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Development of real-time PCR assays for specific detection of *hmsH*, *hmsF*, *hmsR*, and *irp2* located within the 102-kb *pgm* locus of *Yersinia pestis*

Charla E. Gaddy [a], Pedro F. Cuevas [a], Laurie J. Hartman [a, c], Gerald B. Howe [a], Patricia L. Worsham [b], Timothy D. Minogue [a, *]

[a] Diagnostic Systems Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA
[b] Bacteriology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA
[c] ClinicalRM, Inc., Hinckley, OH, USA

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ABSTRACT

Virulent isolates of three pathogenic *Yersinia* species (*Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*) harbor a 102-kb chromosomal region which encodes elements critical for virulence. A 35-kb high pathogenicity island is contained in this region, is a known virulence determinant, contains *irp1* and *irp2* iron-regulating genes. An additional segment, the 68-kb high pathogenicity island, contains genetic elements responsible for conferring the *Y. pestis* pigmentation phenotype on Congo red agar at 28 °C. Collectively, these contiguous segments are referred to as the pigmentation (*pgm*) locus, the absence of which results in strain attenuation and exemption from CDC Select Agent status. In this study, we developed a set of four real-time PCR assays to detect the presence or absence of multiple virulence genes located within this region. Specifically, we designed TaqMan® PCR assays to individually detect three hemin storage genes (*hmsH*, *hmsF*, and *hmsR*) which are genetic elements that confer the pigmentation phenotype, as well as the iron-regulating status of 25 *Y. pestis* isolates (representing 23 different strains), thus establishing a molecular based assay capable of determining the *pgm* status of candidate *Y. pestis* isolates. Included in the validation process, was a comparison of these real-time PCR assays and newly developed conventional PCR assays targeting much larger areas of the 102-kb region (including one assay spanning *hmsR* and *hmsF*, one spanning *hmsH* and *hsmF*, one targeting *hmsF*, and one targeting *irp2*). There was high concordance between the conventional and real-time PCR assays for all *Y. pestis* strains tested. The results from the comparative analysis document the specificity and sensitivity of the real-time PCR assays and further solidify the ostensible benefits of real-time PCR over conventional PCR.

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1. Introduction

Yersiniae are members of the *Enterobacteriaceae* family, a group of Gram negative bacilli capable of causing varying degrees of morbidity and mortality. There are currently 11 identified *Yersinia* species, three of which (*Yersinia pestis, Yersinia pseudotuberculosis,* and *Yersinia enterocolitica*) are known to cause human disease. *Y. pestis* is the causative agent of bubonic, pneumonic, and septicemic plague and is transmitted to humans and animals via bites from flea reservoirs, infected rodents, and other small animals (ex. rabbits, chipmunks, cats, etc.), inhalation of aerosolized droplets, or by direct contact [1,2]. Naturally occurring transmission of *Y. pestis* has been responsible for three pandemics resulting in millions of deaths worldwide [3,4]. While the last pandemic began in excess of 150 years ago, animal to human transmission continues to pose a significant health risk to individuals. This is especially true for those located within plague endemic areas, such as those in sub-Saharan Africa and Asia [5–7].

Accidental inoculation of *Y. pestis* resulting in laboratory-acquired infections (LAIs) has been documented in both clinical and research settings [8–10]. In the clinical laboratory, adherence to conventional microbiological procedures for Biosafety Level 2 (BSL-2) environments minimizes the risk of transmission of most infectious agents to laboratory personnel to include *Y. pestis*.

* Corresponding author. USAMRIID, DSD, 1425 Porter Street, Fort Detrick, MD 21702, USA. Tel.: +1 301 619 6352; fax: +1 301 619 2492.
E-mail address: timothy.d.minogue.civ@mail.mil (T.D. Minogue).

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However, due to high aerosol transmissibility and low infectious dose required for inhalation exposure (100–500 organisms), the probability of LAIs increases dramatically when manipulating *Y. pestis* even under these conditions [11]. To mitigate this hazard, the Centers for Disease Control and Prevention (CDC) recommend BSL-3 containment and safety precautions for *Y. pestis* work [12].

Y. pestis is a Health and Human Services (HHS) and CDC Select Agent. As such, it is subject to the rules and regulations of the CDC Select Agent Program restricting agent possession, use, and transfer [13]. Two exceptions are noted: attenuated *Y. pestis* strains that do not contain the 102-kb chromosomal segment referred to as the *pgm* locus or those that lack the low calcium response (*Lcr*) virulence plasmid [14]. *Y. pestis* strains meeting these criteria are not considered select agents. CDC Select Agent Exclusion Rules and Regulations dictate the use of PCR and/or Southern blot analysis to demonstrate the absence of the *pgm* locus or the *Lcr* virulence plasmid when determining the exempt status of any given *Y. pestis* strain.

The *Y. pestis pgm* locus is a 102-kb chromosomal DNA segment responsible for its pigmented phenotype when grown on Congo red agar (CRA) at 28 °C [15]. Deletion of the *pgm* locus results in loss of the pigmentation phenotype, strain attenuation, and loss of the ability of the *Y. pestis* to cause disease via peripheral routes of infection in the absence of extraneously injected iron [15–18]. The *pgm* locus consists of two proximal regions: a ~68-kb region referred to as the 'pigmentation segment' and a ~35-kb high pathogenicity island (HPI). The pigmentation segment contains the hemin storage (*hms*) locus. This encompasses the *hmsHFRS* operon which contains genes that encode hmsH, hmsF, hmsR, and hmsS proteins, all of which facilitate hemin storage *in vivo* and the binding of Congo red *in vitro* resulting in the pigmented phenotype [15,19–24]. The HPI is proximal to the pigmentation segment and contains genetic elements, such as the yersiniabactin (*Ybt*) locus, *psn*, *irp1*, and *irp2*, all of which transcribe products that facilitate siderophore-dependent iron acquisition and sequestration, a process critical for *Y. pestis* virulence [15,16,21,23,25–30]. Genetic elements located within the HPI encode proteins, including YbtE, YbtS, YbtT, YbtU, HMWP1, and HMWP2, which are required for yersiniabactin biosynthesis, the siderophore required for iron acquisition in virulent *Y. pestis* infections [25,31–35]. HMWP1 and HMWP2 are transcribed from *irp1* and *irp2*, respectively, and both proteins are required for mouse virulence by most routes of infection [36–38].

Spontaneous deletion of the *pgm* locus *en bloc* or various sized deletions within both the pigmentation segment and the HPI has been previously described in *Y. pestis* mutants [16]. The finding of segmental deletion of some *pgm*-associated sequences facilitates the need to target multiple sites within this locus to confirm the presence or absence of the *pgm* locus in its entirety.

In accordance with CDC rules and regulations that dictate the entirety of the *pgm* locus be absent for exemption, we previously adopted and used a set of conventional PCR assays developed by Jenkins et al. as an integral part of our strategy to determine the *pgm* status of *Y. pestis* isolates in our laboratory [39]. However, because of the issues inherent to the use of conventional PCR (i.e. post-PCR analysis, low band resolution, and gel-to-gel variability), we sought to develop real-time PCR assays that indicated the full *pgm* status of *Y. pestis* isolates. Here, we report the development and optimization of 4 real-time TaqMan® PCR assays that target *hmsH*, *hmsF*, and *hmsR*, located within the pigmentation segment, and *irp2*, located within the HPI (Fig. 1). The collective presence or absence of these targets allows for rapid determination of the *pgm* status of candidate *Y. pestis* isolates with high confidence.

2. Materials and methods

2.1. Sample acquisition and nucleic acid preparation

All *Yersinia* isolates were obtained from the Critical Reagents Program (Frederick, MD) or the Unified Culture Collection (UCC) maintained at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID, Ft. Detrick, MD). All isolates were

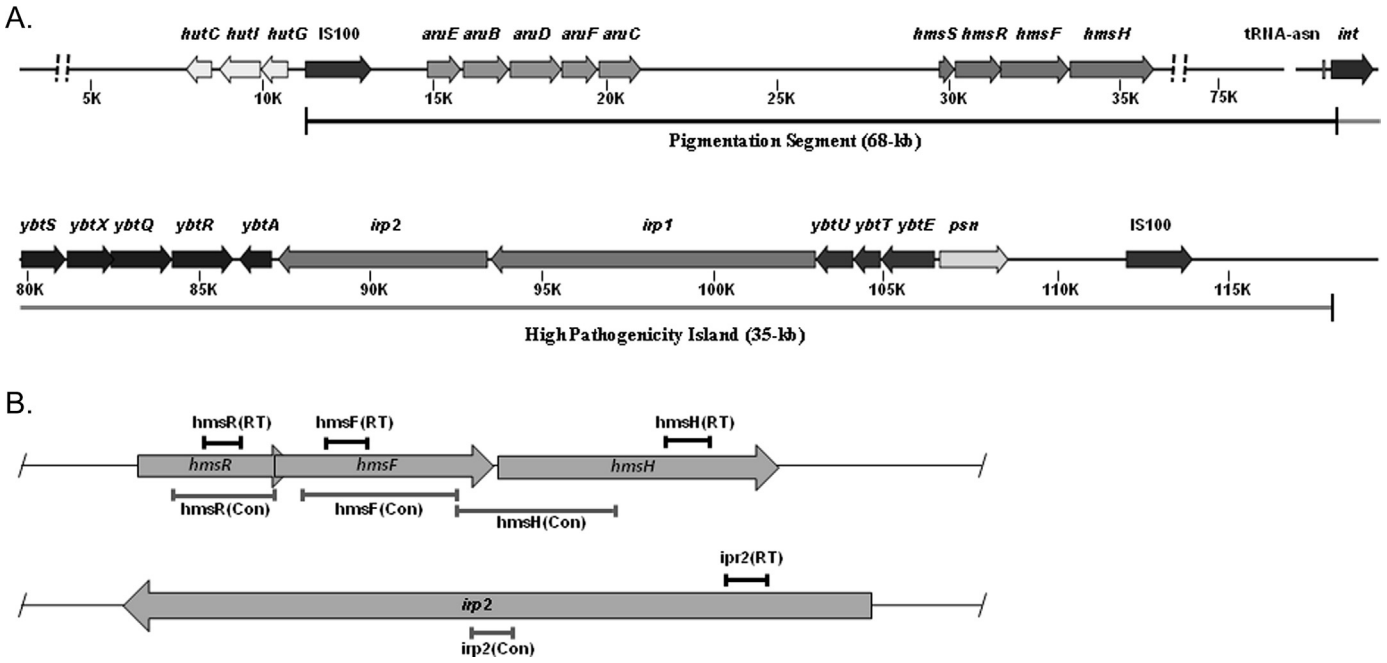

Fig. 1. Genetic organization of the *Y. pestis* 102-kb *pgm* locus. A. Schematic representation displaying major genes located within the 102-kb *pgm* locus including *hms*H, *hmsF*, *hmsR*, and *irp2*. B. Schematic representation displaying location of nucleic acid sequence targeted by conventional and real-time PCR primers. Dark gray bars located above the targeted genes represent real-time PCR amplification products. Light gray bars located below the targeted genes represent conventional PCR amplification products. RT, real-time PCR; Con, conventional PCR.