



Relative immunogenicity and protection potential of candidate *Yersinia Pestis* antigens against lethal mucosal plague challenge in Balb/C mice

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Summary *Yersinia Pestis* outer proteins, plasminogen activator protease and Yop secretion protein F are necessary for the full virulence of *Yersinia pestis* and have been proposed as potential protective antigens for vaccines against plague. In the current study, we used DNA immunization as a tool to study the relative protective immunity of these proteins with a standardized intranasal challenge system in mice. While the natural full-length gene sequences for most of these *Y. pestis* proteins did not display a good level of protein expression *in vitro* when delivered by a DNA vaccine vector, the overall immunogenicity of these wild type gene DNA vaccines was low in eliciting antigen-specific antibody responses and gene sequence modifications improved both of these parameters. However, even modified YopD, YopO and YscF antigens were only able to partially protect immunized mice at various levels against lethal challenge with *Y. pestis* KIM 1001 strain while no protection was observed with either the YopB or Pla antigens. These results demonstrate that DNA immunization is effective in screening, optimizing and comparing optimal antigen designs and immunogenicity of candidate antigens for the development of a subunit-based plague vaccine.

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Introduction

Plague is one of the most feared infectious diseases in humans. It has killed over 200 million humans throughout history and still remains endemic in certain areas of the world. The causative agent of plague, *Yersinia pestis* (*Y.*

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pestis), is also a concern for its potential use as an agent of biological warfare and biological terrorism. Although killed whole-cell vaccines are available for human use in certain countries, poor efficacy has limited their use for prevention of natural or human-inflicted outbreaks [1–3]. Up to this point, the effort to develop next generation plague vaccines has focused mainly on the use of two well-studied protective antigens, F1 and V (LcrV), in the form of subunit-based recombinant protein vaccines [4–8]. However, as shown in a recent study conducted in humans with subunit-based plague vaccines, the immunogenicity of the current vaccine composition is quite weak [9]. Therefore, the importance of identifying additional protective antigens is clear, especially for the highly virulent pneumonic plague.

Previous studies have shown that antibodies to some of the *Yersinia* outer proteins (Yops) are present in convalescence sera from patients infected by *Y. pestis* and in mice that survived experimental challenge [10,11]. Therefore, it was hypothesized that these proteins may serve as protective antigens. Along with F1 and LcrV, Yops and the plasminogen activator protease (Pla) are necessary for the full virulence of *Y. pestis* [12,13] and are encoded by the virulence plasmids [13–15]. Among the 12 secreted Yops, YopB and YopD are known as translocators in the Ysc-Yop type III secretion system involved in the translocation of the Yop effectors across the eukaryotic cell plasma membrane into the target cells [16,17]. The type III secretion apparatus of *Y. pestis* is a conserved mechanism to deliver virulent factors into mammalian host cells [15,18]. Both YopB and YopD are transmembrane proteins and interact with LcrV to form the pore of the Type III secretion apparatus through which the Yop effectors are translocated [13,19,20]. YopO/YpkA is a 81.7 kDa protein kinase with the ability to catalyze autophosphorylation on a serine residue, as shown by *in vitro* experiments, and is targeted to the inner surface of the plasma membrane inside the cells [21].

Recent reports have demonstrated that mice immunized with recombinant YscF, which is a surface localized protein, can produce YscF-specific antibody responses and partial protective immunity against *Y. pestis* by intravenous or subcutaneous challenge [22,23]. These particular results suggest that the antibody responses induced by YscF protein immunization are useful for protection against bubonic plague although the protection mechanism is not quite clear. There is no evidence whether YscF-specific antibody responses are protective for mucosal infection including the pneumonic plague.

Pla is an integral outer membrane protein and a surface protease. It belongs to the omptin family of enterobacterial surface proteins and derives its name from the fact that it can activate the mammalian plasma proenzyme plasminogen into plasmin [24–26]. The importance of Pla for bacterial virulence has been demonstrated *in vivo*. The LD50 for a Pla-negative mutant strain was close to 10^7 bacteria, as compared to less than 50 bacteria for the isogenic Pla-positive strain [12]. This protein is responsible for the highly efficient invasion of plague bacterium from the subcutaneous infection site into circulation [24]. Furthermore, recent data have shown that intranasal infection with a *Y. pestis* strain lacking Pla resulted in the development of a deadly plague infection after 7 days in only 50% of the infected mice when compared to a 100% fatal-

ity rate less than 4 days post-inoculation in the wild type strain indicating that Pla has a significant involvement in the fatal outcome following respiratory infection with *Y. pestis* [27].

The immunogenicity and potential for protection have not been well characterized for the Yop and Pla proteins. In one study using recombinant Yop proteins expressed in *Escherichia coli* (*E. coli*) and purified by the His-tag approach, the recombinant YopO protein significantly prolonged the mean survival time but did not increase the overall survival of mice that had received a subcutaneous challenge of a virulent, non-encapsulated isogenic variant of *Y. pestis* [28]. In this same study, YopD was also shown to be partially protective against death in mice challenged with the non-encapsulated strain but not the wild-type encapsulated *Y. pestis* [28]. No detailed data were provided regarding the antibody responses to individual Yop proteins. The authors also noted that several Yop proteins were denatured before their use as immunogens which may have contributed to the poor protection with Yop proteins.

In recent years, the DNA vaccine approach has been used as an effective tool to study the immunogenicity of candidate antigens against a variety of infectious agents due to its potential to express protective antigens *in vivo*. For example, DNA immunization was used to quickly confirm the Spike protein as the protective antigen against the Severe Acute Respiratory Syndrome – associated coronavirus (SARS-CoV) and to map the protective domains on this Spike protein [29,30]. DNA immunization was also used to effectively identify individual protective antigens in order to formulate subunit-based vaccines against smallpox infection [31]. In a previous study from our laboratory, we successfully established a mouse intranasal challenge model coupled with DNA immunization technology to test the protection efficacy of modified *Y. pestis* antigens against lethal plague challenge [32]. This system provided a standard assay process to screen and score the relative effectiveness of known or novel *Y. pestis* antigens.

In the current study, a molecular antigen engineering approach was used to improve the expression and immunogenicity of Yops, YscF and Pla proteins. Our previous studies, which used DNA vaccines against plague and other infectious agents, have demonstrated that a proper leader sequence is important in eliciting a high level of antibody responses and protective immunity [30,32,33]. Since *yopB*, *yopD* and *yopO* genes do not encode typical signal peptides, the tPA leader sequence was engineered with each of these *yop* genes individually to produce modified *yop* gene inserts for DNA immunization. In addition, certain intragene hydrophobic regions were removed to further improve the immunogenicity of Yop antigens. Our results demonstrate that antigen engineering was effective in improving the immunogenicity of the above Yop, Pla and YscF antigens and that these modified antigens in general induced higher antibody responses than the wild-type gene inserts. In addition, modified YopD, YopO and YscF proteins improved the level of protection. Overall, the protection efficacies with these *Y. pestis* proteins were still low and incomplete when compared to the known protective antigen LcrV, indicating that a search for additional protective antigens against *Y. pestis* is needed.

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