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MINI-REVIEW

Progress on plague vaccine development

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Abstract *Yersinia pestis* (YP), the gram-negative plague bacterium, has shaped human history unlike any other pathogen known to mankind. YP (transmitted by the bite of an infected flea) diverged only recently from the related enteric pathogen *Yersinia pseudotuberculosis* but causes radically different diseases. Three forms of plague exist in humans: bubonic (swollen lymph nodes or bubos), septicemic (spread of YP through the lymphatics or bloodstream from the bubos to other organs), and contagious, pneumonic plague which can be communicated via YP-charged respiratory droplets resulting in person–person transmission and rapid death if left untreated (50–90% mortality). Despite the potential threat of weaponized YP being employed in bioterrorism and YP infections remaining prevalent in endemic regions of the world where rodent populations are high (including the four corner regions of the USA), an efficacious vaccine that confers immunoprotection has yet to be developed. This review article will describe the current vaccine candidates being evaluated in various model systems and provide an overall summary on the progress of this important endeavor.

Keywords Plague · Vaccine · *Yersinia pestis* · Bubonic · Septicemic · Pneumonic plague

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Introduction

History of the plague

Yersinia pestis (YP, the plague bacillus) is one of three gram-negative pathogenic yersiniae and is a member of the *Enterobacteriaciae* family. YP is transmitted among rodents (rats, squirrels, prairie dogs, etc.) and from rodents to humans by flea arthropod vectors (Greenfield et al. 2002). Unlike the other two pathogenic yersiniae, which cause self-limiting gastroenteritis, YP has caused three major human pandemics (Justinian plague—541 C.E.–588 C.E., Black Death pandemic—fourteenth century, and the Indo-China plague—mid nineteenth century) as well as the great plagues of London (1665). The Justinian plague killed

~60% of the North African, European, and South Asian population. Additionally, the Black Death pandemic killed one third of the European population while the Indo-China pandemic killed more than 12 million people in both India and China alone (Inglesby et al. 2000). Historically, plague is one of the most devastating epidemic diseases known to mankind with an overall 200+ million deaths related to the three above-mentioned recorded plague pandemics (Evans et al. 2002).

No other pathogen has shaped our history as dramatically as has YP. In that vein, YP continues to be a global health problem, with 1,000–2,000 human infection cases annually primarily in endemic regions where the rodent populations are very high (e.g., India and China). Furthermore, due to global commerce during the nineteenth century and still the current pandemic, YP was able to establish environmental niches worldwide with the sole exception of Australia (Perry and Fetherson 1997). In fact, one such environmental niche lies in the four corner regions (parts of Arizona, Colorado, New Mexico, and Utah as well as parts of California) of the western USA (Cully and Williams 2001).

YP as a re-emerging infectious agent and a biothreat pathogen

Recently, the World Health Organization (WHO) has categorized plague as a re-emerging infectious disease because of a significant number of cases reported in Zambia, India, Malawi, Algeria, Madagascar, and Congo (2001–2006) (Heymann 2005; Alvarez and Cardineau 2010). Because of these cases, concern exists for a possible fourth pandemic, which is further amplified because of global warming, resulting in an increased prevalence of plague in rodent hosts (Riedel 2005). The current relevance of YP as a bio-weapon is garnered because of its high virulence, the development of multi-antibiotic-resistant strains (Ligon 2006; Alibek and Handelman 2000), and the new specific threats posed by some countries who are rigorously developing offensive biological warfare capabilities (Pearson 1998). In August 2009, Qinghai province in China was quarantined because of 12 human cases and three fatalities resulting from pneumonic plague. Unfortunately, Dr. M.J. Casadaban, Professor, Molecular Genetics, died in Chicago, IL, USA, on September 13, 2009 as a result of infection with an attenuated *Y. pestis* KIM/D27 strain, with which he worked (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6007a1.htm?s_cid=mm6007a1_e&source=govdelivery). More recently (February 2011), the Institute of Pasteur from Madagascar reported the death of 14 patients possibly due to pneumonic plague (http://www.promedmail.org). Although immunization of humans with plague vaccine will discourage the use of YP as a bio-weapon, currently there is no vaccine against plague (Smiley 2008a; Smiley 2008b).

Clinical features of YP

As previously mentioned, in sharp contrast to the fecal–oral route of infection for related pathogenic yersiniae (*Yersinia pseudotuberculosis* [YPT] and *Yersinia enterocolitica* [YE]), YP is transmitted by a dramatically different mechanism, the bite of an infected flea. This disparity exists despite YP only recently (within the last 15,000–20,000 years) having been evolved from YPT as evidenced by genetic divergence (Achtman et al. 1999). More specifically, a genome-wide comparison of YP and YPT revealed that in YP ~13% of YPT genes had been inactivated while 32 novel chromosomal genes had been acquired (Chain et al. 2004). This might have provided selective advantages to YP, allowing for its new lifestyle in fleas and enabling its ability to cause an invasive disease, unlike YPT and YE. With regards to disease, there are three forms of plague in humans: bubonic (swollen lymph nodes or bubos), septicemic (spread of YP through the lymphatics or bloodstream from the bubos to other organs), and finally contagious, pneumonic plague which can be communicated via YP-charged respiratory droplets. The latter two forms of plague can lead to very rapid death if left untreated (50–90% mortality), with pneumonic plague resulting in person-to-person transmission. However, with the administration of streptomycin or gentamicin, mortality can be reduced to ~15% if the antibiotic treatment is initiated within 24–36 h of infection (http://www.cdc.gov/ncidod/dvbid/plague/info.htm).

Antibiotic resistance: making the case for vaccine development

Antibiotic-resistant YP strains have been isolated from human cases of bubonic plague in Madagascar (Galimand et al. 1997; Guiyoule et al. 2001). These plague isolates contained transmissible plasmids; in one case, the plasmid possessed determinants for chloramphenicol acetyltransferase, streptomycin-modifying enzyme, and TEM-1 beta-lactamase. In the other case, the plasmid encoded high-level resistance to streptomycin. Although our recent studies have indicated that levofloxacin is effective in killing all three category A bacterial select agents leading to plague, anthrax, and tularemia (Peterson et al. 2010), the bioweapon programs of the Former Soviet Union likely engineered multidrug-resistant variants of YP (including fluoroquinolone- and tetracycline-resistant forms) (Inglesby et al. 2000). Currently, there are no pre- or post-exposure means of prophylaxis against plague by drugs different from antibiotics administered either alone or in combination with other antibiotics. Therefore,

inhibitors of virulence and/or new immunogenic antigens for recombinant plague vaccines as well as genetically well-defined live attenuated plague bacteria are needed to best combat this disease and in acquiring new countermeasures against this potential agent of biological warfare.

YP virulence genes, gene products, and their activities

In efforts of identifying novel genes that could be deleted for the purpose of developing a highly effective/protective live, attenuated YP vaccine candidate strain(s), an evaluation of recently reported YP virulence mechanisms and virulence-associated genes could provide important leads. YP harbors a well-characterized ~70-kb virulence plasmid (pCD1) that encodes the type 3 secretion system (T3SS) apparatus (the injectisome) as well as the effector protein substrates (*Yersinia* outer proteins—Yops) which it directly translocates/injects into targeted host cells; the T3SS is essential for YP virulence (Dean 2011; Viboud and Bliska 2005; Deng et al. 2002; Cornelis and Van Gijsegem 2000; Perry et al. 1998; Straley and Brubaker 1982). Although significant progress has been made in understanding the disease-causing mechanisms of this pathogen through studies evaluating virulence gene expression and regulation, bacterial iron acquisition mechanisms, prevention of host immune responses via paralysis of phagocytic cells, and suppression and disruption of signal transduction leading to host cell death (Deng et al. 2002), the reasons for the acute character of YP infections are still not fully understood (Deng et al. 2002).

In addition to pCD1, YP has two additional plasmids: pMT1 (110-kb with 115 open reading frames) encodes genes for the anti-phagocytic capsular antigen F1 and murine toxin (Du et al. 2002; Hinnebusch et al. 1998) and a 9.5-kb pPCP1 plasmid encodes three gene products with distinctly different functions. For example, pesticin, encoded for by the *pst* gene, causes cell wall lysis and death of neighboring bacteria by hydrolyzing the bond between *N*-acetylglucosamine and *N*-acetylmuramic acid, thus degrading the glycan backbone of Braun lipoprotein (Ferber and Brubaker 1979). YP uses this bacteriocin to kill the neighboring cells in which the pPCP1 plasmid is absent while, at the same time, protecting itself with pesticin immunity protein, encoded for by the *pim* gene on the pPCP1 plasmid (Sodeinde et al. 1992).

Also present on the pPCP1 plasmid is the gene encoding plasminogen-activating protease (Pla), which interferes with the complement activation cascade and blood coagulation and decreases the extracellular matrix around the foci, thus, helping bacterial dissemination to peripheral organs (Suomalainen et al. 2007). Bacterial surface-bound Pla binds DEC-205 on phagocytic cells, which enhances bacterial uptake and consequently increases initial dissemination at the foci (Zhang et al. 2008). The loss of the pPCP1 plasmid or the *pla* gene attenuates YP administered by the subcutaneous route (Sodeinde et al. 1992; Brubaker et al. 1965; Samoilova et al. 1996; Welkos et al. 1997; Welkos et al. 2002) in mice or guinea pigs and by the intradermal and intranasal routes in mice (Agar et al. 2009b; Sebbane et al. 2006; Lathem et al. 2007). Previous studies also indicated that *pla* mutant strains evoked an earlier inflammatory response than did the wild-type (WT) bacterium, and mice were able to clear the infection sooner, resulting in a higher survival rate in mutant versus WT bacteria-infected animals (Sodeinde et al. 1992; Welkos et al. 1997; Lathem et al. 2007; Agar et al. 2009a). Interestingly, however, these additional plasmids did not appear to be the primary determinants for the exceptional virulence of YP (Williams et al. 1972; Friedlander et al. 1995; Worsham et al. 1995; Welkos et al. 1995; Davis et al. 1996; Andrews et al. 1999; Welkos et al. 1997; Filippov et al. 1990; McDonough et al. 1993). Consequently, the presence of new virulence factors must be looked into in more detail.

The genome of YP is rich in insertion sequences and intra-genomic rearrangements, and there are indications of horizontal gene acquisition, from other bacteria and viruses (Parkhill et al. 2001). Many regions within the YP chromosome show characteristics of islands that were acquired through lateral gene transfer. Among these are several genes acquired from other insect pathogens (Parkhill et al. 2001). Two regulatory systems, namely, PhoPQ reported in *Salmonella* Typhimurium and quorum sensing, were also detected in YP (Parkhill et al. 2001). In fact, loss of PhoPQ from YP leads to a defect in intracellular growth and/or survival (within macrophages), reduced oxidative and acid stress response capacities, and a 75-fold increase in LD_{50} in a mouse model (Oyston et al. 2000), but a more recent study showed that PhoPQ did not seem to play a role in YP virulence by evoking bubonic and pneumonic plague in a murine model (Bozue et al. 2011).YP produces rough lipopolysaccharide (LPS), lacking an O antigen (Parkhill et al. 2001), which protects the bacterial surface from the activity of complement. However, in YP, serum resistance is, in part, also contributed to by the Pla protease which cleaves complement components (Parkhill et al. 2001). It was also noted that YP produces a less-acylated (tetra-acylated) lipid A at 37 °C, reducing the activation of human macrophages as a result of poor induction of host toll-like receptor (TLR) 4-mediated innate immune responses (Kawahara et al. 2002; Kolodziejek et al. 2010). More specifically, when the YP KIM1001's poorly TLR4-stimulating LPS was modified to more strongly induce TLR4, the strain became entirely avirulent even when subcutaneous challenge doses were very high and mice were protected from both YP subcutaneous and pneumonic re-challenges (Montminy et al. 2006).

Other virulence mechanisms of YP

Deletion of the 102-kb pigmentation (*pgm*) locus from the chromosome of YP attenuates its virulence (Huang et al. 2006). Additionally, a transcriptional regulator, RovA, which regulates a variety of genes and is specifically required for the development of bubonic plague in mice by YP, was identified (Cathelyn et al. 2006). More recently, another important global regulator, the transcription factor cyclic AMP receptor protein (Crp), has been shown to regulate the virulence of YP. In YP KIM5-3001 strain, the plasminogen activator gene encoded protein (Pla), required for YP dissemination in human hosts, was found to be directly regulated by Crp (Kim et al. 2007). Furthermore, Crp was found to regulate approximately 6% of all YP bv. microtus strain 201's genes, and an electrophoretic mobility shift assay revealed that 37 genes/operons were direct binding targets of Crp. This included the previously identified *pla* (Kim et al. 2007) and *pst* plasmid-encoded virulence-associated genes, namely, Pla protease and Pesticin, respectively (Zhan et al. 2008). In addition to regulating the *pla–pst* operon, Crp was also found to directly repress *sycO*, *ypkA*, and *YopJ* T3SS-associated genes organized in the *sycO–ypkA–YopJ* operon (Zhan et al. 2009).

Interestingly, a new set of T3SS-dependent proteins has been identified in YP and includes insecticidal-like proteins that are thought to function as transmission factors, contributing to flea morbidity by promoting colonization of the midgut (Gendlina et al. 2007). Additionally, a 3′–5′ exoribonuclease (involved in RNA metabolism/decay), polynucleotide phosphorylase (PNPase—encoded for by the *pnp* gene), was found to be necessary for optimal functioning of the T3SS, and it was also found that this requirement was independent of its catalytic activity but did require a conserved phenylalanine in the PNPase S1 RNA binding domain (Rosenzweig et al. 2005; Rosenzweig et al. 2007). Our subsequent study in mice showed that the *pnp* mutant of YP KIM/D27 (*pgm*-minus) was highly attenuated in animals, an effect that could be complemented by the ectopic expression of the corresponding gene (Lawal et al. 2010). Therefore, the continued investigation of new virulence factors and their interactions with the host machinery is warranted to fully elucidate the mechanisms of YP pathogenesis.

Animal models for plague

Although mice are currently the preferred model among rodents, guinea pigs were often used in the past (Wayson et al. 1946; Donavan et al. 1961; Jawetz and Meyer 1944; Jones et al. 2003). Recent data have shown that mice die during inhalational plague as a result of pneumonia and multi-organ failure. Specifically, liver and kidney damage were noted based on blood chemistry analysis data (Sha et al. 2008). In contrast to mice, which form only enlarged lymph nodes rather than typical bubos, both guinea pigs and rats develop a form of the disease which more closely resembles the human bubonic plague (Wayson et al. 1946; Jawetz and Meyer 1944). Recent studies have shown that the rat model is quite efficacious for characterizing the progression and kinetics of bubonic infection, as well as for examining the host immune response to YP (Chen and Meyer 1974; Sebbane et al. 2005). Moreover, the immunology and genetics of the rat are well characterized, making this animal, which often has been associated with outbreaks of human urban plague, a valuable model in which to study microbial pathogenesis, host response, and the efficacy of new medical countermeasures against plague (Sebbane et al. 2005). We were one of the first groups to fully characterize the rat model of pneumonic plague (Agar et al. 2009b) and also a mouse pneumonic plague model by aerosolization of a highly virulent strain CO92 of YP (Agar et al. 2008). In a remarkably human-like manner, non-human primates are also susceptible to and eventually succumb to an aerosol YP challenge. In particular, cynomolgus macaques (*Macaca fasicularis*) have been often used as an animal model of respiratory plague infection for this reason (Koster et al. 2010). More specifically, cynomolgus macaques, when exposed to YP CO92 respiratory challenge 24 times at LD_{50} (50% lethal dose), experience disease symptoms similar to those in humans, including fever, labored breathing, and heart failure. Most infected animals die 34–92 h post-infection, strongly supporting the use of cynomolgus macaques as the most appropriate (human-like) non-human primate model of plague (Warren et al. 2011).

Vaccines

Overview

At present, there is no plague vaccine; however, until 1999, a formaldehyde-killed, whole-cell vaccine was available for military and laboratory personnel. This vaccine was discontinued because (1) the protection was variable and short-lived, (2) the vaccine was effective against bubonic plague but not against pneumonic plague, and (3) the rate of adverse effects was very high (Deng et al. 2002). Currently, the most promising candidates for subunit plague vaccines are F1 and LcrV (low-calcium-response Vantigen, a component of the T3SS) which are individually immunogenic and have an additive protective effect in combination (Alvarez and Cardineau 2010). Further, F1 and LcrV antigens activate dendritic cells to induce primary T-cell responses essential in developing protective immunity

against plague (Kingston et al. 2007). This vaccine protected mice when given by the subcutaneous and inhalation routes (Williamson 2001). The efficacy of this vaccine has also been shown in a non-human primate model but to a lesser extent than the rodent models (Williamson et al. 2007; Murphy et al. 2007). The protective effect of this vaccine in humans is unknown, and it is of concern that these antigens are weakly immunogenic in humans (Wang et al. 2008). Importantly, this vaccine will not be effective against the non-capsulated strains of YP or those which have different variants of LcrV with diverged amino acid sequence. Therefore, it is crucial that new vaccines/therapeutics be developed and tested against the different clinical forms of plague. A detailed evaluation of the various vaccines developed/in development and characterized/being characterized is discussed in the following paragraphs. For a comparison of various plague vaccines and subunit vaccine candidates under development, see Tables 1 and 2, respectively.

Live attenuated vaccine candidates

From the perspective of immunogenicity, live attenuated vaccines will always be considered best on account of the whole organism being seen by the immune system coupled with its ability to actively replicate, albeit to a lesser extent compared to the WT bacterium. This replication of live attenuated vaccines gives rise to a robust immune response that most closely emulates one elicited by an actual infection. Although a live attenuated *pgm*-minus strain of YP (e.g., EV76) is licensed to be used in humans in the former states of the Soviet Union in spite of some side effects (Feodorova et al. 2007; Zilinskas 2006), the licensure of similar live vaccines in the United States will be met with skepticism by the regulatory agencies. This skepticism is based upon the fact that the EV76 strain is poorly defined genetically and still retains, to a large degree, its virulence potential. Nonetheless, the EV76 strain does illicit a T-cell response that contributes to protection from pneumonic plague in a murine model of infection (Sha et al. 2008). However, uncertainty about the retained virulence potential makes the YP EV76 live attenuated option much less appealing when considering vaccine development.

One way to overcome the retained virulence of the YP EV76 strain is to ectopically express transgenes that could further reduce its virulence potential. In that vein, an *Escherichia coli*-derived transgene encoding the lipopolysaccharide LpxL was over-expressed in the EV76 strain on account of its immunogenicity and ability to strongly activate TLR-4. This resulted in the EV76-LpxL expressing a strain which retain its ability to activate cell-mediated immunity in addition to reducing bacterial load in visceral tissues as well as conferring protection upon a secondary intranasal (IN) challenge with a parental D27 YP strain lacking the *pgm* locus (Szaba et al. 2008). In another clever genetic modification, *crp* gene expression was controlled by the chromosomal arabinose inducible promoter P_{BAD}, allowing for the facile repression of *crp* expression. After an initial subcutaneous challenge with the aforementioned strain, 100% protection against a subsequent subcutaneous re-challenge and 70% protection against a respiratory re-challenge ensued compared to when parental KIM YP was used. These data indicated that control of metabolic use of carbohydrates could be another effective way to generate attenuated mutant vaccine candidate strains (Sun et al. 2010). In fact, the aforementioned strain (Sun et al. 2010) was further attenuated in its virulence by "knocking-in"/incorporating the *E. coli lpxL* gene into the YP chromosome. The *lpxL* gene encodes a hexa-acylated lipid A, a potent TLR4 agonist, LpxL, capable of inducing a strong innate immune response whereas the YP version, a tetra-acylated lipid A, is poorly immunogenic. The aforementioned strain exhibited a 10^7- and 10^4-fold increase in LD_{50} in subcutaneous and IN murine infections, respectively. Furthermore, mice subcutaneously immunized with the aforementioned strain were 100% and 80% protected from subsequent subcutaneous and IN YP re-challenges, respectively (Sun et al. 2011).

However, given the fact there is no plague vaccine, further attenuation of bacteria (through genetic mutations) which can induce protective immune responses may be a viable option. Adhesins, which are required for the intimate binding of YP to its targeted host cell, have also been recently evaluated for their role in virulence using both cell culture and murine models of infection. As it turns out, Ail (attachment invasion locus, also designated as OmpX), plasminogen activator protease (Pla), and pH 6 antigen (Psa) were all required for the optimal binding of KIM/D27 YP to cultured cells as well as virulence in mice. These protein-encoding genes provide additional targets that could be either deleted or mutated in efforts of generating effective live attenuated vaccine strains (Suleyman et al. 2010). In fact, an *ompX* mutant of a fully virulent CO92 YP that was engineered and employed in two pneumonic plague models (mouse and rat) revealed a 2-day delay in the time to death of mice and the complete attenuation of virulence in a rat model of infection at a 10^8 colony forming units (cfu) dose (Kolodziejek et al. 2010). In fact, Δ*caf1* mutants (that cannot make the capsular fraction 1 antigen) and Δ*psaA* mutants had decreased virulence potential during murine infections; however, the degree of attenuation observed with the mutant strains was dependent upon mouse/host factors since different murine strains responded differently (Weening et al. 2011). This illustrates the importance of considering the host immune response

Table 1 List of several categorized plague vaccine candidates

Vaccine	Animal model	Efficacy	References
Live attenuated			
YP EV76 Δ*pgm*	Guinea pigs	Protection against bp	Feodorova et al. 2007
YP EV76-LpxL	Murine	Protection against pp and activation of CMI	Szaba et al. 2008
YP KIM5+ ΔP_{crp21} ::TT *araC* P_{BAD} *crp*	Murine	100% protection against bp and 70% protection against pp	Sun et al. 2010
YP KIM5+ lpxP32::PlpxL lpxL_Pcrp21 ::TT *araC* P_{BAD} *crp*	Murine	10^4–10^7 increase in LD_{50}; 100% protection against bb and 80% protection against pp	Sun et al. 2011
YP CO92 Δ*ail*	Rat	Attenuation in a pneumonic plague model	Kolodziejek et al. 2010
YP CO92Δ*caf1*	Murine	Fully virulent	Quenee et al. 2008
YP Kimberly53 Δ*nlpD*	Murine	Attenuated virulence in bp and pp models	Tidhar et al. 2009
YP KIM5+ Δ*relA*, *spoT*	Murine	100% protection against bp and 60% protection against pp	Sun et al. 2009
YP CO92 Δ Δ*smpA*, *ssrA*	Murine	100% protection against pp and 100 protection against sp	Okan et al. 2010
Live attenuated recombinant			
YPT pYY-	Murine	75–88% protection against bp	Blisnick et al. 2008
ST 14028-LcrV, F1, YopD, orYscF	Murine	100%, 30%, 60%, and 60% protection against pp, respectively	This work
ST RASV-*psaA*	Murine	Humoral anti-PsA response; no protection against pp	Torres-Escobar et al. 2010a
ST RASV-$LcrV_{196}$	Murine	Protection against pp and bb	Torres-Escobar et al. 2010b
ST RASV-*psn*	Murine	Protection against bp and pp	Branger et al. 2010
ST RASV-*caf1*	Murine	Protection against sp	Ramirez et al. 2009
Lactobacillus plantarum-lcrV	Murine	IgA production in remote mucosal linings	del Rio et al. 2010
Plant-based			
Nicotiana tabacum- LcrV–F1	Murine	88% protection against pp	Arlen et al. 2008
Nicotiana benthamiana LcrV–F1	Guinea pigs	Protection against pp	Del Prete et al. 2009
Carrot and lettuce—F1-V	Murine	Strong humoral response	Rosales-Mendoza et al. 2010a and 2010b
DNA-based			
lcrV/caf1/heat-labile enterotoxin	Murine	40% protection against pp using a DNA gene gun	This work
lcrV (encoding 127 residues)	Murine	60% protection against YP IP challenge	Vernazza et al. 2009
il12/lcrV/caf1	Murine	80% protection against pp	Yamanaka et al. 2008
ltn/lcrV/caf1	Murine	Enhanced protection against pp	Yamanaka et al. 2009; 2010
yscF/caf1	Murine	Enhanced protection against YP infections relative to *caf1* alone	Wang et al. 2010a
Virus vector-based			
Vaccinia virus—*lcrV/caf1*	Murine	100% protection against pp	Bhattacharya et al. 2010
Raccoon pox virus-*caf1*	Prairie dogs	38% protection against pp	Rocke et al. 2008
Vaccinia Ankara-$LcrV_{307}$	Murine	85% protection against pp and bp	Brewoo et al. 2010
Adenovirus- anti-LcrV antibody	Murine	80% protection against pp	Sofer-Podesta et al. 2009
VSV-LcrV	Murine	Strong humoral response; 90% protection against pp	Chattopadhyaya et al. 2008

YP Yersinia pestis, *YPT Yersinia pseudotuberculosis*, *ST Salmonella* Typhimurium, *CMI* cell-mediated immunity, *pgm* pigmentation locus, *Δ* deleted, *pp* pneumonic plague, *bp* bubonic plague, *sp* septicemic plague, *VSV* vesicular stomatitis virus

when developing vaccine candidates for plague. A more recent study indicated that the Δ*caf1* mutant of CO92 YP was attenuated for virulence in a bubonic plague but not in a pneumonic plague mouse model when compared to the WT CO92 strain (Sha et al. 2011).

Counterintuitively, the successful generation of strains deleted for genes that are suspected of promoting virulence does not always translate into effective vaccine candidates. In one such example, the *caf*1YP KIM/D27 mutant and *caf1* CO92 (*pgm* locus deleted) mutants were engineered and evaluated for their virulence during initial BALB/c challenges as well as re-challenges of parental YP KIM/D27-immunized mice. Surprisingly, the CO92 *caf1*-deleted mutant strain not only retained its virulence in murine

Table 2 List of several categorized plague subunit vaccine candidates

Vaccine	Animal model	Efficacy	References
LcrV	Murine Swiss-Webster	Protection against *A. hydrophila*	This work
$LcrV_{196-225}$	Murine/BALB/c	Protection against bp	Quenee et al. 2010
F1–$LcrV_{1-270}$	Murine/BALB/c	Protection up to 518 days post-immunization against YP 141	Wang et al. 2010b
F1–LcrV	Murine	Strong anti-LcrV IgG; weak cell-mediated immunity	Qiu et al. 2010
	Cynomolgus macaques	100% protection against pp	Chichester et al. 2009
F1–LcrV powder form	Murine/Swiss-Webster	70% protection against bp	Huang et al. 2009
F1 + cationic liposome nucleic acid complexes	Murine C57/BL6	Protection against pp	Jones et al. 2010
F1–LcrV + CpG oligonucleotides	Murine/BALB/c	Enhanced IgG2a production and protection against pp	Amemiya et al. 2009
LcrV + *Lactococcus lactis* particles	Neonatal mice	100% protection against IV YP challenge	Ramirez et al. 2010
LcrV +Gram positive enhancer matrix	Murine	Enhanced IgG production	Zeng et al. 2009
LcrV +DL-lactide-co-glycolide (microparticle)	Murine/BALB/c	Protection against IP YP challenge	Uppada et al. 2009
LcrV–F1 (microencapsulated)	Murine	Induced cell-mediated immunity	Gupta et al. 2010
LcrV–F1-flagellin	Murine/BALB/c	Robust humoral response; 100% protection against pp	Mizel et al. 2009
	African green monkeys	Robust humoral response	Mizel et al. 2009
	Cynomolgus macaques	Robust humoral response	Mizel et al. 2009
F1 and LcrV + lipid A mimetics	Murine/BALB/c and Rat	63% and 90% protection from pp, respectively	Airharta et al. 2008
Yop EBD complex	Murine/BALB/c	Protection against IV YP challenge	Ivanov et al. 2008
LcrV (pulsed onto dendritic cells)	Murine/BALB/c	Induced humoral and cell-mediated immunity; protection against pp	Do et al. 2010

YP Yersinia pestis, *IP* intraperitoneal, *IV* intravenous, *pp* pneumonic plague, *bp* bubonic plague, *Yop Yersinia* outer protein

subcutaneous and intranasal (IN) infections but also was able to cause lethal infections in KIM/D27 (the non-pigmented vaccine strain)-immunized mice when the re-challenge route was IN. KIM/D27-immunized mice re-challenged with the CO92 *caf1*-deleted mutant strain via the subcutaneous route were 100% protected for all 14 days of evaluation (Quenee et al. 2008). Indeed studies have shown that the deletion of genes encoding *pla*, *pcm*, *yopH*, *lpxM* (an acyl transferase gene which encodes an enzyme involved in LPS acylation resulting in pleiotropic effects including decreased expression of Pla, Caf1, and LcrV antigen), Braun and *nlp* liporoteins (*lpp*), and *guaBA* (an auxotrophic mutant unable to synthesize guanine) significantly attenuates YP, while retaining their ability to prime protective immune responses (Agar et al. 2009a; Sha et al. 2008; Feodorova et al. 2009; Galindo et al. 2009; Oyston et al. 2010; Liu et al. 2010; Szaba et al. 2008; Tidhar et al. 2009).

More specifically, NlpD (encoded for by the *nlpD* gene—part of the *pcm* operon) was found to be essential in the fully virulent Kimberly53 YP strain for the colonization of mouse organs following both aerosol and subcutaneous challenge, and its deletion resulted in a greatly elevated LD_{50} of 10^7 cfu. Interestingly, the *nlpD* mutant suffered from impaired cell separation during division despite no demonstrated impairment in its pure culture growth rate (Tidhar et al. 2009). This study on NlpD corroborated our earlier report on Braun Lpp demonstrating that the deletion of the Braun *lpp* gene attenuated the bacterium in developing both bubonic and pneumonic plague in a mouse model (Sha et al. 2008). Furthermore, this Braun Lpp mutant was quickly cleared from mouse organs on account of its inability to survive within macrophages. Our subsequent studies defined the Lpp mechanism of action in host cells as well as the role of a global stress response gene (*htrA*), which was down-regulated in the *lpp* mutant, indicating reduced survivability of the mutant in macrophages (Galindo et al. 2009; Galindo et al. 2010; Liu et al. 2010). Impaired cell separation associated with the *nlp* mutant and attenuated virulence of both *nlpD* and Braun *lpp* mutants represents attractive vaccine candidates.

In a separate study, the ability to synthesize ppGpp, involved in bacterial stress responses to multiple environmental conditions, was evaluated for its potential role in virulence and whether the ppGpp deletion mutant could serve as an attenuated vaccine candidate strain. To engineer a ppGpp-deficient YP strain, the two genes involved in ppGpp synthesis were deleted, namely, *relA* and *spoT*. The YP KIM5+ double mutant suffered from reduced growth

rate as well as a greater than 1,000-fold reduction in virulence in a murine model of infection. More importantly, the YP KIM5+ *relA* and *spoT* double mutant conferred 100% protection against a subsequent subcutaneous challenge with the virulent parental KIM5+ strain and approximately 60% protection in mice re-challenged with a respiratory KIM5+ infection (Sun et al. 2009).

In addition to Braun *lpp*, *nlpD*, *relA*, and *spoT*, other gene targets have been identified as necessary for optimal YP virulence. The highly conserved bacterial SmpA–SsrA stress response system is fairly well characterized (Karzai, et al. 2000; Keiler 2008). SmpA and SsrA are encoded for by the *smpB* and *ssrA* genes, respectively, and are adjacent to one another in the YP genome. SsrA functions as a unique tmRNA molecule that possesses both transfer and messenger activities that, together with its protein partner SmpB, rescues stalled ribosomes during stress conditions experienced by bacteria (Dulebohn et al. 2007). A YP CO92 double *smpB–ssrA* mutant was highly attenuated in virulence using both IN and intravenous (IV) murine models of infection. Furthermore, mice intranasally immunized with a 10^4-cfu dose of the double mutant produced a robust humoral response resulting in 100% protection from subsequent intranasal and IV challenge with CO92 Δ*pgm* strain (Okan et al. 2010).

Interestingly, putative vaccine candidates that produce the desired immune-protective outcomes do not necessarily exhibit attenuated virulence phenotypes. Further, multiple routes of immune priming must be characterized since different routes could lead to different correlates of protection upon re-challenge with the WT strain. For example, a YP strain in which the YopJ effector protein had been replaced with *Y. enterocolitica* YopP homolog displayed a hyper-virulent phenotype as judged by its ability to induce macrophage cytotoxicity in vitro. However, following a subcutaneous infection, this transgenic YP strain exhibited gross attenuation in virulence as well as conferring protection against subsequent septicemic and pneumonic plague (Zauberman et al. 2009). This seemingly counterintuitive finding, although not fully understood, needs to be taken into consideration when considering live attenuated vaccine strain candidates.

Taken together, when considering the development of live attenuated vaccines, one needs to evaluate whether the attenuated strain has decreased virulence following multiple routes of infection in more than one animal model. Also, one needs to consider whether the attenuated strain confers protection against subsequent re-challenges with various fully virulent strains (including F1 deleted variants) and whether an attenuated strain having ectopically expressed regulatory genes (under the control of inducible promoters) is a truly viable option for a marketed live attenuated vaccine.

Live attenuated recombinant/surrogate vaccines based on *Y. pseudotuberculosis* (YPT), *Salmonella* Typhimurium RASV, and *Salmonella* Typhimurium *lpp*/*msbB* mutants

As alluded to above, whole-cell, live attenuated vaccines elicit optimal immune responses in the host and are thus considered best. However, in reality, many pathogens are not considered optimal as live attenuated vaccine candidates on account of their high virulence potential. Therefore, other approaches were taken including using other closely attenuated surrogate organisms or less closely related attenuated vaccine surrogate strains that are engineered to express a transgene derived from the pathogen in question.

Employing the former approach, an avirulent YPT strain devoid of its high pathogenicity island, type IV pilus, and virulence plasmid (pYV analogous to pCD1) was evaluated for its use as a live attenuated, oral vaccine candidate for a subcutaneous YP challenge. Interestingly, the attenuated YPT strain did confer 75–88% immunoprotection against subsequent subcutaneous YP challenge, demonstrating that a closely related surrogate strain can be readily developed and safely administered via the oral route, resulting in the immunoprotection of vaccinated mice (Blisnick et al. 2008). Earlier studies also revealed similar findings where immunizations with live attenuated YPT provided modest protection against bubonic plague (Wake et al. 1978; Simonet et al. 1985; Taylor et al. 2005). Furthermore, the oral YPT ΔBA strain was also found to confer approximately 50% protection in mice intranasally infected with YP (Okan et al. 2010). By using the more distantly related recombinant strain approach, an adhesion/pili pH 6 antigen (PsaA), recently evaluated for its role in virulence and cell adhesion, was expressed and produced in a live attenuated surrogate vaccine strain of *Salmonella* Typhimurium, RASV, and a stable production of the 15-kDa PsaA was observed in total cell extracts, demonstrating that such an approach is entirely possible to develop (Torres-Escobar et al. 2009). Further, the recombinant RSVA–PsaA strain was determined to elicit a systemic, humoral anti-PsaA IgG immune response; however, the humoral response alone was not sufficient to confer protection against a subsequent pneumonic plague challenge (Torres-Escobar et al. 2010a).

Perhaps PsaA is not the appropriate antigen of choice when considering the use of the surrogate RASV live attenuated strain. Alternatively, PsaA might be an appropriate antigen of choice when co-produced alongside another/multiple YP antigens in the RASV strain, thus producing synergistic immunogenicity. Another limitation to consider when using either the avirulent YPT strain or the attenuated, recombinant RASV strain expressing and producing YP antigens is the route of immunization used. *Salmonella* Typhimurium is an enteric pathogen transmitted via the oral–fecal route, much like YP's closely related

cousins, YPT and YE. Therefore, when attempting to use such a strain to protect against a subsequent YP infection, additional boosting via other routes might also be required for conferring immunoprotection against pneumonic or even bubonic plague (as the organism in these forms of plague are not transmitted via the oral–fecal route).

A recombinant *Salmonella* Typhimurium RASV strain expressing and producing a truncated LcrV ($LcrV_{196}$—residues 131–326) derived from YP was evaluated for its ability to confer immunoprotection against both YPT and YE. Not surprisingly, the production of the LcrV variant in the RASV strain conferred protection against the expected YPT and YE strains; however, what was unexpected was that the RASV strain alone was able to provide immunoprotection against YPT, most likely on account of cross-protective antigens (Branger et al. 2009). Considering the aforementioned surprising finding, whether RASV can confer immunoprotection against other closely related enteric pathogens should also be determined prior to developing recombinant RASV strains expressing and producing foreign transgenes of interest, possibly saving both time and energy. In a similar scenario, we noted that animals immunized with YP LcrV were significantly protected against systemic infections caused by an emerging human pathogen *Aeromonas hydrophila* at a dose of 3×10^7 cfu (Fig. 1). We characterized the T3SS of a diarrheal isolate SSU of *A. hydrophila* (Sha et al. 2005), and its LcrV shares 41% sequence similarity with the LcrV of YP. These data indicated that the immunization of animals and possibly humans with LcrV might also provide cross-protection against other pathogens that have a functional LcrV homologue.

In efforts of fine-tuning LcrV expression and production in the above-mentioned RASV strain, the same truncated LcrV variant ($LcrV_{196}$)-encoding gene as mentioned above was cloned into a plasmid vector equipped with two origins of replication, one optimized for low-copy propagation (pSC101) while the other optimized for high-copy plasmid propagation (pUC). The pUC origin of replication was repressed by arabinose which is unavailable within the mouse, resulting in a switch that allowed for the high expression levels of ectopically expressed LcrV-encoding gene within the mouse, eliciting robust immune responses. In fact, following oral vaccination using the finely controllable recombinant RASV $LcrV_{196}$-expressing and -producing strain, strong Th1 and Th2 responses, which protected the vaccinated animals from both intranasal and subcutaneous challenges with the fully virulent CO92 strain, were observed (Torres-Escobar et al. 2010b).

RASV has drawn considerable attention as a recombinant, live attenuated vaccine candidate strain for YP. However, LcrV is not the sole YP transgene being characterized in the aforementioned surrogate strain. In fact, PsaA was characterized as a YP antigen candidate; however, no protection was observed following a subsequent pneumonic plague challenge (Torres-Escobar et al. 2010a). Despite that setback, other YP-derived antigen vaccine candidates have been evaluated for their immunogenecity when expressed and produced in the recombinant RASV strain.

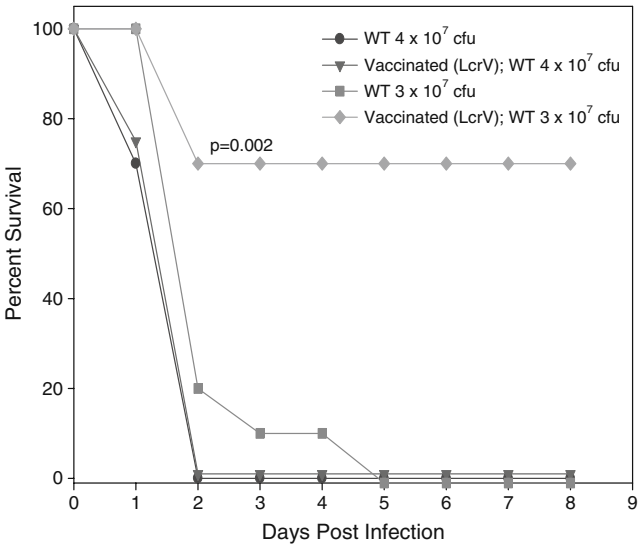

Fig. 1 Antibodies to YP LcrV were protective against a 3×10^7-cfu systemic challenge (IP route) dose of *A. hydrophila* SSU in a mouse model. Female Swiss-Webster mice (n=10/group) were either given only alum or immunized with alum mixed with purified LcrV (10 μg/mouse) by the IP and intramuscular (IM) route (5 μg by each route) on days 0, 15, and 30. The animals were bled via the retro-orbital route, and the sera had high LcrV antibody level by ELISA. On day 45, the animals were challenged with WT *A. hydrophila* SSU at doses of 3×10^7 and 4×10^7 cfu by the IP route. The data were analyzed statistically by using Kaplan–Meier's survival estimates, and p values of $\leq$0.05 were considered significant. Although no protection was noted in animals immunized with LcrV at a challenge dose of 4×10^7 cfu, 70% of the vaccinated animals were protected against a challenge dose of 3×10^7 cfu of *A. hydrophila*

Psn, an outer membrane protein encoded in all three pathogenic yersiniae by their high pathogenicity island, is the receptor for the siderophore yersiniabactin which helps promote bacterial growth within the host. In addition to targeting Psn, another outer membrane protein HmuR, involved in iron scavenging within the host, was also expressed and produced as a transgene in the recombinant RASV strain and evaluated for its protective properties as a live attenuated vaccine candidate for plague. In parallel with Psn and HmuR, the same group also expressed and produced $LcrV_{196}$ and an LcrV variant that was mutated in five key residues required for the activation of TLR-2. Interestingly, oral immunization with RASV-Psn, RASV-LcrV (full length LcrV), and RASV-$LcrV_{196}$ all protected mice from subsequent bubonic and pneumonic plague evoked by CO92 strain of YP. However, in sharp contrast

and like the RASV recombinant strain expressing and producing the LcrV variant that does not activate TLR-2 (for reasons unclear), RASV-HmuR did not confer protection against either of the aforementioned plague infections (Branger et al. 2010). Ultimately, not all outer membrane proteins will serve as ideal vaccine candidate antigens for expression and production in the surrogate RASV strain despite being "visible" to the immune system. A detailed evaluation and characterization of the protective properties of various outer membrane and secreted proteins is fully warranted based upon the above-mentioned findings.

When considering the development of an effective plague vaccine, all segments of the population must be considered under the auspice of herd immunity. Within that societal cross-section, newborns and infants are of particular concern due to their immature immune system and resulting susceptibility to infections. In fact, their immature immune systems also pose challenges to both the quality and magnitude of vaccine responses. In that regard, an attenuated *Salmonella* Typhi strain was engineered to express and produce the YP F1 antigen (with the potential to protect against both plague and typhoid fever) and was used to immunize naive neonatal mice intranasally. Such immunization resulted in balanced IgG subtype response, production of memory B-cells, induction of robust cell mediated immunity, and enhanced activation of the professional antigen-presenting dendritic cells. Furthermore, newborn mice that had been primed with *Salmonella* Typhi (F1) and boosted with F1-alum were protected from a subsequent YP EV76 systemic IV challenge (Ramirez et al. 2009). When considering attenuated surrogate *Salmonella* strains, it becomes important to consider all of the available candidate strains especially when considering herd immunity and to not limit studies to the one strain being predominantly utilized.

Since both LPS and Braun lipoprotein (Lpp) of *Salmonella* Typhimurium function as potent stimulators of inflammatory and immune responses through the activation of TLR-4 and -2, respectively, we evaluated whether *lpp* knockout mutants (in conjunction with attenuation of the lipid A moiety's biological effects via the deletion of the *msbB* gene required for LPS biosynthesis) were effective live attenuated vaccine candidates to deliver YP antigens. We used *Salmonella* Typhimurium 14028 strain that harbors two copies of the *lpp* gene, *lppA* and *lppB*, (Sha et al. 2004), and we constructed a mutant *Salmonella* strain that was deleted for both of these copies. The *lppAB* double-knockout (DKO) mutant was avirulent in mice when given by the intraperitoneal (IP) and oral routes. Since the *lppAB* DKO mutant retained intact LPS that could still induce a significant inflammatory response, we constructed an *Salmonella* Typhimurium 14028 triple-knockout (TKO) mutant in which the *lppA* and *lppB* genes were deleted from a *msbB* gene (encoding an enzyme required for the acylation of lipid A) deletion mutant (Fadl et al. 2005a). Although highly attenuated, the TKO mutant was unable to survive and replicate within host cells in vivo. Concerned with whether such a TKO mutant would be able to generate an immunological memory response, we generated two additional DKO mutants in which either the *lppA* or the *lppB* gene was deleted in conjunction with the *msbB* gene. Such mutants were able to survive intracellularly within the host cells long enough to generate an immune response while inducing an attenuated inflammatory response in a mouse model of salmonellosis (Fadl et al. 2005b). Since our *lppB*/*msbB* mutant still had one functional copy of the *lpp* gene and the modified version of LPS (with much reduced biological activity), it was expected to significantly activate both innate and adaptive immune responses in the host with the induction of a minimal inflammatory response. Indeed such a mutant (e.g., *lppB*/*msbB*) represented an excellent vaccine candidate based on its ability to generate strong humoral and cell-mediated immune responses as well as its rapid clearance from the host tissues (Liu et al. 2008).

Consequently, we cloned and produced YP antigens, such as F1, LcrV, YopD, and YscF (the last three antigens represent components of the T3SS) from a pGEN222 plasmid. The pGEN222 plasmid contains a *hok-sok* system to optimize the expression of heterologous antigens in *Salmonella* strains for delivery to the human immune system (Galen et al. 1999). Recombinant pGEN222 plasmids containing any of the above-mentioned four genes were electroporated into the *lppB*/*msbB* mutant, and the production of these antigens was evaluated by Western blot analysis using specific sera to WT CO92. Subsequently, animals were immunized with these mutants orally, and, after 30 days, these mice were aerosol-challenged with a 10 LD_{50} of WT CO92. We observed 30% protection in animals immunized with the mutant producing F1, 100% with the mutant producing LcrV, 100% with mutants given simultaneously which produced F1 and LcrV, and 60% with mutants given simultaneously which produced YscF and YopD (Fig. 2).

Interestingly, *Salmonella* has not been the sole organism used as a surrogate/recombinant option for expressing and producing YP antigens. The lactic acid bacterium, *Lactobacillus plantarum*, was employed as a safe, well-tolerated recombinant strain for the expression and production of YP-derived LcrV. Since lactic acid bacteria have historically been consumed by humans, the prospects of developing a live attenuated oral vaccine candidate promised much less risk than employing an attenuated *Salmonella* strain. When cloned downstream of the signal sequence of *Borrelia burgdorferi* in the recombinant *L. plantarum* strain, LcrV was positioned within the desired peptidoglycan region, produced a balanced IgG subtype response in mouse serum,

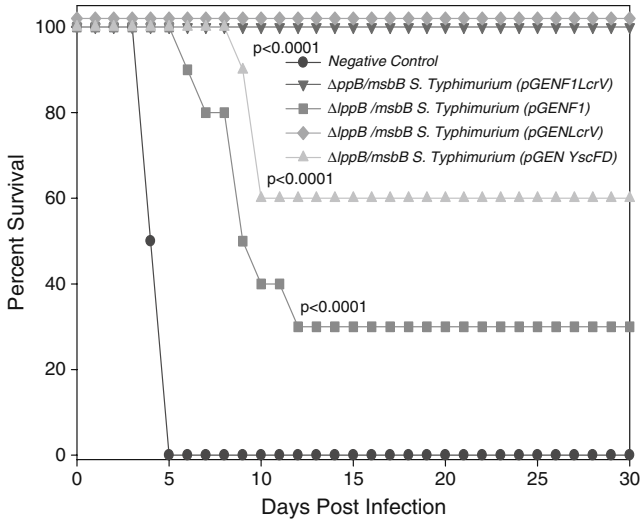

Fig. 2 Female Swiss-Wesbter mice (n=10/group) were orally immunized with *lppB/msbB* mutant strain of *Salmonella* Typhimurium (5×10^6 cfu or 2.5×10^3 cfu when combination was given) with various recombinant plasmids. The animals were given ampicillin in water (40 mg/kg/day) for a period of 7 days to maintain the plasmid. After 30 days, the animals were challenged via the aerosol route (10 LD_{50}) with YP CO92, and protection was observed for a period of 30 days. The data were analyzed by Kaplan–Meier's survival estimates, and p values of ≤0.05 were considered significant

exhibited significant levels of IgA in remote mucosal linings, including the lungs and vagina of inoculated mice, and produced a Th1-skewed pro-inflammatory response with some Th2 involvement (del Rio et al. 2010). Early indications suggest that the aforementioned recombinant lactic acid bacterial strain would be a good live attenuated vaccine candidate; however, no re-challenge studies using virulent YP were conducted. As a result, its true protective efficacy has yet to be determined.

Plant-based plague vaccines

Much effort has been placed into developing recombinant plant-based oral vaccines for plague (Alvarez and Cardineau 2010). Like the recombinant/surrogate bacterial plague vaccines, *lcrV* (encoding LcrV) and *caf1* (encoding F1) have been the transgenes mostly expressed in recombinant plant tissue. Relative to the poor immunogenecity of a surrogate, live attenuated vaccine, recombinant plant-based vaccines are even less immunogenic on account of their lacking key danger signals recognized by innate immune cells through their Toll-like receptors. To overcome this challenge, many researchers first prime with subcutaneous routes of the antigens together with an adjuvant (acting like a subunit vaccine) followed by an oral boost using the transgenic plant expressing and producing the same plague antigens.

Using the above-described methods (in most instances) coupled with the promise of lower costs associated with plant-based vaccine development, several positive results have been reported. In one such study, Swiss-Webster mice were immunized subcutaneously using tobacco leaves from the plant *Nicotiana tabacum* ectopically expressing and producing (from a chloroplast expression vector) an LcrV–F1 (F1-V) translational fusion protein. Surprisingly, 88% of mice boosted orally with the transgenic F1-V translational fusion protein survived a lethal dose of a YP CO92 aerosol challenge compared to only 33% of the mice primed and boosted with the same fusion protein via the subcutaneous route (Arlen et al. 2008). In a separate study, guinea pigs that were administered a transgenic *Nicotiana benthamiana* tobacco plant expressing and producing the F1-V fusion were protected against a subsequent pneumonic plague infection (Del Prete et al. 2009). In fact, efforts have been made to ensure that levels of antigen production within the recombinant plant tissue have been optimized, ensuring the greatest magnitude of immune response. By fusing the N-terminal of the γ-Zein protein (produced in maize by the endoplasmic reticulum which induces protein body formations) to an F1-V fusion antigen, the expression and production of the γ-Zein fusion protein (F1-V) was found to accumulate to levels of up to three times higher than those of the F1-V fusion protein alone in a variety of plant tissues, including those derived from *N. tabacum* (Alvarez et al. 2010).

However, expressing plague transgenes in various plant tissues is a biologically unnatural event that could produce undesirable effects or, as alluded to above, not produce the desired effect at all (i.e., not have plague antigen stably expressed and produced at levels required for eliciting a robust immune response). In one such case, the plague F1-V fusion protein was not produced in transgenic tomato plants that were confirmed to contain the *f1-v* fusion gene as judged by Southern blot analysis. The unapparent F1-V production was found to be a result of RNA silencing, and that reversion of the *f1-v* silencing could be achieved by ectopically expressing P19, a viral suppressor of RNA silencing derived from the tomato bushy stunt virus. By employing P19, vaccine producers can use transgenic tomatoes to express and produce their antigens of interest, ensuring the highest expression levels (Alvarez et al. 2008). In addition to plague, the development of plant-based vaccines for other select agents (variola virus and *Bacillus anthracis*) are currently being evaluated (Rigano et al. 2009); however, the above-mentioned limitations and countermeasures must be taken into consideration when determining the specific plant to employ as well as achieving optimal expression/production levels. Indeed a recent study showed that the fusion of the extracellular domain of human capillary morphogenesis protein 2, a receptor for the protective antigen of *B. anthracis*, with human IgG Fc, when produced in tobacco plant expression

systems, provided protection to rabbits against a lethal challenge with *B. anthracis* spores (Wycoff et al. 2011).

Tobacco leaves and tomato plants alone are not the only putative plant-based plague vaccines being tested. Recently, carrot tap roots and lettuce have also been evaluated for their potential use as plant-based plague vaccines. In the case of both transgenic carrots and lettuce plants, the stable expression and production of an F1-V fusion protein was noted and, following subcutaneous challenges in mice, strong humoral responses were observed, confirming the retention of immunogenecity of the recombinant transgenes in both plant tissues (Rosales-Mendoza et al. 2010a; Rosales-Mendoza et al. 2010b). Taken together, several studies employing various animal models have been carried out to evaluate the protective correlates of plant-based plague vaccines, and these alternative plague vaccines do show promise and offer a more economical means of plague vaccine production. However, additional animal models need to be tested, including non-human primates, before such alternative plague vaccines become more widely championed.

DNA-based plague vaccines

In addition to plant-derived oral plague vaccines, considerable attention has also been given to alternative DNA-based vaccines. Since much stock has been placed in LcrV and F1 antigens, many of the DNA-based plague vaccines have also been engineered to contain either all or part of the open reading frames encoding the aforementioned plague antigens as well. In one such study, a minimal length of DNA required to encode a 127-amino-acid residue region of LcrV was found to elicit a strong humoral response (with an IgG1) bias in mice following three doses delivered via a gene gun. Furthermore, following an intraperitoneal (IP) challenge with virulent YP, mice vaccinated with the truncated *lcrV*-DNA vaccine enjoyed greater than 60% survival compared to only 33% survival in mice vaccinated with the empty vector alone (Vernazza et al. 2009).

Like all vaccines, excluding live attenuated ones, DNA vaccines clearly require boosting as well as the addition of adjuvants to increase their immunogenicity and enhance the overall immune response. In one such study, IL-12 was additionally cloned into the same plasmid containing either the F1 or LcrV antigen-encoding genes and applied to mice intranasally. On account of IL-12 serving as a molecular adjuvant that promoted IgA production in the mucosa, the immunized mice were 80% protected from a subsequent pneumonic YP challenge (Yamanaka et al. 2008). Furthermore, following an intramuscular (IM) vaccination, the DNA vaccine encoding IL-12/LcrV induced a stronger cell-mediated immune response than did the DNA vaccine encoding IL-12/F1-V (fusion protein), and boosting with proteinaceous F1 antigen enhanced the overall magnitude of the humoral response (Yamanaka et al. 2009). However, the molecular adjuvant employed can greatly affect the immune-protective correlates of DNA vaccines in addition to the routes of administration. More specifically, achieving optimal protection following immunization with an F1-V DNA vaccine required low expression levels of the molecular adjuvant IL-12 and, when administered intranasally, conferred 80% protection in mice from a subsequent YP pneumonic challenge. However, poorer protection was observed when IL-12 was over-expressed, strongly suggesting that the expression levels of various molecular adjuvants, like IL-12, need to be optimized prior to their inclusion in DNA-based vaccines (Yamanaka et al. 2008).

When another putative molecular adjuvant, the chemokine lymphotactin, was tested after being expressed and produced in a DNA vaccine co-expressing/producing the F1-V fusion protein, its intramuscular administration conferred better protection against a subsequent pneumonic plague challenge than did an IN administration of the same vaccine (Yamanaka et al. 2010). Although promising, it becomes imperative that putative molecular adjuvants be evaluated by co-expressing multiple plague antigen-encoding genes from DNA vaccines and be administered via multiple routes in animals before definitive statements are made about their potential usefulness in DNA-based plague vaccines. One important consideration for DNA-based plague vaccines is codon optimization for expression in mammalian cells, and most studies have taken this into consideration. Following such codon optimization, LcrV production from a DNA-based plague vaccine was higher in mice and induced a greater magnitude of humoral response, and better protection against a subsequent mucosal challenge of YP was achieved. More importantly, however, DNA-based vaccination with an additional plague antigen (YscF—the T3SS needle tip protein) fused to F1 resulted in better protection against a subsequent YP infection than did vaccination with either F1 or YscF (Wang et al. 2010a). The aforementioned findings emphasize the need to further investigate additional plague antigens for their protective roles in DNA-based vaccines and, by extension, all vaccine types. We cannot limit our studies to F1 and V antigens exclusively.

Our own study has shown that mice immunized with a plasmid vector containing genes for F1 and LcrV by use of a gene gun were protected against pneumonic plague (Fig. 3). To enhance the immunogenicity of the DNA vaccine, we included the gene encoding the heat-labile enterotoxin (LT) of *E. coli* as a molecular adjuvant in our plasmid construct. Although we noted 40% protection of immunized mice, further standardization of the immunization protocols could lead to increased protection. Impor-

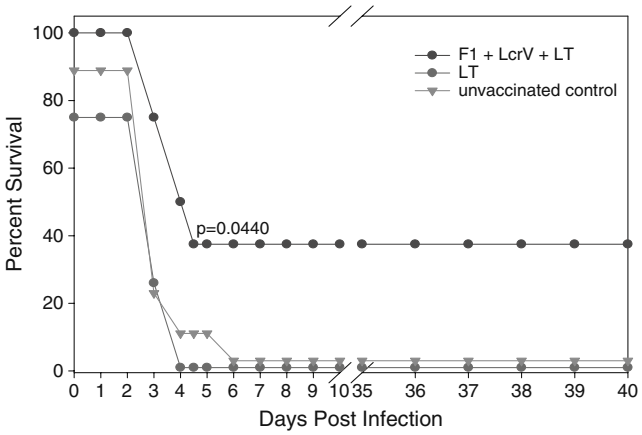

Fig. 3 Mice immunized with plasmid vectors (from PowderMed and now part of Pfized, Inc.) containing genes for F1, LcrV, and LT were protective against pneumonic plague. Female Swiss-Webster mice (n= 12) were immunized with recombinant plasmids coated with 1.6-μm gold particles and shot with the gene gun (Bio-RAD, Hercules, CA, USA) on the ears. The animals were immunized on days 0 and 3 months before intranasal challenge after 8 months following the last boost with a 10 LD_{50} of YP CO92. The mortality data were analyzed by Kaplan–Meier's survival estimates and p values of ≤0.05 were considered as significant

tantly, we noted protection from pneumonic plague 32 weeks after the last immunization of mice with the above-mentioned DNA vaccine.

Virus vector-based vaccines

Live avirulent or attenuated recombinant viruses expressing genes encoding plague antigens can be engineered as easily as the bacterial recombinant plague vaccines and offer several advantages over their bacterial counterparts. Non-enveloped/naked viruses persist for long periods of time in the environment without losing their infectivity, making them attractive options for vaccinating animal reservoirs of YP in their native environments. In fact, the term vaccine is derived from the vaccinia virus (the etiological agent of cow pox) which served as the first vaccine developed by Edward Jenner to protect against the variola virus (the etiological agent of small pox).

In one such case, vaccinia was used to develop a reservoir-based plague vaccine in which plague-derived LcrV and F1 antigens were expressed and produced as an F1-V fusion protein (which has been the strategy for several other vaccines as well). The overall rationale for such a vaccine design is to minimize the spread of the pathogen in the wild among reservoir animals, thereby concomitantly decreasing the incidence of human infection. The recombinant, vaccinia virus-based vaccine was orally administered at a dose of 10^8 pfu to C57/BL6 mice and found to successfully elicit a strong dose-dependent humoral response that provided 100% protection to vaccinated animals against a pneumonic challenge of ten times the lethal dose of YP KIM/D27. Most importantly, protection in vaccinated mice was observed up to 45 weeks following the administration of the oral recombinant F1-V producing vaccinia-based vaccine (Bhattacharya et al. 2010).

However, to truly characterize the efficacy of a reservoir-based plague vaccine, mice are not the best model to employ. Rather, actual YP reservoirs should be used instead (if possible) to better reflect the dynamics in that population. In that vein, a recombinant raccoon pox virus producing plague-derived F1 antigen was orally administered to an actual YP reservoir, prairie dogs (*Cynomys* spp.). To achieve this, wild prairie dogs were captured in South Dakota and orally administered with the recombinant raccoon pox virus in pelleted food. There was an observed dose-dependent humoral response with regards to the number of doses consumed by the prairie dogs. Additionally, the vaccinated animals were 38% protected from a subsequent subcutaneous challenge with the fully virulent YP CO92 strain relative to only 11% in animals that received an empty raccoon pox virus alone (i.e., not producing the YP F1 antigen) at doses closely mirroring those physiologically contracted through a flea bite (Rocke et al. 2008). Furthermore, when the same group orally administered two recombinant raccoon pox viruses producing the F1 antigen and a 307-amino-acid residue truncated form of LcrV, they were able to achieve a stronger humoral response and better protection in both vaccinated mice and prairie dogs compared to animals vaccinated with raccoon pox virus not producing any plague antigens upon challenge with virulent YP CO92 via the subcutaneous route of infection (Rocke et al. 2010a, b).

The generalization of a vaccine response using only one type of vector/vaccine preparation and one route of immunization does not provide a complete picture with regards to correlates of protection. To address this, a separate study that employed a modified vaccinia Ankara recombinant vector expressing and producing either the full-length F1 or the 307 amino acid residue truncated form of LcrV and utilized an IM route of immunization was carried out. As it turned out, IM immunization with 5.0×10^7 pfu of the Ankara recombinant vector producing the truncated form of LcrV provided the best protection (85%) against both an IN and an IP YP CO92 challenge while the recombinant vaccinia vaccine producing F1 provided less protection (50%) (Brewoo et al. 2010).

Poxviruses alone are not the sole viral vector being developed as recombinant vaccines for YP. Recently, modified, non-replicating adenovirus vectors have been evaluated for their potential use as viral-based plague vaccines. In fact, one somewhat counterintuitive study focused on developing a YP recombinant adenovirus-based vaccine that expressed and produced both the heavy and light chains of a previously

identified anti-LcrV protective antibody rather than a plague antigen. Surprisingly, C57/BL6 male mice that were administered the aforementioned recombinant vaccine IV produced significant levels of IgG that persisted for up to 12 weeks. Furthermore, upon IN challenge with a 2×10^4-cfu dose of fully virulent YP CO92, the vaccinated mice enjoyed 80% protection compared to 0% surviving mice who received the replication-deficient adenovirus vector alone (Sofer-Podesta et al. 2009). This less-than-conventional plague vaccine addresses the threat of a true bioterrorist attack (in real time), in which individuals would have little time to seek treatment and/or the delivery of a vaccine. Consequently, the lag time associated with an active immune response (where the patients develop their own neutralizing antibodies) could result in death. To counter that lag, the above-mentioned "quasi-passive immunity"-based vaccine results in the production of highly effective neutralizing anti-LcrV antibodies within 1 day of administration, achieving peak antibody levels within 3 days (Sofer-Podesta et al. 2009). In a more conventional manner, the same adenovirus vector was employed for the development of an F1- or LcrV-expressing and -producing fusion protein tethered to an adenovirus capsid protein. The aforementioned recombinant adenovirus vectors were administered at a dose of 10^9–10^{11} pfu via the IM route. Four weeks following the immunization, a boost via the same route and dose was administered, and all mice were intranasally challenged 6 weeks later with a 2×10^4-cfu dose of fully virulent YP CO92. Immunized mice were significantly better protected than mice that were immunized with only the purified F1 protein plus a conventional adjuvant (Boyer et al. 2010).

An earlier study by Chiuchiolo et al. (2006) also showed that a single administration of a replication-defective adenoviral gene transfer vector encoding the LcrV antigen could stimulate protective immune responses after a single IM immunization. Furthermore, immunized mice were protected from a lethal IN challenge of YP CO92 from 4 weeks up to 6 months after immunization. In addition, Van Blarcom et al. (2010) reported that affinity maturation of a neutralizing LcrV antibody delivered by the adenoviral vector system conferred increased protection to mice against pneumonic plague.

Pox viruses and adenovirus have also been joined by the innocuous human vesicular stomatitis virus (VSV) as a potential viral candidate for the development of a novel plague vaccine. VSV was engineered to express and produce either a secreted or non-secreted form of the plague antigen LcrV. Following IM vaccination of female BALB/C mice with the recombinant VSV, strong humoral responses with an IgG2a bias (more pronounced than when employing the non-secreted form of LcrV) were observed. Furthermore, animals immunized with recombinant VSV expressing and producing the non-secreted from of LcrV demonstrated 90% protection from a subsequent YP CO92 IN challenge with 10,000 cfu, ten times the LD_{50} (Chattopadhyaya et al. 2008). Taken together, viral-based plague vaccines have shown promise, especially for plague reservoirs, and have the advantage of the virion itself, possessing intrinsic adjuvant properties and resulting in a more robust immune response.

Subunit plague vaccines

The majority of resources and energy invested in plague vaccine development seems to have been disproportionately applied to the pursuit of various plague subunit vaccines, especially F1, LcrV, and F1–LcrV fusion subunit vaccines (Smiley 2008a and references therein), and even a reverse LcrV–F1 fusion protein has been characterized (Goodin et al. 2010). LcrV, in particular, has been heralded as the most important protective plague antigen, and reductionist studies have revealed that amino acid residues 196–225 are sufficient and essential in protecting BALB/C mice from a subsequent YP CO92 subcutaneous challenge (Quenee et al. 2010). Other studies have revealed a protective role played by LcrV residue N255 which lies just beyond the aforementioned protective region (Hill et al. 2009). Subunit plague vaccines comprised of capsular antigen F1 and T3SS-associated LcrV are in clinical trials, and these proteins are well tolerated by humans and appear safe (Williamson et al. 2005). In fact, IM administration of an F1-truncated LcrV (270 residues) fusion protein to BALB/C mice resulted in the long-term maintenance of protective antibodies to both antigens up to 518 days after the initial immunization and they were protected at that same time point by a YP 141 challenge (Wang et al. 2010b). These vaccines provide substantial protection in animal models against pneumonic plague; however, non-human primates are much less protected, and the immunogenicity of such vaccines in humans is weak (Smiley 2008a; Williamson et al. 2005; Wang et al. 2008; Cornelius et al. 2008). More importantly, the immune correlates of protection are unknown; therefore, the extent of protection provided by such vaccines in humans cannot be predicted (Titball and Williamson 2004; Bashaw et al. 2007).

Like DNA-, plant-, and inactivated virus-based vaccines, subunit vaccines also suffer from compromised immunogenicity. In one recent study using IM routes of immunization, subunit vaccine conjugates of LcrV–F1 were compared directly to live attenuated YP EV76. LcrV–F1 subunit vaccine preparations were more effective than YP EV76 in eliciting anti-LcrV IgG but were less effective in inducing the Th1 cytokine, IL-12, suggesting a weaker cell-mediated immune response (Qiu et al. 2010). The compromised immunogenicity of subunit vaccines can be overcome, in many cases, through the use of an adjuvant.

Cationic liposome–nucleic acid complexes is one such adjuvant that, when combined with F1 and administered orally to C57/BL6, produced a greater magnitude of anti-F1 antibodies than did the delivery of the same preparation via the subcutaneous route as well as conferring protection against a subsequent pneumonic challenge with a fully virulent strain MG05 of YP (Jones et al. 2010). In another study, cytosine-phosphate-guanine (CpG) oligonucleotides (an adjuvant/immunomodulator) were included in a F1–LcrV subunit vaccine administered to BALB/C mice via the subcutaneous route, resulting in significantly enhanced IgG2a antibody production and protection against a subsequent subcutaneous challenge of YP CO92 (5×10^7 cfu) or aerosol challenge (22 times the LD_{50}) (Amemiya et al. 2009). However, the F1-minus strains of YP are highly virulent, and different strains of this pathogen might have variants of LcrV (Smiley 2008a; Titball and Williamson 2004). Further, full-length LcrV might be immunosuppressive in nature and cause Th2 cytokine polarization (Murphy et al. 2007), although several studies have suggested the contrary (Reithmeier-Rost et al. 2007; Pouliot et al. 2007). Therefore, such F1–LcrV-based vaccines might not be optimal. Additionally, since protection against pneumonic plague is not fully dependent on the humoral immune response alone, there is an additional concern of cell-mediated immunity triggered by the current vaccine subunit vaccine components, which play an important role in protection (Smiley 2008a; Smiley 2008b).

Despite that fact, LcrV has been given much attention as both a subunit vaccine candidate as well as a preferred antigen for expression in surrogate/recombinant live attenuated strains. In that vein, LcrV was immobilized on non-living *Lactococcus lactis* particles and administered intranasally to newborn mice modeling the immunization of human newborns. What ensued was the induction of humoral and cell-mediated immunity in addition to enhanced dendritic cell maturation that secreted Th1-biased cytokines. Furthermore, 100% of *L. lactis* particle-immobilized LcrV-immunized animals were protected from a 30X lethal IV dose of YP EV76 compared to only 20% survival of animals immunized with either LcrV or *L. lactis* particles alone (Ramirez et al. 2010). Based on the aforementioned data, LcrV could potentially serve as an effective subunit vaccine candidate if conjugated to a well-characterized protein partner. In addition to LcrV, the F1 antigen also receives a plethora of attention regarding its status as a subunit vaccine candidate. However, since F1 is not required for the pathogenesis of pneumonic plague in mice and non-human primates, additional immunogenic antigens must be examined for the new recombinant plague vaccine which will not only provide a more balanced adaptive immune response but also protect the host against the F1-minus strains of YP (Murphy et al. 2007).

However, as seen in plant-, DNA-, and viral vector-based plague vaccines, LcrV–F1 fusion proteins have also been commonly characterized as a fusion protein subunit vaccine. For example, 250 μg of an LcrV–F1 fusion protein, expressed in and purified from the tobacco plant *N. benthamiana*, was administered via the subcutaneous route to cynomolgus macaques and boosters at days 14 and 28 (via the same route at the same dose) were also administered. An analysis of the vaccinated animals' sera revealed strong humoral responses replete with anti-LcrV and anti-F1 antibodies (primarily of the IgG1type), and immunized mice were 100% protected following 100 times the LD_{50} IN challenge with YP CO92 (Chichester et al. 2009). Still, novel methods of F1-V vaccine delivery are always being developed to further improve the subunit vaccines' correlates of protection. Recently, an F1–LcrV subunit vaccine in powder form was developed to produce a more stable vaccine (than the more conventional liquid preparation) and for it to be potentially delivered intranasally. When the IM, IN, and intradermal (ID) routes of immunization with 10 μg of powder-based F1–LcrV vaccine were compared for their efficacy in female Swiss-Webster mice, 70–90% protection was achieved from a subsequent subcutaneous challenge with 1,000 times the LD_{50} of YP CO92 following both IM and ID routes, and similar levels of protection were also observed for the IN route of immunization after an additional boost (Huang et al. 2009).

Alternatively, the LcrV antigen of YP was fused to an anchor protein which enabled loading onto a Gram-positive enhancer matrix (GEM) particle and was characterized for its immunogenicity. Using near-field scanning optical microscopy and atomic force microscopy (both providing resolution at the individual molecule level), the LcrV fusion protein was found to cluster and bind tightly to the GEM particle, and it was also found that this interaction was only disrupted at acidic pH (comparable to that in the phagosome environment within macrophages). Furthermore, when delivered to mice via the IN route, a significant difference in the IgG humoral response was observed compared to anti-LcrV levels in mice immunized with either LcrV alone or GEM alone (Zeng et al. 2009). Similarly, biodegradable polymeric delivery systems have been employed to stabilize plague antigens in subunit vaccine preparations (Carrillo-Conde et al. 2010), and various LcrV epitopes enveloped in poly (DL-lactide-co-glycolide) microparticles were successfully delivered intranasally to BALB/C mice, resulting in the protection against a 10^6 lethal dose by IP challenge with YP 195/p (Uppada et al. 2009). Furthermore, the IN delivery of microencapsulated F1 and LcrV protective antigens yielded ten conjugates that showed a strong Th1 cytokine profile response to the vaccine in peripheral, secondary lymphoid tissues, demonstrating the ability to involve cell-mediated immunity in the vaccine immune response (Gupta et al 2010).

Flagellin has also been characterized as a potent adjuvant of vaccines (since it binds to TLR-5 on multiple innate immune cell types) and was used to make a flagellin-F1–LcrV fusion protein. BALB/C mice, African green monkeys, and cynomolgus monkeys all demonstrated robust humoral responses to plague antigens when immunized and boosted with the flagellin fusion protein through either the IN or the IM route. Furthermore, immunized mice were 100% protected against a subsequent YP CO92 respiratory challenge, strongly suggesting that the inclusion of flagellin into a plague subunit vaccine can greatly augment vaccine efficacy (Mizel et al. 2009). Similarly, lipid A mimetics have been used as molecular adjuvants that are potent activators of the innate immune system through interactions with TLR-4. Following an IN immunization and priming with F1- and LcrV-purified antigens (coupled with lipid A mimetics) in both BALB/C mice and Sprague–Dawley rats, 63% and 90% protection from a subsequent YP CO92 respiratory challenge was achieved using 100 and 1,000 times LD_{50}, respectively (Airharta et al. 2008).

However, as alluded to earlier, the exploitation of other potentially protective plague antigens (other than LcrV and F1) is fully warranted and could reveal even better candidates for subunit vaccine development. In addressing this need, a Yop BDE complex, derived and purified from YPT, was used to subcutaneously immunize BALB/C mice, which resulted in a robust humoral response with anti-Yop E, B, and D IgG antibody production. Furthermore, immunized mice were shown to be protected against a lethal dose of a YP KIM5 $F1^-$ strain administered intravenously. Unfortunately, the immunized mice were not protected against an $F1^+$ YP challenge (Ivanov et al. 2008), dampening the enthusiasm regarding this plague vaccine candidate. In a more promising report, LcrV pulsed directly onto antigen-presenting dendritic cells was successful in inducing both humoral and a cell-mediated immunity that conferred protection to IP immunized BALB/C mice upon an IN YP CO92 challenge at a 100 times the LD_{50} (Do et al. 2010).

Discussion

With the threat of bioterrorism, the need for effective, prophylactic vaccines against select agents is of paramount importance. YP is one such select agent that is an attractive candidate for bioterrorism on account of its facile cultivation, facile genetic malleability (including the introduction of antimicrobial resistance genes), and air-borne communicability via charged respiratory droplets, resulting in person–person transmission. In the event of a bioterrorist attack, rapid dissemination of the most effective vaccine will not be enough to prevent disease and death to those that have already been exposed. Therefore, a combined therapeutic approach will need to be taken including: vaccination, passive immunization or DNA vaccine-expressing protective antibodies against plague antigens (Sofer-Podesta et al. 2009), and treatment with antibiotics like ciprofloxacin or levofloxacin. In fact, one such study that employed a subunit vaccine with either F1 or LcrV antigens coupled with ciprofloxacin treatment resulted in mice being protected against an aerosol challenge of YP strain GB carried by both large (12 μm) and small (1 μm) aerosol particles (Thomas et al. 2009). Based upon a study using Swiss-Webster mice as an animal model, protective antibody titers in immunized individuals could be measured and used as a gauge to approximate disease onset (Little et al. 2010), thus helping healthcare professionals better manage individual cases/patients.

Additionally, unconventional attacks, including bioterrorist attacks, are exceedingly difficult to predict. In fact, such an attack could include more than one microbial pathogen. Considering such a scenario, one group studied the efficacy of a plague/anthrax combined subunit vaccine. Mice and rabbits both exhibited robust antibodies to plague F1 and LcrV antigens as well as anthrax-protective antigen. Furthermore, mice were protected from a 10^7 subcutaneous dose of YP strain 141, and rabbits were fully protected against a 1.2×10^5-cfu subcutaneous challenge of *B. anthracis* spores (Ren et al. 2009).

Currently, there is no effective plague vaccine that presents limited host toxicity, so investigations are ongoing to evaluate multiple plague vaccine platforms including the development of live attenuated vaccine strains, surrogate/recombinant live vaccine strains, plant-based vaccines, DNA-based vaccines, viral-vector based vaccines, and protein subunit vaccines (as mentioned above). Despite these efforts, no single vaccine has shown promise in all animal models tested (including non-human primates) and, as a result, further studies are needed to not only identify new immunogens for recombinant plague vaccines but also develop live attenuated vaccines; both approaches could, and perhaps should, be coupled in one vaccine preparation. One of the biggest hurdles is vaccine safety, and since plague is such a deadly infection, live attenuated vaccines, although the most immunogenic, are generally frowned upon and instead safer subunit vaccines are preferred (with no threat of reversion to full virulence). As an outgrowth to generating safer live attenuated plague vaccines, a recombinant *Salmonella* Typhimurium RASV strain or a *lppB/msbB* mutant strain expressing plague antigenic transgenes has been used. Although a safer option than using the attenuated YP EV76 strain, *Salmonella* Typhimurium is an enteric pathogen transmitted via the oral–fecal route, much like YP's closely related cousins, YPT and YE. Therefore, additional boosting with acellular YP antigens (via some other route, e.g., IM) might also be needed to synergisti-

cally improve the immune response since a YP infection does not follow the same oral–fecal route of transmission.

Another limitation is that most pneumonic plague animal models of infection employ clinically derived, fully virulent YP strains and require bio-safety level (BSL) 3 containment facilities for associated studies. Unfortunately, not all researchers have access to such facilities. However, recently, a BSL-2 murine model of pneumonic plague infection that uses the attenuated YP KIM5 strain, which has a large chromosomal deletion in the pigmentation locus and lacks the siderophore yersiniabactin, has been developed (Galvan et al. 2010). However, given the availability of BSL3 facilities and more clinically relevant strains of YP (e.g., CO92), the physiological relevance of using a BSL2 model for vaccine development becomes less attractive to the vaccine development community.

Finally, when developing plague vaccine platforms, it is also important to consider the use of multiple animal models when testing vaccine efficacy, and many of the included studies have achieved that in testing murine, rat, and non-human primate models. One recent study compared mouse, guinea pig, and rabbit models of infection for an LcrV–F1 fusion subunit vaccine candidate. The guinea pigs exhibited greater variability in protection against virulent YP strain 141 challenge following vaccination, and the authors suggested using rabbit as an alternative model when characterizing vaccine efficacy (Qia et al. 2010). However, that raises bigger issues with the physiological relevance of using non-human primate models when animal models that are more closely related to humans exist. However, considering the high costs associated with non-human primate models, exploratory vaccine studies with greater risk might be more appropriately tested in more cost-effective animal models.

Ultimately, despite progress being made on multiple fronts of vaccine development for YP, the goal of producing a widely adopted plague vaccine still has yet to be achieved. Despite the various technical challenges described above, we are presently in a greater need of a highly effective plague vaccine than ever before. With the threat of bioterrorism and YP, being designated as a re-emerging pathogen by the WHO, a widely available, cost-effective vaccine is the only true panacea and protective measure. However, in achieving this, the research community must not limit their resources to the exclusive study of F1 and LcrV as potential plague vaccine candidate antigens. Additional plague antigens need to be identified and characterized for their potential employment in subunit vaccine design, and the development of a safe, attenuated YP vaccine strain should not be discouraged since live attenuated vaccines have the greatest immunogenicity. Perhaps the best plague vaccine will be comprised of a combination of live attenuated strain coupled with either subunit or DNA-based vaccine boosts. Regardless, the plague predicament will not "go away" until effective prophylactic measures (e.g., the development of a plague vaccine) are in place to protect the masses.

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