmed the locus to be unique to *Y. pestis*. The associated transcript and protein were produced throughout growth with the highest abundance occurring in stationary phase and MRM MS conclusively identified the YPO1670 protein in cell extracts.

Conclusions and clinical relevance: These findings validated YPO1670 as a reliable candidate biomarker for *Y. pestis* and that a dual DNA and protein targeting approach is feasible for the development of next-generation assays to accurately differentiate pathogens from near neighbors.

Keywords:
Chromosomal and protein biomarker / MRM MS / Pathogens / *Yersinia pestis*

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

The single chromosomes of *Yersinia pestis* and *Yersinia pseudotuberculosis* share 97% nucleotide identity and molecular studies strongly suggest that *Y. pestis* emerged from *Y. pseudotuberculosis* [1, 2]. *Yersinia pestis* strains generally harbor three plasmids while *Y. pseudotuberculosis* strains typically have one plasmid [3]. *Yersinia pseudotuberculosis* is a ubiquitous soil- and water-borne microbe capable of causing mesenteric adenitis and septicemia [4]. In contrast, *Y. pestis* is a blood-borne pathogen that can cause life-threatening bubonic and pulmonary plague that can be lethal within days if left untreated, meriting the concern that this pathogen may be used as a biological weapon [4–7]. Bubonic plague is a zoonotic disease that can be transmitted to humans by infected animals or insects and pulmonary plague can be transmitted from person to person via airborne infectious particles. Plague has been responsible for at least three pandemics and persists today with humans and animals being infected throughout the world [5, 8, 9]. Since *Y. pestis* is classified as a select agent, the presumptive identification of this pathogen prompts an investigation by public health authorities with corresponding assessment using molecular assays.

Current DNA-based molecular assays are dependable when used in routine clinical applications. However, misidentification has occurred when assays were challenged with mixtures of clinical and environmental samples. Increased specificity has resulted from the use of multiple

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Abbreviations: BHI, brain-heart infusion; **TSB**, tryptic soy broth

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genomic targets using PCR-based platforms to extend functionality and confirm identity of rare organisms. Due to the low prevalence of disease caused by *Y. pestis* in industrialized countries, even a highly specific test generates an unacceptable number of false-positive results. Further, differentiation methods that focus on plasmid targets are unreliable since plasmids can be eliminated. Therefore, next-generation assays for the identification of infectious organisms are expected to detect multiple signatures for improved specificity, increasing the need for species-specific chromosomal-derived targets.

Detection of protein mass patterns by MS has been used to characterize pathogenic and environmental microbes in which highly abundant proteins are predominantly represented in the peptide mass fingerprints, masking the detection of unique proteins in low abundance [10]. MALDI TOF biotyping in clinical microbiology laboratories has been reported to rapidly identify the bacterium directly from a single colony, but enrichment of the microbe is typically required [10]. Although MALDI-based biotyping is fast, a major disadvantage of this approach is the difficulty in deciphering the identity of the peaks within a differential pattern. Shotgun mass mapping utilizing LC MS/MS has also been exploited to define a set of conserved bacterial proteins [11, 12], but acquisition of the results requires several days. In comparison to these approaches, MRM MS provides both rapid and accurate identification of microbes by detecting species-specific biomarkers, following validation of potential targets derived from genomic or proteomic sequencing.

The goal of this project was to utilize a combinational approach targeting both chromosomal DNA and protein biomarkers using PCR and MRM MS, respectively, to reliably differentiate *Y. pestis* from closely related microbes such as *Y. pseudotuberculosis*. The study presented here validates a candidate *Y. pestis*-specific chromosomal and protein biomarker for clinical diagnostics and environmental research, as well as describes a comprehensive strategy that can be applied to accurately distinguish other pathogens from near neighbors that share considerable genomic content.

2 Materials and methods

2.1 *Yersinia* sp. strains and culture conditions

Yersinia pestis strains were transferred to the University of Nebraska Medical Center in Omaha following the requirements of the Select Agent Program as outlined in the Animal and Plant Health Inspection Service/Centers for Disease Control and Prevention (CDC) Form 2, Guidance Document for Request to Transfer Select Agents and Toxins [13]. Manipulation of viable culture material was performed by authorized individuals within a biosafety level 3 laboratory certified for select agent work by the United States Department of Health and Human Services using laboratory biosafety criteria as described by the National Institutes of Health and CDC [14]. Prior to removal of *Y. pestis* cell preparations, lysate, or nucleic acids from the biosafety level 3 facility, sterility was confirmed by plating 10% of the sample onto sheep blood agar or chocolate agar plates and incubating the plates for 3 days at 37°C with ambient air. *Yersinia* species examined in the inclusivity and exclusivity test panels to assess specificity are shown in Supporting Information Table S1. Confirmation of species identity was obtained by biochemical and PCR analyses. Culturing procedures are also described in the Supporting Information.

2.2 PCR and quantitative real-time PCR amplification of DNA target sequences

Isolation of *Yersinia* genomic DNA, as well as PCR and quantitative real-time PCR of the locus tag identifier YPO1670 was performed as described in the Supporting Information.

2.3 RNA isolation and RT-PCR

Culturing of *Y. pestis* and *Y. pseudotuberculosis* for RNA analysis and the methodology used for RNA isolation and RT-PCR is detailed in the Supporting Information.

2.4 Production of recombinant YPO1670 protein

The recombinant YPO1670 protein was constructed with a C-terminal hexahistidine tag using *Y. pestis* CO92 DNA as the template and pET-30 (Novagen, Madison, WI, USA) as the expression vector. Details of the cloning, expression, and purification procedures utilized are provided in the Supporting Information. After purification and dialysis, the recombinant YPO1670 protein was greater than 98% pure as determined by 12% (w/v) SDS-PAGE analysis. Aliquots of the purified recombinant protein were stored at 4°C.

2.5 Antibody production and Western blot analysis

Procedures used for antibody production and Western blot analysis are detailed in the Supporting Information.

2.6 Sample preparation for LTQ-Orbitrap MS and data analysis

To prepare *Yersinia* proteins for LTQ-Orbitrap MS analysis, the bacteria were cultured to stationary phase (22–24 h) in brain-heart infusion (BHI) broth (Supporting Information Fig. S1). Bacterial cells were then pelleted and resuspended in chilled lysis buffer comprised of 50 mM Tris (pH 7.5),

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5% (v/v) glycerol, 200 mM NaCl, 0.1 mM PMSF, 0.1 mM DTT, and EDTA-free protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). Cells were lysed in a BeadBeater (Biospec Products, Bartlesville, OK, USA) using a 50-mL chamber lined with ice and containing chilled 0.1-mm glass beads. The recovered cell lysates were centrifuged for 1 h at 45 000 × *g* and 4°C. To remove viable bacteria, cell extracts were filtered through glass fiber prefilters and sterile 0.45 μm acetate filters (Nalgene, Rochester, NY, USA). The protein concentration in the filtrates was quantified using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

To enrich for the endogenous YPO1670 protein, sterile *Y. pestis* supernatants were immunopurified using the IgG mAbs F340–2 (14A3) and F340–3 (17E3) obtained in this study. The antibody affinity column was prepared by crosslinking 5.5 mg of IgG to 1 mL of agarose beads. After loading the sterile supernatants onto the affinity column, the captured proteins were eluted with glycine buffer (pH 2.5) and fractionated by 12% (w/v) SDS-PAGE. The denaturing gel was then stained with CBB G-250 and the proteins in the size range of 36–38 kDa were excised. To prepare the proteins for MS analysis, the excised proteins were reduced, carbamidomethylated, and trypsin digested as previously described [15].

The LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA, USA) used for analysis of the *Y. pestis* proteins was equipped with an Eksigent nano-LC system. The salt-free, trypsin-digested protein samples were loaded on a PicoFrit C18 column-emitter (New Objective, Woburn, MA, USA) that was equilibrated with 0.1% formic acid. Peptides were eluted with a 60-min linear ACN gradient (0–60%) containing 0.1% formic acid and then electrosprayed into the LTQ-Orbitrap XL mass spectrometer. The LTQ-Orbitrap XL mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra (from m/z 375 to 1575) were acquired in the Orbitrap with resolution R = 100 000. The most intense ions (up to five, depending on signal intensity) were sequentially isolated for fragmentation in the linear ion trap using CID with normalized collision energy of 30% and a target value of 5000. Former target ions selected for MS/MS were dynamically excluded for 75 s. MS files were submitted to the UNiquant software pipeline for peak list generation [16]. Peptides and the associated proteins were identified in the open access International Protein Index peak list using the Mascot 2.1.04 software package (Matrix Science, London, UK) [17].

2.7 Sample preparation for mass spectrometric MRM analysis

Cell lysates of *Y. pestis* were prepared for MRM MS by growing the bacteria in BHI broth at 25°C and 225 rpm. The bacterial cells were pelleted after 22–24 h (OD_{600} = 5.5, Supporting Information Fig. S1) and then resuspended in chilled lysis buffer containing 50 mM Tris (pH 7.5), 5% (v/v) glycerol, 200 mM NaCl, 0.1 mM PMSF, 0.1 mM DTT, and EDTA-free protease inhibitors (Roche Applied Science). Lysis, centrifugation, and filtration to remove viable bacteria were performed as described above, except that a 15-mL chamber was used in the BeadBeater (Biospec Products) to lyse the cells. Proteins in the extracts were separated by 12% (w/v) SDS-PAGE and the presence of an antibody-reactive 37 kDa protein was confirmed by Western blot analysis. After CBB G-250 staining of the fractionated *Y. pestis* proteins, protein bands with a molecular mass of approximately 37 kDa were excised. The gel slices containing *Y. pestis* proteins in the 36–38 kDa range were reduced, carbamidomethylated, and then digested with trypsin as previously described [15].

The MRM method used to detect and confirm the presence of the YPO1670 protein was developed by performing in silico analysis to predict the resulting trypsin digested peptides and by utilizing LC-MS/MS of the trypsin digested recombinant protein. Theoretical peptide masses were calculated with the MS-Digest function in the Protein-Prospector program developed by the University of California, San Francisco and directed by Dr. Alma Burlingame (http://prospector.ucsf.edu/prospector/mshome.htm). For analysis of the digested recombinant protein, the LTQ-Orbitrap or Q-TRAP4000 hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) was used. The identified peptides and MS/MS spectra were then utilized to establish the MRM assay on the Q-TRAP 4000. Parent and daughter ion masses used for MRM analysis are listed in Table 1.

For the MRM assay, trypsin digested *Y. pestis* peptides derived from the gel band were separated on an Eksigent nanoflow LC system with a 75 μm diameter × 150 mm long C18 PepMap reversed-phase column (LC Packings, San Francisco, CA, USA). Elution of the peptides was achieved with a 90-min linear ACN gradient (2–60%) containing 0.1% formic acid at a flow rate of 0.3 μL/min. Peptides were then electrosprayed into the Q-TRAP 4000 that was set to operate in the MRM mode. MS source parameters were as follows: capillary voltage, 1.8 kV; source temperature, 75°C; and curtain gas flow rate, 20 L/h. Nitrogen was used as curtain gas and collision gas. Declustering, collision cell exit, and entrance potentials were set at 75, 15, and 10 V, respectively. The dwell time for each transition was 40 ms and the retention time window was set at 5 min for every transition. The collision energy and expected elution time for each precursor ion are listed in Table 1.

MS data were acquired with Analyst software version 1.5 (Applied Biosystems) and independent experiments were performed to validate reproducibility. The search engine Mascot (Matrix Science) was used to determine the MOWSE score for the mass fingerprint obtained. The MOWSE score is a statistical calculation that estimates the probability of a correct identification, whereby the higher the score, the higher the confidence level that the correct protein has been identified. More specifically, a MOWSE score greater than 67 has a 95% probability that the protein identification is correct.

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Table 1. MRM transitions for detecting hypothetical protein YPO1670 in the *Y. pestis* cell lysate

Peptide	Parent ion mass	Ion charge	Daughter ion mass	Ion charge	Collision energy (V)	Elution time (min)
TPPFDSSR	453.4	$y8^{2+}$	708.3	$y6^{1+}$	28	17
TPPFDSSR	453.4	$y8^{2+}$	611.3	$y5^{1+}$	28	17
NLYC(CAM)EIDTR[a)]	592.3	$y9^{2+}$	956.6	$y7^{1+}$	34	22
NLYC(CAM)EIDTR[a)]	592.3	$y9^{2+}$	793.3	$y6^{1+}$	34	22
MoxDIFDFIDK[b)]	580.3	$y9^{2+}$	784.4	$y6^{1+}$	34	48
MoxDIFDFIDK[b)]	580.3	$y9^{2+}$	522.4	$y4^{1+}$	34	48
GQYEPLEYPR	626.3	$y10^{2+}$	774.4	$y6^{1+}$	36	15
GQYEPLEYPR	626.3	$y10^{2+}$	1066.5	$y8^{1+}$	36	15
IKPLEYYYPDK	714.9	$y11^{2+}$	1187.6	$y9^{1+}$	40	30
IKPLEYYYPDK	714.9	$y11^{2+}$	977.2	$y7^{1+}$	40	30
IGLETESYHLFFQQGK	949.0	$y16^{2+}$	754.4	$y6^{1+}$	44	51
IGLETESYHLFFQQGK	949.0	$y16^{2+}$	892.4	$y15^{2+}$	44	51
LAVENHEDETADEIIQIVK	722.7	$y19^{3+}$	991.5	$y17^{2+}$	44	25
LAVENHEDETADEIIQIVK	722.7	$y19^{3+}$	957.6	$y8^{1+}$	44	25

a) Carbamidomethylation of cysteine (CAM) added a mass of 57 daltons.
b) Oxidation of methionine (Mox) added a mass of 16 daltons.

3 Results

3.1 Experimental design and optimization of culture conditions

The hypothesis tested in this study was that a combinational approach targeting both chromosomal DNA and protein biomarkers unique to *Y. pestis* would reliably differentiate this pathogen from closely related microbes such as *Y. pseudotuberculosis*. To be considered a reliable biomarker, the target must be stable and present throughout growth in all strains of the pathogenic species of interest. Therefore, to determine the impact of media, temperature, and the growth phase of the bacteria on the targeted gene products presence and abundance, experiments were performed using various conditions and media. Bacterial growth was assessed in tryptic soy broth (TSB), BHI, and RPMI 1640 with and without aeration at 25°C or 37°C, temperatures reflecting that of a flea or mammalian host, respectively.

Interestingly, *Y. pestis* grew in all three media, whereas *Y. pseudotuberculosis* only grew in TSB and BHI (Supporting Information Fig. S1). The highest cell densities obtained for both bacterial species grown in either TSB or BHI occurred when the cultures were aerated, with BHI providing fourfold higher cell densities for *Y. pestis* (maximum $OD_{600} = 5.5$) than TSB (maximum $OD_{600} = 1.3$) at 25°C. Calcium supplementation to TSB enhanced *Y. pestis* growth (maximum $OD_{600} = 2.0$) as was previously reported [18], however, growth was still threefold higher in BHI even without calcium supplementation. Conversely, *Y. pseudotuberculosis* grew to high cell densities in both TSB (maximum $OD_{600} = 10.4$ at 37°C and 6.7 at 25°C) and BHI (maximum $OD_{600} = 10.5$ at 25°C) regardless of whether calcium was added or not.

Using the culturing conditions described in the Supporting Information section, *Y. pestis* and *Y. pseudotuberculosis* entered stationary phase at 25°C in TSB after 8 and 32 h, respectively. At 37°C in TSB, *Y. pestis* and *Y. pseudotuberculosis* entered stationary phase after 4 and 10 h, respectively. When *Y. pestis* and *Y. pseudotuberculosis* were cultured in BHI at 25°C, both bacteria entered stationary phase after approximately 16–18 h. These results indicated that either TSB or BHI could be used to propagate *Y. pestis* and *Y. pseudotuberculosis*. However, when higher cell densities of *Y. pestis* were needed, BHI was the preferred medium.

3.2 In silico analysis of *Y. pestis*-specific genes

Computational analyses of chromosomal sequences available for *Y. pestis* and *Y. pseudotuberculosis* revealed genes unique to *Y. pestis*, one of which was identified as YPO1670 and was predicted to encode a protein with a molecular mass of 37 kDa. Mining of the NCBI nonredundant 2011 database using default parameters identified 45 records with different accession numbers and indicated that all amino acid sequences were 100% identical to YPO1670 from the different *Y. pestis* strains. Three different names were submitted for this protein that included a putative *L*-xylulose-5-phosphate 3-epimerase, an AP endonuclease family 2, and a conserved hypothetical protein.

Yersinia. pestis YPO1670 was chosen for further validation for several reasons. Homology-based in silico analyses suggested that this locus identifier encoded a 37 kDa cytosolic protein that was unique to this pathogen. Therefore, analysis of the endogenous and recombinant YPO167 protein would be presumably less problematic than studying a hydrophobic membrane protein. The predicted size and composition of the YPO1670 protein would provide a sufficient number of trypsin-digested peptides and therefore, conclusively identify *Y. pestis*. The nucleotide sequence and transcript associated with this locus were noted by others to be present in *Y. pestis*, but absent in *Y. pseudotuberculosis* [19, 20]. In addition, no other bacterial species contained this nucleotide

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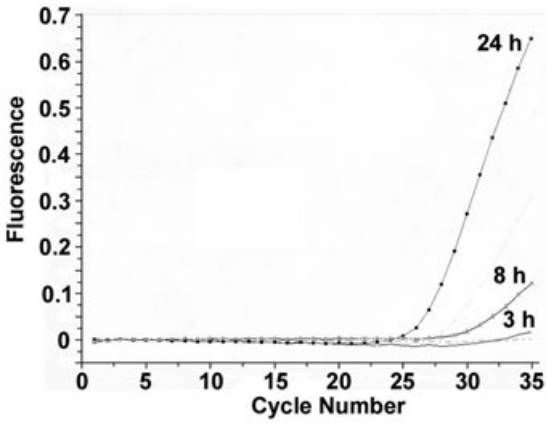

Figure 1. Representative RT-PCR showing differential expression of YPO1670 mRNA. *Yersinia pestis* RNA was analyzed using YPO1670-specific primers after 3, 8, and 24 h of growth at 37°C in tryptic soy broth.

sequence, supporting our hypothesis that YPO1670 and the products encoded by this locus would provide a *Y. pestis*-specific biomarker.

3.3 PCR amplification and transcription of *Y. pestis* YPO1670 locus

To confirm that the YPO1670 ORF was unique to *Y. pestis*, the presence of this DNA target in *Y. pestis*, *Y. pseudotuberculosis*, and other *Yersinia* species was assessed. Initially, primers specific to the YPO1670 locus were utilized in PCR amplification against an in-house collection of various *Yersinia* species that include *Y. enterocolitica, Y. frederiksenii, and Y. rohdei* (Supporting Information Table S1). Only the *Y. pestis* strains examined produced the expected 479 bp amplicon. For a more comprehensive analysis of the YPO1670 ORF, quantitative real-time PCR was used to evaluate a large test panel of genomic DNA extracted from numerous *Y. pseudotuberculosis* strains (Supporting Information Table S1). These data demonstrated that the chromosomal YPO1670 locus was unique to *Y. pestis* and absent in other *Yersinia* species, including *Y. pseudotuberculosis*.

Transcription of the YPO1670 ORF was evaluated by performing RT-PCR with RNA extracted from *Y. pestis* and *Y. pseudotuberculosis* grown in TSB after 3 h, 8 h, and 24 h at 37°C. As shown in Fig. 1, *Y. pestis* mRNA associated with the YPO1670 locus was observed at all three time points examined with the highest expression after 24 h of growth. In contrast, no transcription of this gene was detected in *Y. pseudotuberculosis* (data not shown). To verify that the RNA isolated from *Y. pseudotuberculosis* was of high integrity, RT-PCR was performed using primers for the *Y. pseudotuberculosis*-specific gene *mdoG*. These reactions resulted in the expected 62 bp product from extracted *Y. pseudotuberculosis* RNA, providing additional evidence that the YPO1670 ORF is absent in this species since the RNA isolated was of high quality. Together

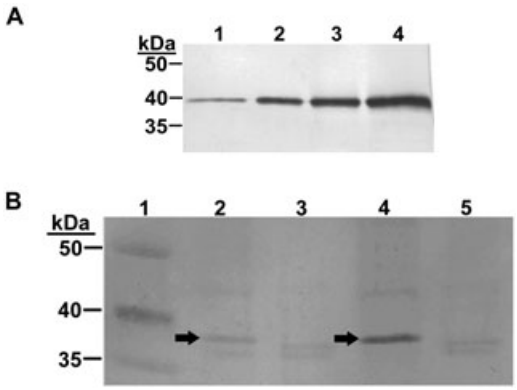

Figure 2. Western blot analysis of recombinant and endogenous *Y. pestis* YP1670. (A) Recombinant *Y. pestis* YP1670 at 5 ng (lane 1), 25 ng (lane 2), 50 ng (lane 3), and 100 ng (lane 4) was assessed by Western blot analysis using polyclonal antiserum. (B) Whole cell extracts from *Y. pestis* and *Y. pseudotuberculosis* grown in brain-heart infusion at 25°C were analyzed by Western blot analysis using YPO1670-specific mAbs. Molecular mass markers (lane 1), *Y. pestis* cultured for 2 h (lane 2), *Y. pseudotuberculosis* cultured for 2 h (lane 3), *Y. pestis* cultured for 24 h (lane 4), and *Y. pseudotuberculosis* cultured for 24 h are shown.

these data, as well as the DNA sequencing results obtained for the amplified products, confirmed that the YPO1670 locus is unique to *Y. pestis* and transcriptionally expressed. More importantly, these results demonstrated that the transcript associated with YPO1670 is present throughout growth and is most abundant during the stationary phase.

3.4 Western blot analysis detects *Y. pestis*-specific YPO1670 protein

Although the gene and transcript corresponding to the YPO1670 locus were exclusively detected in *Y. pestis*, Western blot analysis was needed to determine if and when production of the predicted 37 kDa protein occurred. To achieve this goal, polyclonal antisera and mAbs were generated against peptides and the recombinant protein derived from the predicted YPO1670 ORF sequence. Results demonstrated that both the polyclonal and YPO1670-specific mAbs had an LOD that was approximately 5 ng (Fig. 2A and data not shown). Western blot analyses using extracts of *Y. pestis* and *Y. pseudotuberculosis* grown in TSB or BHI at 25°C demonstrated that the 37 kDa YPO1670 protein was exclusively expressed by *Y. pestis* throughout the growth cycle with the highest level of production occurring during stationary phase (Fig. 2B, lanes 2 and 4), in agreement with transcript abundance (Fig. 1). Two faint bands at approximately 36 kDa were observed for *Y. pseudotuberculosis* that did not change in concentration throughout growth and during stationary phase, suggesting that the antibodies nonspecifically bound to these proteins (Fig. 2B, lanes 3 and 5). These findings supported the potential that the *Y. pestis* YPO1670 protein may serve as a unique biomarker

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Clinical Relevance

Current DNA-based molecular assays are dependable when used in routine clinical applications, but misidentification has occurred when assays were challenged with mixtures of clinical and environmental samples. Due to the low prevalence of disease caused by *Yersinia pestis* in industrialized countries, even a highly specific test generates an unacceptable number of false-positive results. Further, differentiation methods that focus on plasmid targets within pathogens are unreliable since plasmids can be eliminated. Therefore, next-generation assays for the identification of infectious organisms are needed to detect multiple signatures for improved specificity based on species-specific chromosomal-derived targets with protein products. The goal of this project was to utilize a combinational approach targeting both chromosomal DNA and protein biomarkers to reliably differentiate *Y. pestis* from closely related microbes such as *Y. pseudotuberculosis*. The study presented here validates a candidate chromosomal and protein biomarker specific to *Y. pestis* using PCR and MRM MS, respectively. This comprehensive strategy can be applied to accurately distinguish other pathogens from near neighbors that share considerable genomic content.

that is present throughout growth and during stationary phase.

3.5 MS detects and confirms the identity of the *Y. pestis* 37 kDa protein as YPO1670

Although Western blot analyses demonstrated reactivity with the antibodies developed against the *Y. pestis* 37 kDa protein target, the possibility remained that this was due to cross-reactivity. Confirmation that the antibodies were specifically binding to the YPO1670 protein was investigated by MS analysis of the immunoaffinity purified protein. Following 24 h growth of *Y. pestis* in conditions that produced the highest abundance of the 37 kDa protein based on Western blots analyses, cell lysates were subjected to immunoaffinity chromatography. The mAb-captured proteins were analyzed using the LTQ-Orbitrap MS instrument. Two independent experiments identified the same *Y. pestis* peptide (LAVENHEDETADEIIQIVK) using this MS method, which generated an unambiguous match to YPO1670. These results confirm that the 37 kDa protein detected and captured by the antibodies was the *Y. pestis* YPO1670 protein and therefore, this target protein is produced and unique to this pathogen.

A more sensitive proteomic-based methodology was needed to detect and identify the endogenous *Y. pestis*-specific YPO1670 protein when present in whole cell lysates at biological concentrations. The MRM MS methodology previously described was selected for this purpose [21]. This approach provides the ability to rapidly search for candidate peptides obtained from the mass fingerprints that match with the target of interest. Twenty-eight pairs of parent and daughter ions were included in the MRM method and 14 of these were detected using a Q-TRAP 4000 mass spectrometer. Table 1 lists the 14 MRM transitions that gave positive results for tryptic peptides of the YPO1670 protein and representative positive hits for MRM transitions are shown in Fig. 3A in which masses 580.3/522.4 and 580.3/784.4 were obtained. A third MRM transition with masses 580.3/637.4 was also acquired. A parent ion with an *m/z* equivalent to 580.3 eluted at 50.1 min; this parent ion had daughter ions with an *m/z* equal to 784.4 and 522.4. This MRM hit triggered acquisition of the representative fragmentation spectrum shown in Fig. 3B. The y-ion series from y2 at 262.2 to y8 at 1012.7 supported the premise that the target protein contained the

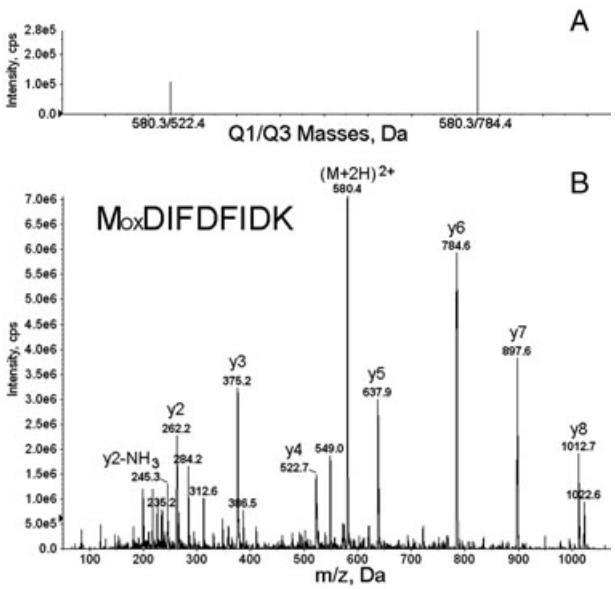

Figure 3. Representative MRM transition and MS/MS spectrum of a peptide detected in the trypsin digested cell lysate of *Y. pestis*. (A) MRM detected two parent/daughter ion pairs corresponding to theoretical masses for peptide MoxDIFDFIDK, where Mox indicates oxidized methionine. (B) The MS/MS spectrum of the doubly charged parent ion with an *m/z* equivalent to 580.4 yielded fragment ions whose masses support the sequence MoxDIFDFIDK, a peptide in the *Y. pestis* YPO1670 protein.

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  1 MKIGLETESY MLFFQQGKMD IFDFIDKAWS LGLDGVEINI IPDEGLHPEF
 51 GVLSSDDPTY LARVRHAIEQ RNLYCEIDTR FTSVKAISKA VQFASALGAD
101 VIRTYMFRRG QYEPLEYPRI IHELKSLVPL LQANRIRLAV ENMEDETADE
151 IIQIVKAVDS LWVGAHCDIG NGMMAWEEPV VTVSQLAPFT YSTHFKDHIV
201 TQMDEQLVVC GVPIGEGSID IDNCFRILVE KSGITRVNIE TCFPYASAFS
251 RPCNPNEVST LKGTFEVKTP PFDSSRIKPL EYYYPDKISP ELLQELMQAQ
301 DHCVYTSVQV LKALRDKYCP NTTTGIVE
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Figure 4. *Yersinia pestis* YPO1670 protein sequence. Peptides in bold letters were identified in the *Y. pestis* cell lysate by MS using MRM.

peptide sequence MoxDIFDFIDK, where Mox indicates oxidized methionine. As expected from the results shown in Fig. 3A, the y6 ion at 784.6 Da and the y4 ion at 522.7 Da were both present in Fig. 3B. The peak intensity of y6 was higher than that of y4, concurring with the relative peak heights of these ions shown in Fig. 3A. The MS/MS spectra for the six additional peptides identifying the YPO1670 protein and the MRM chromatographic profiles are shown in the Supporting Information section. Figure 4 shows the protein sequence for YPO1670 and the seven peptides identified by MRM MS.

Overall, MRM MS identified the target YPO1670 from a *Y. pestis* extract, confirming the ability to detect this unique protein at biological concentrations. Out of 328 amino acids, 82 were identified in seven peptides with a MOWSE score of 1101, indicating high confidence in the identity of the 37 kDa protein as YPO1670. Although two peptides are typically sufficient to confidently identify a protein using MRM MS, seven matching peptides were reproducibly obtained, covering 25% of the YPO1670 protein.

4 Discussion

The capability for acquisition of multidrug resistance in *Y. pestis* [22], and the ease at which this highly virulent pathogen can be transmitted by arthropods or infected mammals emphasizes the importance of this select agent pathogen. Since the prevalence of plague is low, even assays with excellent sensitivity and specificity will have a relatively high false-positive rate. Combinational approaches that utilize both DNA and protein targets into one assay may improve diagnostic accuracy. To achieve this goal, the temporal expression of targeted gene products and the stability of the differentiating biomarkers must be considered. *Yersinia pestis* presents a special problem since many DNA targets used in current assays are located on plasmids that may be lost or transferred to other bacterial species. Further, the difficulty in acquiring mAbs with high specificity to a target protein makes reliable immunological assays arduous and costly to develop. Therefore, in contrast to PCR- and antibody-based assays, which comprise the majority of the current methodologies, the use of MRM MS to identify species-specific protein biomarkers is less likely to incur cross-contamination issues and background problems, respectively.

A comparison of 59 *Y. pestis* and 39 *Y. pseudotuberculosis* genome sequences identified eight chromosomal loci specific to *Y. pestis*, one of which included the YPO1670 gene [23]. Each *Y. pestis*-specific loci was individually deleted and the resulting mutant strain was fed to fleas via a blood meal or used to infect mice [23]. The data obtained from these mutational analyses showed that none of these unique genes contributed to virulence by itself, since no differences were noted compared to wild-type *Y. pestis*. Carniel and associates proposed three possible explanations for their findings. (i) The deletion of several or multiple genes was needed to impair virulence, (ii) the extreme pathogenicity of this microbe in the mouse model obscured minor virulence defects, and/or (iii) these acquired loci may participate in the long-term persistence of this pathogen outside of an insect vector or mammalian host. However, for a more complete analysis of gene function, additional studies are needed to determine whether these *Y. pestis*-specific loci produce a protein product.

Although previous studies have identified proteins expressed by *Y. pestis*, no comparison was made to the proteome of *Y. pseudotuberculosis* [24–26]. Therefore, YPO1670 appears to be the first confirmed chromosomally encoded protein unique to *Y. pestis*. Identification of *Y. pestis*-specific chromosomally encoded proteins by a proteomic approach is difficult, since the target proteins may require special conditions for expression and/or are produced at low levels for a brief period of time. The studies described here provide evidence that the YPO1670 protein is expressed exclusively by *Y. pestis*, but not the closest relative *Y. pseudotuberculosis*. Further, the production of the *Y. pestis* YPO1670 protein throughout growth and especially during stationary phase supports the premise that this protein would serve as a reliable candidate biomarker for this pathogen. As a result of these studies, the hypothetical protein associated with the locus tag YPO1670 in the *Yersinia* database has been updated to be annotated as an expressed *Y. pestis* protein.

This study provided proof of concept that species-specific proteins can be used as biomarkers to distinguish closely related microbes within a mixture of other proteins. MRM MS proteomic profiling can also provide insight into the pathophysiological status of the host by the detection of disease-specific biomarkers, unique protein/peptide patterns, and/or protein modifications. MS/MS analysis of intact proteins has even identified misannotated sequences and suggested homology-based classification of microbes with no or limited sequence information [27]. We anticipate that the dual use of DNA and protein biomarkers has utility in the future for pathogen identification in both clinical and field applications.

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