

## REVIEW

# Plague vaccines: current developments and future perspectives

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Despite many decades of intensive studies of *Yersinia pestis*, the causative agent of plague, there is no safe and efficient vaccine against this devastating disease. A recently developed F1/V subunit vaccine candidate, which relies mainly on humoral immunity, showed promising results in animal studies; however, its efficacy in humans still has to be carefully evaluated. In addition, those developing next-generation plague vaccines need to pay particular attention to the importance of eliciting cell-mediated immunity. In this review, we analyzed the current progress in developing subunit, DNA and live carrier platforms of delivery by bacterial and viral vectors, as well as approaches for controlled attenuation of virulent strains of *Y. pestis*.

*Emerging Microbes and Infections* (2012) 1, e36; doi:10.1038/emi.2012.34; published online 7 November 2012

**Keywords:** Plague; protective antigens; vaccine; *Yersinia pestis*

## INTRODUCTION

Plague is a devastating infectious disease caused by *Yersinia pestis* that by estimate has claimed about 200 millions of human lives throughout history.<sup>1</sup> It is a zoonotic disease that remains endemic in many parts of the world, exists in the form of natural reservoirs and causes periodic outbreaks in susceptible rodents.<sup>2</sup> Humans are accidental hosts, because they can contract the disease through bites from fleas containing *Y. pestis*, by contact with tissue and blood from infected animals, or more rarely by direct transmission through aerosol droplets. The latter type of transmission can result in a highly lethal pneumonic form of plague. The possibility of aerosolization of the agent, including human-to-human spread, is of particular concern in the case of naturally occurring epidemics or acts of bioterrorism and use of *Y. pestis* as a bioweapon.<sup>3</sup> There is no currently licensed plague vaccine in the Western world, and the countries of the former Soviet Union and China still use a live plague vaccine created in the 1920s to immunize plague workers and populations at risk.<sup>4</sup> If recognized early, plague is treatable with antibiotics, and there have been only two reports referring to the emergence of plasmid-mediated, antibiotic-resistant strains of *Y. pestis* in Madagascar that have not caused any disease.<sup>5,6</sup> Nevertheless, a recent study of *Y. pestis* isolates in Mongolia showed the existence of naturally occurring, multi-drug-resistant variants of the plague microbe.<sup>7</sup> Moreover, whole-genome sequencing of the *Y. pestis* organisms that caused the Black Death 14th century Europe revealed little difference between these and currently circulating strains.<sup>8</sup> Taking into account that the strains responsible for the Black Death likely originated in China in the regions near Mongolia,<sup>9</sup> we certainly need to develop a prophylactic option in the event highly virulent and antibiotic-resistant *Y. pestis* strains suddenly

emerge. In this respect, creation of an efficient and safe plague vaccine could be considered an immediate priority.

## BRIEF HISTORY OF PLAGUE VACCINES (KILLED WHOLE-CELL (KWC) AND LIVE WHOLE-CELL (LWC))

The search for vaccines to prevent plague began in 1895 when French scientist Alexandre Yersin tested plague immunity in laboratory animals (rabbits, mice, rats) after repeated immunization with either whole-cell, heat-killed, and agar-grown cultures of *Y. pestis* or live *Y. pestis* strains which had lost their virulence after multiple *in vitro* passages.<sup>10</sup> This study prompted the development of two types of plague vaccines, namely, KWC or LWC vaccines derived from virulent *Y. pestis* strains. The KWC preparations contained microbial cells inactivated by controlled procedures, such as heating or addition of different disinfectants. These vaccines contained no live pathogen, were unconditionally safe, and produced immunity in animal models to bubonic, but not pneumonic, plague after a single injection.<sup>11</sup> Human immunization with the KWC vaccine, such as plague vaccine USP (Cutter Biological Inc., Berkeley, CA, USA) during the Vietnam War indirectly confirmed the efficiency of this vaccine.<sup>12</sup> However, multiple and long-lasting immunization schedules (from months to years) were needed to induce plague immunity in humans with the KWC vaccines. Live attenuated variants of *Y. pestis* were obtained from wild-type isolates by continuous passage on routine bacteriological media at an ambient temperature and consequently used as the LWC plague vaccines. These vaccines were found to elicit the prompt (over several days) development of plague immunity against both bubonic and pneumonic forms of plague that correlated with the ability of bacteria to colonize and temporarily proliferate in tissues

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Received 3 July 2012; revised 12 September 2012; accepted 13 September 2012

and organs of the mammalian host. Therefore, the use of the LWC plague vaccine was generally associated with a risk of the development of an uncontrolled infectious process due to the existence of residual virulence. Hence, fatal cases of plague have been seen in small-animal models and non-human primates following administration of live vaccines.<sup>12,13</sup> Nevertheless, decades of use of live plague vaccines in humans did not result in any reported vaccine-related casualties, although several millions were vaccinated with the LWC by the middle of the twentieth century.<sup>14</sup> Currently, LWC plague vaccine is licensed for human use in the countries of the former Soviet Union and China.<sup>4</sup> The vaccine used for human immunization in these countries is the derivative of the EV76 attenuated strain of Girard and Robic.<sup>10</sup> The major reason for attenuation is the loss of the unstable 102 kb pigmentation locus (Pgm) containing the Ybt (yersiniabactin) iron acquisition system.<sup>15,16</sup> Other live plague vaccine candidates selected from virulent *Y. pestis* strains of different origins (some of them tested in humans) were described in our recent review.<sup>17</sup> Although the efficacy of both KWC and LWC human vaccines has been proven by the decades of their use, a number of local and systemic, vaccine-related side effects were observed in vaccinees, such as a strong pain at the site of injection, swelling, local erythema, regional lymphadenopathy, malaise, headache, giddiness, anorexia, weakness and mild fever with an elevated body temperature of up to 38.5–39.5 °C. Both vaccines require annual boosting, prompting the development of less reactogenic, and more safe and efficient vaccines for plague.<sup>4</sup>

#### CURRENT STRATEGIES FOR VACCINE DEVELOPMENT

Progress in recombinant technology has made major protective antigens of *Y. pestis* available in a highly pure form to allow an evaluation of the vaccine potential of the subunit and other types of next-generation vaccines. Importantly, these vaccines eliminated risk factors associated with the use of live vaccine and significantly reduced undesirable side effects linked to the massive administration of a mixture of numerous antigens existing in whole-cell killed vaccines. Nevertheless, intensive clinical trials are necessary to prove that these vaccines are safer and can provide better immunity than KWC and especially LWC vaccine.

#### Recombinant subunit vaccines in small animal models

Two antigens, namely, capsular subunit protein F1 and the low-calcium response V antigen (LcrV) were proven to be best in eliciting protection against plague in different animal models. The recombinant F1 demonstrated similar levels of protection against either subcutaneous or aerosol challenge of mice with the wild-type *Y. pestis* CO92 in comparison with that of F1 extracted from the plague bacterium.<sup>18</sup> The recombinant V antigen as a fusion of LcrV of *Y. pseudotuberculosis* with the immunoglobulin G (IgG)-binding domain of staphylococcal protein A provided both passive and active protection against intravenous challenge with the attenuated Pgm<sup>-</sup> strain of *Y. pestis* KIM.<sup>19,20</sup> A highly purified LcrV antigen expressed as a poly-histidine fusion peptide provided an increased level of protection.<sup>21</sup> The combination of these two antigens provided stronger protection than that elicited with each antigen alone, as well as that induced by killed and live plague vaccines. Moreover, analysis of sera for the IgG subclasses revealed the prevalence of IgG1 over IgG2a directed against both F1 and LcrV.<sup>22</sup> In the past decade, two major formulations of subunit vaccines consisting of F1 and LcrV antigens were developed by such methods as mixing both purified antigens (F1+V) or fusing them together (F1-V). To immunize animals, alhydrogel was most often the adjuvant of choice, although poly-L-lactide microspheres, MPL,

CpG, TiterMax, flagellin, and so forth, were tested as well. Typically, outbred Swiss-Webster or inbred BALB/c and C57BL/6 mice were used to evaluate protection, although other small animal species such as brown Norway rats, Hartley guinea pigs, and New Zealand white rabbits, were used as animal models. Intramuscular (i.m.) and subcutaneous route immunization were the most popular. A table listing vaccine formulation, type of adjuvant, animal model, route of immunization and challenge with *Y. pestis* was provided in our recent review on this subject.<sup>17</sup> The results of the testing of the F1/V subunit vaccine were described in several reviews<sup>4,17,22–28</sup> that can be summarized in the following statements: (i) the F1/V vaccine provided strong and long-lasting (over one year in mice) protection in small animal species against challenge with several fully virulent strains of *Y. pestis* administered by the most commonly used routes, such as subcutaneous, intranasal and aerosol; (ii) protection was mediated mostly by humoral immunity, with antibodies to LcrV playing a major role. Usually the addition of F1 improved protection, although, in some studies, the contribution of F1 was negligible; (iii) in many cases, the level of protection correlated with the titers of antigen-specific IgG1. However, generally, the protection was due to a mixed Th1/Th2 immune response; (iv) protection provided by anti-LcrV antibodies relied mostly on blocking the type 3 secretion system (T3SS). In this respect, it was possible to disarm unwanted immunomodulating activity of LcrV,<sup>29–31</sup> and (v) both F1+LcrV and F1-V vaccine candidates are in phase II of clinical trials.

Non-encapsulated strains of *Y. pestis* are still substantially virulent,<sup>4,17</sup> and there is a polymorphism among *lcrV* gene in *Y. pestis*.<sup>32,33</sup> Thus, the addition of other protective antigens to the F1/V subunit vaccine is highly desirable. Among antigens tested in protection studies, such as Pla, LPS, PsaA, YadBC, YscF, YpkA, YopH, YopE, YopK, YopM, YopN, YopD and YscC, limited success was achieved with the polymer subunit of T3SS needle YscF, and the T3SS translocator protein YopD. The latter was efficient mainly in protection against capsule-negative variants of *Y. pestis*.<sup>17</sup>

#### Subunit vaccines in non-human primates

The cynomolgus macaque pneumonic plague model is considered standard for testing the development of plague vaccines and therapeutics. In this model, the F1/V subunit vaccines administered i.m. with alhydrogel provided both passive and active protection against aerosol challenge with fully virulent *Y. pestis*.<sup>34–37</sup> In contrast, this F1-V vaccine protected African green vervets poorly despite eliciting a robust antibody response, although a fusion F1-V with a potent adjuvant flagellin likely can provide better protection in this and the cynomolgus macaque model.<sup>38</sup> The F1/V vaccine was protective against bubonic plague in rhesus macaques<sup>39</sup> and baboons.<sup>40</sup> Importantly, the non-human primate model has allowed the initiation of studies on establishing immune correlates of protection that are crucial for evaluating vaccine efficacy in humans.<sup>28,41</sup> In addition to passive immunity in mice, immune correlates were evaluated by using *in vitro* assays, such as a competitive enzyme-linked immunosorbent assay with protective MAb7.3; also examined were the effects of inhibition of *Yersinia*-mediated cytotoxicity in macrophages, and the contribution of tumor-necrosis factor- $\alpha$  and interferon- $\gamma$  cytokines in immune defense.<sup>36,42–44</sup>

#### DNA vaccines

The initial attempts to develop DNA plague vaccines had very limited success, generating low-serum titers to the F1 or LcrV. To achieve notable protection, DNA vaccination required boosting with a homo-

logous protein antigen. The inclusion in the constructs of the molecular immunopotentiator interleukin-12, or the use of mucosal adjuvant CT (cholera toxin), did not significantly improve the immunity. Subsequently, *Y. pestis* antigens were expressed with the signal sequence of tPA (human tissue plasminogen activator) that improved the secretion of soluble proteins. This well-developed system for DNA vaccination with LcrV allowed elicitation of a significant antibody response and provided protection against intranasal challenge with *Y. pestis*. Similar constructs expressing F1 demonstrated much lower protection.<sup>45</sup> Other antigens, such as Pla, YopB, YopD, YpkaA and YscF, were tested in this system, and several constructs produced modest protection.<sup>46</sup> Importantly, it was possible to improve LcrV DNA vaccine by enhancing its performance with the help of the F1–YscF construct.<sup>47</sup> Our recent review provides a more detailed description of DNA plague vaccines.<sup>17</sup> Overall, the outcome of DNA vaccination was strongly influenced by the DNA vaccine construct, and this perspective technology must be optimized to allow its use in vaccination against plague.

### Bacterial, viral and plant platforms

Development of plague vaccines based on the expression of protective antigens of *Y. pestis* in live carriers began in parallel with the testing of subunit vaccines. There are obvious limitations to this type of vaccines, since they require a precise level of attenuation, especially when expressing protective antigens, which often are factors of virulence. This is particularly important due to the risk of causing complications in immunocompromised populations. Preparation and storage of the vaccine stockpile also have to be strictly controlled to ensure viability of the delivery vector. However, the beneficial part of the live vaccines is the possibility of large-scale production, low cost, and, most importantly, their capability to elicit robust mucosal and cell-mediated immunity.

**Heterologous bacterial delivery systems.** A *Salmonella*-based live carrier platform is one of the most characterized bacterial delivery systems. Not surprisingly, it was used first to express capsular substance F1, LcrV, and then both antigens in attenuated *Salmonella enteritica* serovars Minnesota, Typhimurium, and Typhi. Oral immunization with these live vaccine candidates elicited predominantly IgG2a antigen-specific antibodies and generally provided only partial protection against *Y. pestis*. Immunity could be improved by a combination of intranasal immunization and parenteral boosting with purified antigens; however, the level of protection was notably lower than that typically provided by vaccination with recombinant antigens.<sup>17</sup> A new generation of *Salmonella* vectors with enhanced immunogenicity, defined control of attenuation, increased stability in expression and delayed *in vivo* synthesis of heterologous antigens has allowed improvements in protective properties of the vaccine primarily based on modified LcrV.<sup>48</sup> This vaccine still has not achieved the level of immunity comparable with that elicited by the subunit F1/V vaccines. Nevertheless, this system is well suited for expression of antigens located on the cell surface of *Y. pestis*, and some of them, such as outer membrane protein HmuR and PsaA adhesin, have been tested as vaccine candidates.<sup>17</sup> Additional information on the subject could be found in a recent review.<sup>49</sup> Another attempt to use a heterologous delivery system was the expression of LcrV from *Y. pseudotuberculosis* in the non-invasive, non-colonizing species *Lactococcus lactis*. This vaccine failed to protect against *Y. pestis*, although this failure could be explained by the choice of an incorrect version of LcrV.<sup>50</sup>

**Viral delivery of plague antigens.** There are several well-established viral delivery platforms that were tested starting from a decade after F1/V subunit vaccine was first described. One of them was a replication-deficient adenoviral vector Ad5 expressing human codon-optimized LcrV in a secreted form (AdsecV). A single administration of AdsecV viral particles in mice by the i.m. route resulted in robust protection in a pneumonic plague model.<sup>51</sup> This is a very promising system, which, however, has one major disadvantage. Thus far, the best adenoviral vectors are based on a virus of serotype 5 to which the majority of the human population has neutralizing antibodies. This can significantly reduce the efficacy of Ad5-derived vaccines. The use of adenoviral vectors of other serotypes that are rarer in humans were less successful due to their reduced expression of foreign antigens. A potential solution may be to develop of efficient Ad vectors from primates. Nevertheless, it was shown recently, that intranasal immunization with Ad5 vectors producing Ebola glycoprotein can bypass pre-existing immunity.<sup>52</sup> Another delivery platform arose from the concept that there are no neutralizing antibodies in the human population to the vesicular stomatitis virus, a natural pathogen of livestock, and to raccoon poxvirus. Consequently, both of these delivery platforms were used to express F1/LcrV antigens that provided a suitable level of protection.<sup>53,54</sup> Recently, secreted F1 and LcrV were expressed in a smallpox-modified vaccinia, Ankara, which protected mice against *Y. pestis*.<sup>55</sup> Additional details on viral delivery for plague vaccination were provided in two recent reviews.<sup>17,49</sup> Overall, viral platforms probably provide the best possibilities for the creation of next-generation plague vaccines; however, their safety and efficacy will require prolonged testing.

**Attenuation of *Y. pestis*.** *Y. pestis* attenuated strain EV NIEG has been widely used in Russia as a human live plague vaccine. This strain is a subculture of the original EV76 reference strain that was deposited in the culture collection of the Scientific Research Institute of Epidemiology and Hygiene (Russian abbreviation—NIEG, Kirov).<sup>56</sup> The vaccine is produced by growing EV NIEG on routine bacteriological media at 28 °C; it is then lyophilized, and typically applied intradermally by scarification. The undesirable side effects of this vaccination resulted mostly from the massive toxicity from the LPS of dead bacteria in vaccine preparation. To reduce the toxicity, lipid A of EV NIEG was modified by deleting the *lpxM* gene encoding late acyltransferase to yield a less toxic, penta-acylated form of lipid A. This *lpxM* mutant possessed enhanced protective properties in guinea pigs and outbred and BALB/c mice, due to its prolonged survival in vaccinated animals and the balanced expression of major protective antigens.<sup>57,58</sup> Another approach to vaccine development was introduction of the *lpxL* gene of *Escherichia coli* encoding another late acyltransferase absent in *Y. pestis*. The resulting strain constitutively produced highly potent hexa-acylated lipid A that seems able to overstimulate innate immunity at an early stage of infection with *Y. pestis* leading to its elimination.<sup>59</sup>

Attenuation of the wild-type *Y. pestis* is another approach in the construction of vaccine candidates. For example, inactivation of the *nlpD* and *lpp* genes encoding for outer membrane lipoproteins resulted in the excellent ability of the mutants to elicit protective immunity to *Y. pestis*.<sup>60,61</sup> There were a number of other mutations in the wild-type *Y. pestis* that reduced virulence to a certain degree. Although many of them were not initially created as potential vaccine candidates, the knowledge of mechanisms underlying the pathogenesis of *Y. pestis* can help in creating vaccine candidates with desired properties. Among the mutants tested in protection studies were those

carrying lesions in the following genes: *yopH* for T3SS effector protein, aromatic-dependent *aroA* and guanine nucleotide biosynthesis *guaBA* mutants; *crp* for the cyclic adenosine monophosphate receptor protein; *relA* and *spoT* for the enzymes involved in the synthesis of ppGpp affecting the stringent response; *smpB-ssrA*, encoding housekeeping functions for the translational machinery; and *dam* for DNA adenine methylase. The protective properties of these mutants were described in detail in two recent reviews.<sup>17,49</sup>

**Expression of *Y. pestis* antigens in plants.** Thus far, plants have been used to express F1/V proteins as an economical way to produce these protective antigens, and these plant-derived plague vaccine candidates were successfully tested in protection experiments in different animal models, including non-human primates. To obtain these antigens, three strategies, such as a plant virus-based expression system, as well as nuclear and chloroplast transformation, were employed. Detailed data for engineering plant-derived vaccines for prevention of bubonic and pneumonic plague were provided in recent reviews.<sup>17,62</sup> We included the plant-derived expression of protective antigens in the carrier platform section of the review, since this approach has the possibility of future development of these vaccines for mucosal delivery in an edible form.

In summarizing the entire section on different delivery platforms, it is necessary to indicate that live vaccines are instrumental in eliciting cellular immunity, the component which is largely missing in the subunit F1/V vaccine candidate that relies primarily on humoral immunity. The necessity of including a dominant protective antigen that can prime *Y. pestis*-specific memory T cells was proposed in the studies of Steve Smiley *et al.* Production of type 1 cytokines, such as tumor-necrosis factor- $\alpha$  and interferon- $\gamma$ , contributes significantly to defenses against plague.<sup>43,63–65</sup> In this respect, the most promising strategy for searching for human dominant T-cell proteins/epitope(s) might be that of reverse vaccinology, a high-throughput approach in combination with screening for interferon- $\gamma$ -secreting cells, similar to that described recently for mice immunized with live plague vaccine.<sup>66</sup> Recently, the involvement of the CD8<sup>+</sup> T cell-mediated immune response in protection against *Y. pestis* infection was demonstrated in mice immunized with the LcrV-based DNA vaccine. The unraveling of protective T-cell epitopes within the LcrV antigen is crucial for the creation of a vaccine that can elicit optimized humoral and cell-mediated immunities.<sup>67</sup>

A hallmark of plague is its ability to modulate immune responses at early stages of infection by suppressing the production of pro-inflammatory cytokines.<sup>68</sup> Mechanisms underlying this immunosuppression include such factors as a less stimulatory lipid A structure that interferes with the toll-like receptor pathways,<sup>69,70</sup> action of LcrV and Yop effectors of the T3SS,<sup>29,31</sup> as well as unidentified determinant(s) that can create a localized, dominant anti-inflammatory state in the lungs of infected animals.<sup>71</sup> Thus, a successful vaccination against plague ideally should result in the restoration of early pro-inflammatory responses to infection with *Y. pestis*.

### Future prospects

Most likely within the next several years, an F1/V subunit plague vaccine that is delivered by i.m. inoculation with alhydrogel will be available to those having a high risk of exposure to *Y. pestis*. Since this vaccine relies mostly on humoral immunity, it will be necessary to periodically boost vaccinees. It is hoped that by the time this vaccine hits the market, we will know the universally accepted immune correlates of protection. Ideally, the development of personalized medicine

should help us to identify human genetic factors, which may influence the range of the individual response to the vaccine (from high responders to non-responders), as well as those with counter-indications for vaccination. We expect that a direct search or a reverse vaccinology approach will allow us to identify powerful T-cell dominant epitope(s) to be included in an F1/V formulation, or in an improved polyvalent vaccine with additional synergistic protective antigens. One of the more probable scenarios leading to the optimal immunization strategy of next-generation vaccines would be a prime-boost schedule in which DNA or live carrier vaccination are followed by boosts with a polyvalent subunit vaccine.<sup>72</sup> Countries that still use live plague vaccines have a unique opportunity to begin clinical trials of a prime-boost vaccination strategy in the very near future.

### ACKNOWLEDGMENTS

This work was supported by a grant from the Defense Threat Reduction Agency (HDTRA1-11-1-0032) to Vladimir L. Motin and by a subcontract with the University of Texas Medical Branch at Galveston (No 11-082) to Valentina A. Feodorova. We thank Ms Mardelle J Susman for editing the review.

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