HHS Public Access

Author manuscript

Adv Exp Med Biol. Author manuscript; available in PMC 2019 June 11.

Published in final edited form as:

Adv Exp Med Biol. 2016 ; 918: 313–360. doi:10.1007/978-94-024-0890-4_12.

Chapter 12 Plague vaccines: status and future

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Abstract

Three major plague pandemics caused by the Gram-negative bacterium *Yersinia pestis* have killed nearly 200 million people in human history. Due to its extreme virulence and the ease of its transmission, *Y. pestis* has been used purposefully for biowarfare in the past. Currently, plague epidemics is still breaking out sporadically most of parts of the world, including the United States. Approximately 2,000 cases of plague are reported each year to the World Health Organization. However, the potential use of the bacteria in modern times as an agent of bioterrorism and emergence of a *Y. pestis* strain resistant to eight antibiotics brings out severe public health concerns. Therefore, prophylactic vaccination against this disease holds the brightest prospect for its long-term prevention. Here, we summarize the progress of current vaccines development for counteracting plague.

Keywords

Yersinia pestis; plague; vaccines

1. Brief history of plague vaccines

Y*ersinia pestis*, the causative agent of plague, is an aerobic, non-motile, gram-negative bacillus. Plague is a deadly disease that has impacted humans for at least 1,500 years [1] and it continues to be a disease of significant concern. The destructive potential of plague is evident from three major pandemics: the Justinian plague of the 6th and 7th centuries that affected North Africa, Europe, Central and Southern Asia, and Arabia; the second pandemic in Europe, which killed one third of the Western European population (including the Black Death of 1347–1351 A.D.); and the third pandemic, which originated in China in 1855 and spread around the world via ship-borne rats [2]. Overall, *Y. pestis* is estimated to have killed 100–200 million individuals throughout history, making it one of the worst human infectious diseases. It is also considered a reemerging disease [3, 4], and most of the several thousands of human cases each year are now reported from Madagascar and other countries in Africa.

Although it does not match the “big three” (malaria, HIV/AIDS, and tuberculosis) in number of people annually affected in the contemporary era, it is far more pathogenic and has the potential to spread much more rapidly than these other diseases [5]. Plague remains as one

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of the top five bioterrorism threats [6] and a CDC Tier 1 Select Agent pathogen. Therefore, there is an urgent need for effective means of pre-exposure and post-exposure prophylaxis. Owing to the short incubation period, treatment with antibiotics, and possibly monoclonal antibodies and drugs inhibiting mediators of pathogenicity, offers the best prospect for post-exposure prevention of disease. However, *Y. pestis* strains resistant to multiple drugs have been isolated from plague patients in Madagascar, which may spread multiple antibiotic resistance encoding genes to plague reservoirs [7–9]. For longer-term protection and to counter drug resistance, vaccination is believed to be crucial [10, 11]. The development of vaccines got an early start in 1897, when Waldemar Haffkine (1860–1930) showed that a heat-killed culture of plague bacteria protected rabbits against experimental infection. This preparation was tested in humans in India, with >20 million doses being given, resulting in observations of reduced incidence and mortality in immunized persons [12]. In an effort led by Meyer, starting in 1939 [13, 14], the US Army developed a formalin-killed *Y. pestis* vaccine that was given to more than a million American servicemen deployed to Vietnam [14]. Plague Vaccine (USP), a formalin preparation of the fully virulent strain *Y. pestis* 195/P, was the first FDA licensed plague vaccine for human use in the United States and the United Kingdom [13, 14]. Controlled clinical trials have not been reported, but studies of United States military personnel during the Vietnam War strongly suggest that formalin-killed, whole-cell vaccines protect against bubonic plague [15, 16]. However, these vaccines cause significant adverse reactions, particularly after booster injections, which are needed to maintain protection [17]. Moreover, they generally fail to protect mice and non-human primates against pulmonary *Y. pestis* challenge, and several humans contracted pneumonic plague despite immunization with this vaccine [18]. In the US since 1999, lack of effective protection against pneumonic plague, adverse reactions such as fever, headache, lymphadenopathy and the need for booster injections eventually resulted in diminished interest of the USP vaccine [19–21]. Currently, USP vaccine is still used for research only in UK [22, 23]. Thus, killed whole cell vaccines are probably not suitable for defense against weaponized pneumonic plague.

In 1931, Georges Girard and Jean Robic developed a live attenuated non-pigmented strain of the plague bacillus in Madagascar called EV [24]. This vaccine or similar live attenuated bacteria with designations including EV76, EV NIIEG and Tjiwide were administered to millions of people in Madagascar, Indonesia, Vietnam, and the Soviet Union [24, 25]. *Vaccinum pestosum vivum siccum* on the base of the strain EV line NIIEG is still used and commercially available in Russia (http://www.epidemiolog.ru/catalog_vac/?SECTION_ID=&ELEMENT_ID=476) and Kazakhstan (http://pharmprice.kz/annotations/vakcina-chumnaya-zhivaya-suhaya/) [26]. By the end of the 20th century, these vaccines were rarely used outside of Russia due to their strong adverse reactions. Although considerable progresses have been made for developing safe effective vaccines against plague for human use, a licensed plague vaccine has not been released into commercial market yet. Here, we summarized current progresses in development of plague vaccines.

2. Subunit vaccines

Searching for new antigens from *Y. pestis* is a continuous endeavor for developing plague vaccines. Table 2 listed *Y. pestis* antigens that were evaluated for vaccine purpose.

Lipopolysaccharide (LPS) is an integral component of the outer membrane of Gram-negative bacteria and can be used as an immunogenic molecule [27–34]. Prior et al showed that the LPS extracted from *Y. pestis* strain GB stimulated the production of TNF-α and IL-6 from mouse macrophages, but was less active in these assays than LPS isolated from *Escherichia coli* strain 0111. They also indicated that an antibody response to LPS in mice was primed by LPS immunization, but this response did not provide any protection against 100 MLD of *Y. pestis* strain GB [35]. The pH 6 antigen (PsaA) was initially described in 1961 as an antigen synthesized by *Y. pestis* and formed fimbria-like structures on the bacterial surface at the temperature close to body temperature of mammals (35–41 °C) and acidic pH values (5.8–6.0) close to the pH of abscesses or phagolysosomes in macrophages [36]. PsaA serves as an important adhesin in the establishment of *Y. pestis* infections [37–39]. The lack of PsaA synthesis in the *Y. pestis* KIM5 strain caused virulence reduction and an increase in the LD_{50} of at least 100 fold in mice after retroorbital injection [40]. However, the loss of synthesis or constitutive production of pH 6 antigen in the fully virulent wild-type strains 231 and I-1996 did not influence their virulences or the average survival time of subcutaneously inoculated BALB/c mice [41]. Rabbits immunized with a live EV76 vaccine strain primed high levels of anti-PsaA (IgG) at 42 days after initial immunization [42] and also mice immunized with the EV76 produced a strong T-cell response to PsaA [43]. Schifferli's group showed that mice immunized with 40 μg of PsaA adjuvanted with alhydrogel primed a strong humoral immune response and provided a significant protection (70%) against a intranasal infection with *Y. pestis* KIM5 (Pgm-) in the iron dextran-treated mouse model [44]. However, no protection was shown in the case of immunization with PsaA protein against subcutaneous infection with fully virulent *Y. pestis* strains 231 and I-1996 [41].

Benner et al determined the humoral immune response to *Y. pestis* antigens in mice that survived lethal *Y. pestis* aerosol challenge after antibiotic treatment, such as F1, V antigen, YpkA, YopH, YopM, YopB, YopD, YopN, YopE, YopK, plasminogen activator protease (Pla), and pH 6 antigen as well as purified lipopolysaccharide [45]. Their results indicated that the major antigens recognized by murine convalescent sera were F1, LcrV, YopH, YopM, YopD, and Pla [45]. Andrews et al purified the recombinant proteins (YpkA, YopD, YopE, YopH, YopK, and YopN) and evaluated the role of *Yersinia* outer proteins (Yops) in conferring protective immunity against plague in mice injected with above these proteins. Most Yop-vaccinated animals succumbed to infection with either wild-type encapsulated *Y. pestis* or a virulent, nonencapsulated isogenic variant. Vaccination with YpkA significantly prolonged mean survival time but did not increase overall survival of mice challenged with the nonencapsulated strain. Only immunization with YopD provided significant protection for mice against challenge with the nonencapsulated *Y. pestis* strain [46].

Straley's group firstly found that *Y. pestis* YadB and YadC, two new members of the Oca (oligomeric coiled-coil adhesins) family of proteins [47, 48], have the ability to form trimers and correlate with invasion of *Y. pestis* into epithelioid cells [49]. Loss of *yadBC* caused a modest loss of invasiveness for epithelioid cells and a subtle decrease in virulence for bubonic plague but not for pneumonic plague in mice [49, 50]. But immunization with the GST-$YadC_{137–409}$ protein, which fused YadC aa 137 to 409 to C terminal of glutathione S-transferase (GST), provided partial protection against $F1^-$ *Y. pestis* challenge in mice and

was found to stimulate mixed Th1/Th2 responses [51]. However, Sun et al showed that YadC810 protein immunization could not provide any protection against subcutaneous and intranasal challenge of virulent *Y. pestis* CO92 [52]. The explanations for this contrary result: 1) the higher challenge dose we used; 2) the YadC protein (aa 32–551) used for immunization in our studies was different with the YadC protein (aa 137–422) used by Murphy et al [51]. The variation of amino acid sequences might change the configuration of the YadC protein in the two cases [52]. YscF is a surface localized protein that is required both to secrete Yops and to translocate toxins into eukaryotic cells [53–56], which suggested that YscF was required for virulence and might be a potential protective antigen. Matson et al showed that a robust antibody response to YscF primed by immunization with was able to afford significant protection to immunized mice following challenge with *Y. pestis* [57].

Yang's group employed the high-throughput screening to identify new protective antigens of *Y. pestis*. Total 261 genes from *Y. pestis* were selected on the basis of bioinformatics analysis and were expressed in *Escherichia coli* BL21(DE3). After purification, 101 proteins were qualified for examination of their abilities to induce the production of gamma interferon in mice immunized with live vaccine EV76 by enzyme-linked immunospot assay. Thirty-four proteins were found to stimulate strong T-cell responses. The protective efficiencies for 24 of them were preliminarily evaluated in mice. In addition to LcrV, nine proteins (YPO0606, YPO1914, YPO0612, YPO3119, YPO3047, YPO1377, YPCD1.05c, YPO0420, and YPO3720) provided partial protection against challenge with a low dose (20 times the 50% lethal dose [20× LD_{50}]) of *Y. pestis* 201, but only YPO0606 could partially protect mice from infection with *Y. pestis* 201 at a higher challenge dosage (200× LD_{50}) [43].

Recently, Chopra's group [58] indicated that immunizations with OmpA, Ail/OmpX, Pla and F1-V by intramuscular (i.m.) route induced different amounts of antibody titers against above antigens. The titers of anti-Pla and anti-OmpA antibodies were the lowest, while the anti-Ail/OmpX antibody titers were similar to that of F1-V antigen. Mice immunized with the F1-V antigen were completely protected by s.c. challenge with 500 LD_{50} Δ*caf1* mutant strain of *Y. pestis* CO92 which corresponded to approximately 75 to 100 LD_{50} of the WT *Y. pestis* CO92 [59]. Antibodies to both OmpA and Ail/OmpX provided protection to mice resulting in 40 to 50% mice survival, respectively, while antibodies to Pla did not provide any protection to mice. All of the unimmunized, naive control mice died by day 20, although 90% of mice immunized with Pla antigen died by day 10. Comparing with the protective effects of Ail/OmpX alone, they did not observe any additive or synergistic effect on protection against bubonic plague in mice immunized with Ail/OmpX, OmpA, and Pla cocktail. In a pneumonic plague mouse model, F1-V immunization clearly provided complete protection for mice against i.n. challenge. Interestingly, immunization with Pla provided 60% protection to animals when challenged with 15 LD_{50} [7,500 colony-forming units (CFU)] of Δ*caf1* mutant of CO92 despite Pla immunization not protection against a bubonic plague challenge. Neither Ail/OmpX nor OmpA immunizations protected mice from developing pneumonic plague despite immunizations with both of the aforementioned antigens providing protection against bubonic plague in mice. In rat model, immunization with OmpA, Ail/OmpX, Pla and F1-V in rats also produced somewhat comparable antibody titers. The F1-V immunization provided complete protection for rats against i.n. challenge

with 8.5 LD_{50} of wild-type CO92, Ail/OmpX immunization provided 50% protection, while OmpA and Pla immunization failed to provide any protection. In addition, immunization with Ail/OmpX, Pla, or OmpA did not provide any protection to rats against s.c. challenge with 7 LD_{50} of *Y. pestis* CO92. Although results indicated that none of immunization with above antigens provided better protection against *Y. pestis* challenge in mice than that with F1 and/or LcrV antigens, these antigens may be useful to be the combination with F1 and/or LcrV to augment protective immunity of such subunit vaccines. Following, we will emphasize on summarizing the progresses in F1 and/or LcrV subunit vaccines.

Y. pestis produces a specific capsule composed of the fraction I, the biosynthesis of which is temperature dependent. The monomer of the protein capsule is 17.5-kDa, i.e. F1 antigen, confers resistance to phagocytosis [60], has good immunogenic properties in *Y. pestis*, and is secreted onto the bacterial surface by the Caf1 system, which consists of Caf1A as an anchor, Caf1M as a chaperone, and several Caf1 (F1) structural proteins [61, 62]. Meyer et al indicated that the anti-F1 human serum afforded significant passive protection in mice [63]. Mice immunized with F1 antigen purified from the *E. coli* recombinant [64, 65] and *Y. pestis* [65] were protected by lethal *Y. pestis* challenge. However, protective efficacy against plague challenge correlated with titer of F1 antibody was discrepancy in these studies [64, 65]. Immunization with one dose of F1 formulated in poly(lactide-co-glycolide) (PLG) microparticles and liposomes induced high F1 antibody titers in mice and provided great protection against 10^5 CFU of *Y. pestis* GB strain [66].

Since F1-negative strains have been isolated from natural sources and caused experimental fatal disease [67]. Davis et al indicated that African green monkeys inhaled F1-negative and/or F1-positive strains of *Y. pestis* died at 4 to 10 days postexposure and had lesions consistent with primary pneumonic plague [68]. Moreover, Quenee et al also confirmed that Δ*caf1 Y. pestis* was not only fully virulent in animal models of bubonic and pneumonic plague but also broke through immune responses generated with live, attenuated strains or F1 subunit vaccines [69]. The same group indicated that immunization of mice and guinea pigs with the recombinant F1 generated robust humoral immune responses. Sixty percent of immunized mice survived pneumonic plague challenge with 100 MLD *Y. pestis* CO92 [70], but only 12.5% guinea pigs survived pneumonic plague challenge with 250 MLD *Y. pestis* CO92 [71]. These studies suggested that plague subunit vaccines shouldn't be solely based on the F1 antigen, although F1 antigen is a good protective immunogen.

LcrV is a multifunctional virulence protein encoded on these 70-kb pCD1 plasmid, which codes for a virulence-associated type III secretion system (T3SS) necessary for the translocation of Yops into eukaryotic target cells [72] and is the core of the *Yersinia* pathogenicity machinery that targets cells of the immune system [73, 74]. LcrV is exported to the bacterial surface by the T3SS, localizes to the tip of the T3SS needle structure, and can be secreted into the extracellular milieu [75–78]. LcrV also interacts with the Ysc gate protein LcrG [72, 79] and cooperates with YopB and D to form a channel or translocon for delivering Yops into eukaryotic cells [77, 80].

LcrV as a primary antigen or passive anti-LcrV antibodies are demonstrated to protect bubonic or pneumonic *Y. pestis* infection in many research articles and reviews [11, 18, 19,

71, 81–86]. Anderson et al demonstrated that immunization with the recombinant full-length LcrV antigen protected mice from lethal bubonic and pneumonic challenge by a wild-type *Y. pestis* CO92 ($F1^+$ strain), or by the isogenic $F1^-$ strain C12 [82]. As described above, vaccination of mice with F1 partially protects mice and rats from s.c. challenge with *Y. pestis*, and macaques against pneumonic plague by passive transfer of sera collected from F1-vaccinated rabbits [69], whereas there is some evidence that F1 has adjuvantising activity on the immune response to the co-administered V antigen and that this effect is specific and not due to LPS contamination [87]. Thus, including F1 into LcrV antigen combination might augment protective immunity against *Y. pestis* strains.

Williamson et al showed that co-immunization with the purified culture-derived F1 and the recombinant LcrV sub-units afforded a greater level of protection than with either sub-unit alone [88]. Also they showed that the antibody titers to F1 and V were correlate with protection [89] and immunization with a single dose of alhydrogel-adsorbed F1+V vaccine afforded great protection against aerosolised *Y. pestis* challenge [87]. Their studies demonstrated the potential of the combined F1+V vaccine to be developed as a human prophylactic for pneumonic plague. The Defense Science and Technology Laboratory (Dstl) at Porton Down (United Kingdom) has developed the subunit vaccine rF1 + rV, which is comprised of purified recombinant F1 (rF1) as well as recombinant LcrV (rV, derived from GST-LcrV) conjugated with alhydrogel [87, 90]. Avecia Biologics Ltd. initially licensed the technology to manufacture rF1 + rV vaccine in GMP level for phase I and II trials. Then, PharmAthene Inc., acquired an exclusive license for rF1 + rV, which is now manufactured as RypVaxTM, and continues vaccine development towards FDA licensure. In a phase I clinical trial, GMP manufactured rF1 + rV (RypVax) was administered to 24 healthy adult males in a double blind, ascending dose design study, where groups of six individuals received vaccine at dose levels of 5, 10, 20 or 40 μg protein in a volume of 0.5 ml, administered intramuscularly with two doses, prime (day 1) and booster (day 21). RypVax immunized volunteers developed rF1- and rV-specific antibodies on day 14 and increased titers after the booster injection [91]. PharmAthene Inc., conducted three phase I trials and reported vaccine safety in humans. Levels of antibodies varied considerably among members of each vaccine group. A phase II efficacy trial for RypVax was launched in 2003, however experimental tools and generated data are currently not available [86].

Since the United States Department of Homeland Security and Department of Defense require an effective vaccine to protect state and local emergency response and rescue teams, as well as scientists and members of the World Heath Organization during laboratory and field work with *Y. pestis* [92], the United States Army Medical Research Institute for Infectious Diseases (USAMRIID) developed the rF1-V fusion protein as a vaccine. The rF1-V vaccine protected experimental mice against pneumonic as well as bubonic plague caused by either an $F1^+$ or $F1^-$ strain of *Y. pestis*, and provided better protection than F1 or V alone against the $F1^+$ strain [93]. Moreover, vaccination with F1-V fusion antigen provided similar protective efficacy against *Y. pestis* challenge as vaccination with F1 + V combination [92, 93]. Under contract with the United States Department of Defense (DOD), Dynport Vaccine Company developed rF1-V towards FDA licensure. The progress of DynPort Vaccine Company towards licensure of the rF1-V vaccine was posted on the National Institute of Health clinical trial website (www.clinicaltrials.gov). Two clinical trials were reported for

the rF1-V plague vaccine. The first trial was designed as a phase I, open-label, dose-escalating study for safety, tolerability and immunogenicity in healthy volunteers. The second trial was designed as a phase II, dose-blinded, block-randomized, multi-center study to select dosage and schedule of rF1V, examining immune responses up to 210 days, with additional immunogenicity and safety/reactogenicity data collection through 540 days. Both trials have been completed, however results are not yet available. According to a DynPort Vaccine Company press release, 44 healthy volunteers between the ages of 18 and 40 were enrolled in the phase I trial and no vaccine related serious adverse events were identified. The phase II trial tested 400 healthy volunteers between the ages of 18 and 55 [86].

LcrV was reported to trigger the release of interleukin-10 by host immune cells through its interaction with TLR2/CD14 [94–96] and also to suppress tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) production [97, 98]. However, several other groups demonstrated that *Y. pestis* LcrV could not efficiently activate TLR2-signaling and that TLR2-mediated immunomodulation did not play a major role in pathogenesis of plague [99–101]. Arguments about immunomodulatory features of LcrV need to be resolved in further study, but potential concerns have been dispelled in the vaccine design. Schneewind's group from University of Chicago developed a nontoxigenic LcrV vaccine, rV10, a variant with a deletion of LcrV residues 271–300, removing potential epitope of interaction with TLR2/CD14. Immunization with recombinant purified rV10 conjugated with alhydrogel elicited immune responses that protected mice against a lethal challenge with the fully virulent *Y. pestis* strain CO92 [102, 103]. Compared to rLcrV immunization, rV10 immunization provided equal levels of vaccine protection to mice [102]. The rV10 displayed a reduced ability to release interleukin-10 or prevent the release of tumor necrosis factor alpha from lipopolysaccharide-stimulated primary macrophages [103]. Immunization with rV10 also provided great protection against bubonic and pneumonic plague challenge in rats, guinea pigs and non-human primates [104]. Eighty seven percent of guinea pigs immunized with the rV10 plague vaccine survived pneumonic plague challenge with 250 MLD *Y. pestis* CO92, while only 50% of guinea pigs immunized with rLcrV survived this challenge [71]. Additionally, guinea pigs vaccinated with rV10 or F1-V had similar survival by the intranasal challenge with 1,000 MLD *Y. pestis* CAC1 (*caf1A*::IS*1541*) [104]. In an NIAID supported program, University of Chicago investigators use GMP manufactured rV10 to demonstrate preclinical efficacy in animals [105]. The rV10 vaccine is currently undergoing US Food and Drug Administration (FDA) pre-Investigational New Drug (pre-IND) authorization review for a future phase I trial [86].

Additionally, Yang and Wang's group developed a new subunit vaccine consisting of F1 and rV270 (a recombinant LcrV variant lacking amino acids 271 to 326 to reduce its potential immunosuppressive activity) being evaluated in different animal models including mouse, guinea pigs, rabbits and Chinese-origin rhesus macaque [106–110]. Mice immunized with the subunit vaccines and EV76 vaccine were achieved complete protection against challenging with 10^6 CFU of virulent *Y. pestis* strain 141. Immunization with the subunit vaccine and EV76 vaccine provided good protection against challenge with the similar CFU of *Y. pestis* in Guinea pigs and rabbits, respectively. Immunization with subunit vaccines stimulated significantly higher anti-rV270 and anti-F1 IgG titers in mice than in guinea pigs and rabbits, and guinea pigs developed significantly higher IgG titers than the rabbits, but

the anti-F1 response in guinea pigs was more variable than in the mice and rabbits. All above animals immunized with EV76 developed a negligible IgG titer to rV270 antigen. Analysis of IgG subclasses demonstrated that subunit vaccines induced strong predominant IgG1 responses, whereas those receiving EV76 immunization primed IgG1 and IgG2a balanced responses [106]. They further compared immunogenicity of the subunit vaccine SV1 (20 μg F1+10 μg rV270), SV2 (200 μg F1+100 μg rV270) and EV76 in Chinese-origin rhesus macaques, *Macaca mulatta*. Similar like other animal models described above, the macaques immunized with SV1 or SV2 developed higher anti-rV270 IgG titer, while animals immunized with EV76 elicited a negligible IgG to rV270 antigen. No significant antibody titer differences were observed between SV1 and SV2 immunized groups. Also there were no statistical differences for CD4/CD8 ratios, IL-4 and CD69 levels between the three-vaccine immunized groups. However, the EV76 immunized animals produced a significant higher level of IL-12 than the subunit immunized groups, indicating that EV76 had an advantage over SV in respect of cellular immunity. Immunization with SV and EV76 provided a similar protective level against s.c. challenge with 6×10^6 CFU of *Y. pestis* in Chinese-origin rhesus macaques [107]. They also evaluated the long-term immune responses, transmission modes of maternal antibodies and protective efficacy in mice vaccinated with the subunit vaccine SV1. Their studies demonstrated that antibodies to F1 and rV270 were detectable over a period of 518 days and the complete protection against 10^6 CFU of *Y. pestis* 141 by s.c. challenge was achieved up to day 518 after primary immunization [110]. The rV270- and F1-specific antibodies could be transmitted to newborn mice from their mothers until 10 and 14 weeks of age. There was no difference in antibody titers between the parturient mice immunized with SV1 (PM-S) and the caesarean-section newborns from the PM-S or between the lactating mice immunized by SV1 (LM-S) and the cross-fostered mice (CFM) during 3 weeks of lactation. Seventy two percentages of newborn mice survived against s.c. challenge with 4,800 CFU of *Y. pestis* strain 141 at 6-week age, but none of these mice survived against 5,700 CFU of *Y. pestis* challenge at 14-week age. Eighty four percentages of CFM could survive against 5,000 CFU of *Y. pestis* challenge at 7-week age. Their study showed that maternal antibodies induced by SV1 subunit vaccine in mother mice could be transferred to their offspring (newborn mice) by both placenta and lactation. Passive antibodies from the immunized mothers could persist for 3 months in newborn mice and provided early protection for newborn mice [109].

3. Live attenuated *Yersinia* vaccines

Attenuated *Y. pestis* strains that effectively protected albino mice against experimental plague were developed in 1895 by Yersin and in 1903–1904 by Kolle and Otto but were not tested in humans owing to fears of reversion to virulence. The first vaccination of humans with live plague vaccine was done in Manila, The Philippines in 1907, but reliable evidence of its efficacy was not obtained as there were no plague cases in the city at that time [111]. Subsequently, the EV76 strain, a spontaneous *pgm* mutant, was developed from the EV strain isolated by Girard and Robic from a human case of bubonic plague in Madagascar in 1926 [112]. In 1936, a subculture of the EV76 vaccine strain was established at the NIIEG (designation based on the Russian abbreviation of the Scientific-Research Institute for Epidemiology and Hygiene, Kirov, Russian Federation) in the former USSR [112]. This

strain was employed for the development of the live vaccine designated as *"Vaccinum pestosum vivum siccum*", which is manufactured in the former USSR from 1940 [26, 113]. The EV NIIEG strain has been used as a live plague vaccine for the protection of plague researchers and people living in territories endemic for plague in the countries of the former USSR and some Asian countries and is still in use today [113, 114]. Nevertheless, a single dose of the EV NIIEG live vaccine conferred a prompt (day 7 post-vaccination) and pronounced immunity in vaccinees lasting for 10–12 months against bubonic and, to some extent, pneumonic plague [11, 113].

However, EV76 vaccine strain can cause disease in some non-human primates, raising questions about its suitability as a human vaccine [115]. The live Pgm^- strain conferred greater protection against bubonic and pneumonic plague than killed vaccines in animals, but it sometimes caused local and systemic reactions [20, 23, 115, 116]. In addition, a live Pgm^- strain retains virulence when administered by the intranasal (i.n.) and intravenous (i.v.) routes [18, 115, 117]. Variable virulence of the live vaccine strains in animal models and reactogenicity in humans has prevented this vaccine from gaining worldwide acceptance, especially in the US and Europe [15, 118]. Although licensing live attenuated *Y. pestis* as a vaccine will undoubtedly be a long and arduous process, it does not extinguish researchers' passion to explore new attenuated *Y. pestis* mutants as vaccines. Table 2 lists recent developments of live, rationally attenuated *Y. pestis* mutants as vaccines against plague.

In *Salmonella, ΔrelA ΔspoT* mutants are attenuated [119] and *crp* mutants are attenuated and immunogenic [120]. It has also been established that *Y. pestis crp* mutants are attenuated for virulence [121]. In our laboratory, we examined the vaccine potential of *Y. pestis ΔrelA ΔspoT* [122] and Δ*crp* mutants [123]. Mice vaccinated subcutaneously (s.c.) with 2.5×10^4 CFU of the Δ*relA* Δ*spoT* mutant developed high anti-*Y. pestis* serum IgG titers, were completely protected against s.c. challenge with 1.5×10^5 CFU of virulent *Y. pestis* and partially protected (60% survival) against pulmonary challenge with 2.0×10^4 CFU of virulent *Y. pestis* [122]. Results indicate that ppGpp represents an important virulence determinant in *Y. pestis* and the Δ*relA* Δ*spoT* mutant strain is a promising vaccine candidate to provide protection against plague.

The *Δcrp* mutant was completely attenuated (s.c. $LD_{50} > 10^7$ CFU) and partially protective against bubonic plague but no protective against pneumonic plague [123]. The strategy of regulated delayed attenuation was developed in *Salmonella*, in which the virulence gene expression of bacteria is dependent on the presence of sugars (arabinose, mannose or rhamnose). When cells are grown in the presence of sugar, the virulence gene is expressed. Once the cells invade host tissues where free arabinose is not available, virulence gene expression ceases and the cells become attenuated [124]. This strategy was applied to *Y. pestis*, constructing a strain with *crp* under transcriptional control of the *araC* P_{BAD} promoter [123]. The resulting strain was partially attenuated ($LD_{50} = 4.3 \times 10^5$ CFU) and protective against both bubonic and pneumonic plague [123].

One strategy used by *Y. pestis* to evade the host immune system is to produce lipid A that is not recognized by toll-like receptor 4 (TLR4). This is accomplished due to the temperature-

regulated expression of a key gene in the acylation pathway, *lpxP* encoding palmitoleyltransferase, and a non-temperature sensitive lipid A synthesis gene, *lpxM* encoding myristoyltransferase, which result in hexa-acylated lipid A at 28°C. At 37°C, the body temperature of mammalian hosts, *lpxP* is not expressed, resulting in tetra-acylated lipid A [125], which is not recognized by TLR4 [126] that preferentially recognizes hexa-acylated lipid A [127–129]. In 2006, Montminy et al. reported that a *Y. pestis* strain engineered to produce hexa-acylated lipid A at 37°C by constitutive expression of the *E. coli lpxL* gene from a multicopy plasmid, is attenuated [130]. Based on those knowledge, we constructed a strain χ10015(pCD1Ap) (Δ*lpxP32*::P_{lpxL} *lpxL*), that expresses *E. coli lpxL* from the chromosome of *Y. pestis* KIM6+(pCD1Ap), providing greater genetic stability than plasmid expression. The χ10015(pCD1Ap) was highly attenuated by s.c. administration, but χ10015(pCD1Ap) stimulated a strong inflammatory reaction, which results in mice sick and ruffed in early infection stage, and also retained virulence via intranasal infection. Heterologous expression of the lipid A 1-phosphatase, LpxE, from *Francisella tularensis* in *Y. pestis* yields predominantly 1-dephosphorylated lipid A that might reduce hyper-inflammation of χ10015(pCD1Ap) and the virulence of χ10015(pCD1Ap) by i.n. infection. Results indicated that expression of LpxE on top of LpxL provided no significant reduction in virulence of *Y. pestis* in mice when it was administered intranasally, but actually reduced LD_{50} by three orders of magnitude when the strain was administered subcutaneously [131].

The strain, χ10030(pCD1Ap), produces hexa-acylated lipid A at 37°C and carries the arabinose-regulated *crp* gene [132]. Our results demonstrated an increase in the LD_{50} of χ10030(pCD1Ap) by s.c. and i.n. inoculation of more than 1.5×10^7 and 3.4×10^4-fold, respectively, in Swiss Webster mice, compared to the wild-type virulent *Y. pestis* KIM6+ (pCD1Ap) strain. Both s.c. and i.n. immunization with strain χ10030(pCD1Ap) induced significant protection against both bubonic and pneumonic plague with minimal reactogenicity in mice, attributes consistent with our goal of designing a live safe *Y. pestis* vaccine. However, this strain was still able to induce IL-10 early in infection, a known strategy used by *Y. pestis* to evade detection by the host [75]. Also, due to safety concerns surrounding a live plague vaccine, we consider it prudent to identify and include an attenuating deletion mutation in our final vaccine. Therefore, we plan to enhance the safety and efficacy of χ10030(pCD1Ap) by including a yet to be identified deletion mutation and eliminating its ability to elicit IL-10 early in infection.

Other mutations that affect genes specific for *Yersinia* have also been examined as a basis for attenuating *Y. pestis*. Of note, a *Y. pestis ΔyopH* mutant is attenuated and provides a high level of protection against bubonic and pneumonic plague in mice [133]. Studies showed that *Δpcm and ΔnlpD* mutants were attenuated and elicited protective immunity in mice [134, 135], but the immunization with *ΔnlpD* mutants failed to protect guinea pigs [136].

YscN, an ATPase of *Y. pestis*, has a critical role for virulence factor delivery. Bozue et al indicated that introduction of the *yscN* gene into the *Y. pestis* CO92 led to attenuation following s.c. mice challenges. No mice succumbed to challenge with 4.44×10^4 or 4.44×10^6 CFU of the Δ*yscN* mutant by s.c. route. The attenuation of the *Y. pestis* Δ*yscN* strain suggested the possible use of the strain as a live vaccine. The mice immunized s.c. twice

with 10^7 CFU of the *Y. pestis* Δ*yscN* strain provided 90% protection against s.c. challenge with 180 CFU of the wild-type CO92 strain [137].

Sha et al. showed that the Δ*lpp* Δ*msbB* double mutant *Y. pestis* CO92 strain was grossly compromised in its ability to disseminate to distal organs in mice and in evoking cytokines/chemokines in infected animal tissues. Additionally, mice that survived challenge with the Δ*lpp* Δ*msbB* double mutant, but not the Δ*lpp* or Δ*msbB* single mutant, in a pneumonic plague model were significantly protected against a subsequent lethal wild-type CO92 rechallenge. Thus, the Δ*lpp* Δ*msbB* double mutant might provide a new live-attenuated background vaccine candidate strain [138]. Identification of other attenuating mutations that target unique *Y. pestis* virulence genes will be of significant interest for developing safe attenuated *Y. pestis* vaccines.

Zhang et al. constructed the Δ*yscB* mutant *Y. pestis* biovar *Microtus* strain 201 that is avirulent to humans, but virulent to mice. The evaluation of virulence, immunogenicity and protective efficacy of the Δ*yscB* mutant showed that the Δ*yscB* mutant was severely attenuated, elicited a higher F1-specific antibody titer and provided protective efficacy against bubonic and pneumonic challenge with *Y. pestis* 141 strain (Antigua biovar) in mouse model. The Δ*yscB* mutant could induce the secretion of both Th1-associated cytokines (IFN-γ, IL-2 and TNF-α) and Th2-associated cytokines (IL-4 and IL-10) [139]. The same group evaluated the protective efficacy of the *Y. pestis* Microtus strain 201 as a live attenuated plague vaccine candidate. Their results showed that this strain was highly attenuated by subcutaneous route, elicited an F1-specific antibody titer similar to the EV76 and provided a similar protective efficacy with the EV76 against bubonic plague in Chinese-origin rhesus macaques. The immunization with *Y. pestis* Microtus strain 201 induced elevated secretion of both Th1-associated cytokines (IFN-γ, IL-2 and TNF-α) and Th2-associated cytokines (IL-4, IL-5, and IL-6), as well as chemokines MCP-1 and IL-8. However, the protected animals developed skin ulcer at challenge site with different severity in most of the 201-immunized and some of the EV-immunized monkeys [140].

Recently, Chopra's group employed high-throughput signature-tagged mutagenic means to identify novel virulence factors from *Y. pestis* CO92. In this study, they found *rbsA* that codes for a putative sugar transport system ATP-binding protein, and *vasK*, a component of the type VI secretion system, exhibited attenuation at 11–12 LD_{50} in a mouse model of pneumonic plague. Combining Δ*rbsA* and Δ*vasK* genes into either the Δ*lpp* single or the Δ*lpp*Δ*msbB* double mutant augmented the attenuation to provide 90–100% survivability to mice in a pneumonic plague model at 20–50 LD_{50}s. The Δ*lpp*Δ*msbB* Δ*rbsA* triple mutant-infected mice at 50 LD_{50} were 90% protected upon subsequent challenge with 12 LD_{50} of *Y. pestis* CO92 [141]. They also evaluated whether the deletion of *ail* gene affected virulence of *Y. pestis* CO92. Results indicated that the *ail* single mutant was slightly attenuated compared to the WT bacterium in a mouse model of pneumonic plague, however combining Δ*ail* into Δ*lpp* single mutant strain and Δ*lpp* Δ*msbB* double mutant strain increased their attenuation. LD_{50} of the triple mutant (Δ*ail* Δ*lpp* Δ*msbB*) was equal to 6,800 LD_{50} of *Y. pestis* CO92. The mutant-infected animals developed balanced Th1- and Th2-based immune responses based on antibody isotypes. The triple mutant was cleared from mouse organs rapidly, with concurrent decreases in the production of various cytokines and histopathological lesions.

Animals surviving from infection with the triple mutant were partially protected against subsequently challenged on day 24 with the bioluminescent *Y. pestis* CO92 strain (20 to 28 LD_{50}s) by intranasal route, however efficient clearing of the invading pathogen was visualized in real time by in vivo imaging [142].

Y. pseudotuberculosis, a recent ancestor of *Y. pestis* [143], is much less virulent and typically causes an enteric disease that is rarely fatal. Its lifestyle as an enteric pathogen should facilitate its use as an oral vaccine. With the exception of two additional plasmids carried by *Y. pestis* (pPCP1 and pMT1), the two species share >95% genetic identity and a common virulence plasmid with a conserved co-linear backbone [144]. Based on these similarities, the use of avirulent *Y. pseudotuberculosis* strains as a plague vaccine has been explored. Oral immunization with attenuated *Y. pseudotuberculosis* strains stimulates cross-immunity to *Y. pestis* and provides partial protection against pulmonary challenge with *Y. pestis* [145–147]. While protection was not stellar, it was significant, demonstrating the feasibility of using this approach. Derbise et al. showed that an encapsulated *Y. pseudotuberculosis* IP32953 was generated by cloning the *Y. pestis* F1-encoding *caf* operon and expressing it in the attenuated strain. The new V674pF1 strain produced the F1 capsule *in vitro* and *in vivo*. Oral inoculation of V674pF1 allowed the colonization of the gut without lesions to Peyer's patches and the spleen. Vaccination induced both humoral and cellular components of immunity, at the systemic (IgG and Th1 cells) and the mucosal levels (IgA and Th17 cells). A single oral dose conferred 100% protection against a lethal pneumonic plague challenge (33 × LD_{50} of the fully virulent *Y. pestis* CO92 strain) and 94% against a high challenge dose (3,300 × LD_{50}). Both F1 and other *Yersinia* antigens were recognized and V674pF1 efficiently protected against a F1-negative *Y. pestis* [148].

Recently, Sun et al constructed a *Y. pseudotuberculosis* mutant strain with arabinose-dependent regulated delayed-shutoff of *crp* expression (*araC* P_{BAD} *crp*) and replacement of the *msbB* gene with the *E. coli msbB* gene to attenuate it. Then, we inserted the *asd* mutation into this construction to form χ10057 (Δ*asd-206* Δ*msbB868*::: P_{msbB} *msbB* $_{(EC)}$ ΔP_{crp21}*::*TT *araC* P_{BAD} *crp*) for adapting with an balanced-lethal Asd^+ plasmid to facilitate antigen synthesis. A hybrid protein composed of YopE (1–138aa) fused with full-length LcrV of *Y. pestis* ($YopE_{Nt138}$-LcrV) was synthesized in χ10057 harboring an Asd^+ plasmid (pYA5199, $yopE_{Nt138}$-*lcrV*) and could be secreted through type III secretion system (T3SS) in vitro and vivo. Animal studies indicated that mice orally immunized with χ10057(pYA5199) developed similar titers of IgG response to whole cell lysates of *Y. pestis* (YpL) and LcrV as χ10057(pYA3332, empty plasmid). The χ10057(pYA5199) induced higher level of protection (83% survival) against intranasal (i.n.) challenge with ~130 LD_{50} (1.3×10^4 CFU) of *Y. pestis* KIM6+ (pCD1Ap) than induced by χ10057(pYA3332) (40% survival). Splenocytes from mice vaccinated with χ10057(pYA5199) produced significant levels of IFN-γ, TNF-α, and IL-17 after restimulation with LcrV and YpL antigens [149].

Additionally, a *Y. pseudotuberculosis* mutant strain combined with chromosome insertion of *caf1R-caf1A-caf1M-caf1* operon and deletions of *yopJ* and *yopK*, χ10068 [pYV-ω2 (Δ*yopJ315*Δ*yopK108*) Δ*lacZ044*:: *caf1R-caf1M-caf1A-caf1*] was constructed. Results indicated that gene insertion and deletion did not affect the growth rate of χ10068 compared to wild-type *Y. pseudotuberculosis* cultured at 26°C and also F1 antigen in χ10068 was

synthesized at 37°C (mammal temperature), not at regular culture temperature (26°C). Immunization with χ10068 primed both antibody responses and specific T-cell responses to F1 and YpL. A single dose of oral immunization with χ10068 provided 70% protection against a subcutaneous (s.c.) challenge with ~2.6 × 10^5 LD_{50} of *Y. pestis* KIM6+ (pCD1Ap) and 90% protection against an intranasal (i.n.) challenge with ~500 LD_{50} of *Y. pestis* KIM6+ (pCD1Ap) in mice (manuscript in preparation). As a naturally occurring enteric pathogen, live attenuated *Y. pseudotuberculosis*-based vaccines may be used as an oral vaccine delivered by baits to wild animals, which might reduce the transmission of sylvatic plague to humans by controlling it or eradicating it in its natural rodent hosts.

4. Live vectored plague vaccines

In the process of attenuation, an infectious agent is altered so that it becomes harmless or less virulent, while retaining its ability to interact with the host and stimulate a protective immune response [150]. There are many examples of successful live attenuated vaccines delivered by injection, including the current bacterial vaccine for tuberculosis (BCG) [151] and viral vaccines for measles, mumps, rubella, chicken pox and yellow fever [152]. Rabies vaccines are now available in two different attenuated forms, one for use in humans, and one for animals [153]. There are also a number of mucosally delivered live vaccines. These include oral vaccines against poliovirus [154], cholera [155], rotavirus [156] and typhoid fever [157] and the nasally delivered vaccines against influenza [158–160].

Most pathogens gain entry to the host via mucosal surfaces [161, 162]. Thus, parenterally administered vaccines, which may be limited in their capacity to induce mucosal immune responses, may not be the most appropriate form of vaccination for many infections. In contrast, mucosally delivered vaccines have the potential for inducing both systemic and mucosal immunity. Ideally delivered by the oral or intranasal (i.n.) route, such vaccines also offer the advantage of being easier and safer to administer than needle-based delivery [163]. Therefore, live attenuated vaccines have advantages over subunit vaccines as they are typically taken orally, still inducing strong mucosal and durable immunity [26, 162, 164]. In addition, they are often less expensive to manufacture than subunit vaccines. The major disadvantages of live vaccines include inadequate attenuation, particularly in the case of immunocompromised individuals and the potential to revert to virulence. However, application of modern molecular techniques in conjunction with a detailed understanding of the virulence attributes of the delivery vector or in some cases, of the pathogen itself prior to attenuation make the latter unlikely in a well characterized rationally attenuated vaccine. Thus, development of live vaccines against plague at this time represents an underutilized strategy for preventing this disease.

Live bacterially vectored plague vaccines

The commensal, non-pathogenic bacterium *Lactococcus lactis* has been used to deliver LcrV [165, 166] with some success. However, most of the studies examining the use of live bacterially vectored vaccines for plague, including work in our laboratory, have focused on exploiting live attenuated *Salmonella* to deliver *Y. pestis* antigens. Live attenuated *Salmonella* have attracted considerable attention as vectors for the delivery of a variety of

heterologous vaccine antigens. After delivery by the oral route the bacteria enter the intestinal sub-epithelium via M-cells and are trafficked via mesenteric lymph nodes to fixed macrophages in the spleen and liver [167–169]. This colonization pathway results in the induction of mucosal and systemic immune responses. Table 3 summarizes a number of recent studies utilizing live attenuated *Salmonella* vaccines to deliver *Y. pestis* antigens.

With a few exceptions, all the studies listed in Table 1 used *Salmonella* to deliver F1, LcrV or both. Titball's group has done numerous studies in this area, constructing strains that produce F1-V fusion protein [170], LcrV [171] and F1 capsule on the surface of the cell [119]. Pascual's group took the effort one step further and constructed a *Salmonella* strain that produced F1 as an extracellular capsule and LcrV as a soluble cytoplasmic protein [119]. Sizemore et al demonstrated that attenuated *S. typhimurium* strains expressing cytoplasmically localized F1-V and V antigen antigens were more immunogenic than strains that secreted or localized plague antigens to the outer membrane [172]. In all of these studies, *S.* Typhimurium vaccine strains synthesizing F1 and/or LcrV or fragments of LcrV were demonstrated to elicit humoral and/or cellular immunity against the vectored antigen and to provide some level of protective immunity against either subcutaneous and/or intranasal challenge with *Y. pestis*. Interestingly, some authors noted that immunization with attenuated *Salmonella* alone (no *Y. pestis* antigens) could provide a low level of protection [173–175], indicating that the use of *Salmonella* as a plague vaccine may provide an additional benefit.

Studies have also described *S.* Typhi constructs as candidates for human vaccines. In one study, an *S.* Typhi strain synthesizing capsular F1 was demonstrated to elicit protective immunity when used to intranasally immunize mice [176]. A similar vaccine strain was administered intranasally to 7-day old mice [177]. Immunized mice developed mucosal antibody and IFN-γ secreting cells and were efficiently primed for a later injection of F1 plus alum adjuvant. The *Salmonella* vaccine provided more potent priming than an F1 plus alum prime, demonstrating the potential for using a *Salmonella*-vectored plague vaccine in a prime boost scenario. Recently, Galen et al used a live attenuated *S.* Typhi strain to create a bivalent mucosal plague vaccine that produces both the protective F1 capsular antigen of *Y. pestis* as well as the LcrV protein required for secretion of virulence effector proteins. To reduce metabolic burden associated with the co-expression of F1 and LcrV within the live vector, we balanced expression of both antigens by combining plasmid-based expression of F1 with chromosomal expression of LcrV from three independent loci. The serum antibody responses to LPS induced by the optimized bivalent vaccine were indistinguishable from those elicited by the parent strain, suggesting adequate immunogenic capacity maintained through preservation of bacterial fitness. Importantly, mice receiving the optimized bivalent vaccine were fully protected against lethal pulmonary challenge [178].

Our philosophy with regard to *Salmonella*-vectored vaccines for plague is that F1 and LcrV, while highly effective in laboratory models, may not be sufficient to protect against all strains of *Y. pestis*. For example, non-encapsulated (F1 negative) *Y. pestis* mutants can cause chronic, lethal infections in laboratory rats and mice [179, 180]. However, the relevance of these observations has been brought into question by a recent study showing that the impact of the F1 capsule on *Y. pestis* virulence depends on the strain and genotype of mouse used

for testing [181]. On the other hand, this concern appears to be relevant to humans as an F1 negative strain of *Y. pestis* has been implicated in an acute fatal human infection [182]. Additionally, there are known polymorphisms of LcrV [183] and F1 [Clin Microbiol Infect, 2008. **14**(5): 429–36] that may influence protective efficacy. Therefore, using only two antigens for presentation by *Salmonella* might be insufficient to combat weaponized or naturally occurring *Y. pestis*, leading us to evaluate additional antigens. In addition to LcrV, our group has used *Salmonella* to vector three other *Y. pestis* antigens, Psn [175], HmuR [175], PsaA, also called pH 6 antigen [36], which forms a fibrillar structure on the *Y. pestis* cell surface [184] and YadC, a member of the oligomeric coiled-coil adhesins [52]. Psn and HmuR are outer membrane proteins involved in iron acquisition [185, 186]. The role of PsaA in virulence is not clear [37, 40, 41], but available data indicates it may serve as an adhesin [187] and an antiphagocytic factor [188]. We demonstrated that *Salmonella* delivering Psn elicited significant protective immunity against subcutaneous challenge [175]. We observed partial protection against intranasal challenge, although this did not achieve statistical significance. PsaA was highly immunogenic, eliciting strong serum IgG and mucosal IgA antibodies. However, immunized mice were not protected from subcutaneous challenge and, similar to what we observed with Psn, some immunized mice were protected from intranasal challenge, but the result was not statistically significant [184]. When delivered by our *Salmonella* strains, HmuR was poorly immunogenic and did not confer protection against either challenge route [175]. Mice immunized with *Salmonella* synthesizing YadC or YadC810 are afforded 50% protection but no protection by immunization with the *Salmonella* strain synthesizing YadBC by s.c. challenge with ~230 LD_{50} of *Y. pestis* CO92. None of these provided protection against i.n. challenge with ~31 LD_{50} of *Y. pestis* CO92 [52]. Recently, we optimized expression of three antigens (LcrV196, F1 and Psn) in our newly improved *Salmonella* strain. Oral immunization with the *Salmonella* strain delivering three antigens provided complete protection against s.c. challenge with 5700 CFU of *Y. pestis* CO92 and 60% protection against i.n. challenge with 5000 CFU of *Y. pestis* CO92 (manuscript in preparation).

Virally vectored live plague vaccines

Replication-deficient adenovirus (Ad) vectors are excellent candidates for vaccine platforms as they transfer genes effectively to antigen-presenting cells (APCs) in vivo, with consequent activation of APCs, thus conveying immune adjuvant properties and inducing strong, rapid humoral and cellular immune responses against the transgene product [189]. Crystal's group developed a replication-deficient adenovirus (Ad) gene-transfer vector encoding V antigen and demonstrated that a single injection of the recombinant virus elicited strong anti-LcrV serum antibody responses, LcrV-specific $CD4^+$ and $CD8^+$ responses and protective immunity against an intranasal *Y. pestis* challenge [190, 191]. In a subsequent study, they fused either F1 or LcrV to the C terminus of adenovirus capsid protein, IX. Both constructs elicited strong humoral immunity in mice immunized intramuscularly with greater efficacy than an injection of adjuvanted purified V or F1 [191]. In addition, they also employed adenovirus (Ad) to deliver monoclonal antibodies (MAbs) specific for the *Y. pestis* LcrV antigen, which provided good protection for immunized mice against intranasal challenge with 363 LD_{50} of *Y. pestis* CO92 [192, 193].

Rose's group devised a vaccine utilizing recombinant vesicular stomatitis virus (VSV) vectors expressing the *Y. pestis lcrV* gene [194]. Two intranasal doses elicited high titers of anti-LcrV IgG and protected immunized mice against intranasal challenge. In a follow-up study, the virus was modified to encode a secreted form of LcrV [195]. A single intramuscular dose of 10^9 PFU was sufficient to protect 90% of the immunized mice from a lethal *Y. pestis* challenge. The secreted LcrV was a more potent vaccine that the previous vaccine that encoded the non-secreted form and the authors showed that a high level of protection was dependent on $CD4^+$ but not $CD8^+$ cells and correlated with increased anti-LcrV antibody and a bias toward IgG2a and away from IgG1 isotypes [194, 195]. In addition, Vaccinia virus (VACV) is the vaccine for smallpox and a widely-used vaccine vector for infectious diseases and cancers [196]. Several groups demonstrated that a vaccinia viral vector expressing either *lcrV* or *caf1* (gene encoding F1) as vaccines which are highly immunogenic in BALB/c mice and safe in immunocompromised SCID mice [196–198].

Barton *et al* reported that latent infection of mice with either murine gammaherpesvirus 68 or murine cytomegalovirus results in an increased resistance to both intranasal and subcutaneous infection with either *Listeria monocytogenes* or *Y. pestis* [199]. Latency-induced protection is not antigen specific but involves prolonged production of the antiviral cytokine interferon-γ and systemic activation of macrophages, which upregulates the basal activation state of innate immunity against lethal challenge of plague [199]. This observation might be translated into a proactive approach to provide immunity against plague or other pathogens.

A number of reports described studies to develop viral-vectored bait vaccines to be used to control environmental sources of plague. One group constructed a recombinant vaccinia virus to direct synthesis of an F1-V fusion protein with promising results [197, 198]. Orally immunized mice developed high serum antibody titers against the F1-V antigen and achieved 90% protection against a challenge of 10 LD_{50} of *Y. pestis* [197, 198]. Researchers at the United States Geological Survey's National Wildlife Health Center have been developing a recombinant raccoon poxvirus (RCN) that directs synthesis of the F1 antigen (herein designated RCN-F1) as a bait vaccine to protect Prairie dogs (*Cynomys* spp.). Prairie dogs are highly susceptible to *Y. pestis*. In initial studies, the vaccine protected mice from virulent plague challenge [200] and black-tailed prairie dogs (*Cynomys ludovicianus*) vaccinated intramuscularly with RCN-F1 survived subcutaneous challenge with virulent *Y. pestis* [201]. To provide a more practical approach for field vaccination, the RCN-F1 vaccine was incorporated into palatable, edible bait and offered to black-tailed prairie dogs. Antibody titers against *Y. pestis* F1 antigen increased significantly in vaccinated animals, and their survival was significantly higher upon challenge with *Y. pestis* than that of negative controls [201, 202], demonstrating that oral bait immunization of prairie dogs can provide protection against plague.

5. Other vaccines for plague

DNA vaccines.

Recently, a novel methodology of DNA vaccines has been developed in which genes encoding protein antigens are delivered into host cells for enabling antigen production to

occur in vivo. There are several advantages about DNA vaccines, such as ease of construction, low cost of mass production, high levels of temperature stability, and the ability to elicit both humoral and cell-mediated immune responses [203, 204]. Bennett et al first reported that a DNA vaccine vector encoding a fusion of the *Y. pestis* V antigen and glutathione *S*-transferase (GST) under the CMV promoter could induce V antigen-specific antibody in mice [205], which suggested that there was potential for the development of a DNA vaccine against plague. Grosfeld et al constructed three plasmids expressing the full-length F1, F1 devoid of its putative signal peptide (deF1), and F1 fused to the signal-bearing E3 polypeptide of Semliki Forest virus (E3/F1). Among them, intramuscular vaccination of mice with the plasmid expressing deF1 induced the most effective in eliciting anti-F1 antibodies. Immunization with deF1 DNA conferred complete protection against s.c. challenge with 4,000 LD_{50} of the virulent *Y. pestis* Kimberley53 strain [206]. Garmory et al reported that immunization with the plasmid containing CMV-TE eukaryotic promoters for driving expression of V antigen induced higher IgG2a titers than other five different eukaryotic promoters, but alteration of the codon usage of the *lcrV* gene was not found to improve the anti-LcrV antibody responses [207]. The comparison of DNA vaccines delivered via intramuscular injection with gene-gun administration indicated that gene-gun delivery induced significantly higher antibody responses to F1 or LcrV and also afforded the highest level of protection against *Y. pestis* challenge [206, 207].

Williamson et al reported that mice primed with a combination of plasmid DNA encoding either protective antigen of *Bacillus anthracis* or LcrV antigen of *Y. pestis* and boosted with a combination of the recombinant proteins were fully protected (6/6) against challenge with *Y. pestis*. However, mice primed only with plasmid DNA encoding the V antigen and boosted with rV, which were partially protected (3/6) against *Y. pestis* challenge or mice primed and boosted with plasmid DNA encoding the V antigen which were poorly protected (1/6) against *Y. pestis* challenge. This protective enhancement may be due to the effect of CpG motifs known to be present in the plasmid DNA construct encoding protective antigen of *B. anthracis* [208]. Recently, Albrecht et al evaluated the efficacy of multi-agent DNA vaccines consisting of a truncated gene encoding *B. anthracis* lethal factor (LFn) fused to either *Y. pestis* V antigen (V) or *Y. pestis* F1. Mice immunized with above DNA vaccine by gene gun developed predominantly IgG1 responses to LFn, V, and F1 respectively, were fully protected against a lethal aerosolized *B. anthracis* spore challenge but were partially protected against a lethal aerosolized *Y. pestis* [209]. In addition, Wang et al demonstrated that a novel DNA vaccine expressing a modified V antigen (LcrV) of *Y. pestis*, with a human tissue plasminogen activator (tPA) signal sequence, elicited strong V-specific antibody responses in BALB/c mice. The tPA-V DNA vaccine provided better protection against intranasal challenge with lethal doses of *Y. pestis* than a DNA vaccine only expressing the wild-type V antigen in mice. Additionally, oligomers formed spontaneously by tPA-V primed a higher IgG2a anti-V antibody response in immunized mice, which tends to induce Th1 type cellular immune response [210]. The same group found that an LcrV DNA vaccine was able to elicit CD8+ T cell immune responses against specific epitopes of LcrV antigen and induced protective immunity against i.n. challenge with *Y. pestis* [211].

Mixture of IL-12 with protective antigens might enhance vaccine efficacy, because IL-12 has a central function in initiating and regulating cellular immune responses by stimulating

gamma interferon (IFN-γ) production in both natural killer (NK) cells and helper T cells [212, 213]. Therefore, Yamanaka et al construct two bicistronic plasmids encoding a F1-V fusion protein and IL-12 with different copy numbers to produce high or low level of IL-12. Animal experiments indicated that mice immunized with IL-12(Low)/F1-V vaccine were provided the best protective efficacy (80% survival) against pneumonic challenge of *Y. pestis* compared to mice immunized with IL-12(Low)/F1, IL-12(Low)/V, or IL-12(Low) vector DNA vaccines. However improved expression of IL-12 resulted in lost efficacy when using the IL-12(High)/F1-V DNA vaccine. Although there were differences in the amount of IL-12 produced by the two F1-V DNA vaccines, antibody responses and Th cell responses to F1- and V-Ags were similar [212, 214].

Nanovaccines.

In order to improve efficacy of subunit vaccines, nanotechnology platforms have recently been incorporated into vaccine development to overcome certain concerns about vaccines such as, the weak immunogenicity, intrinsic instability in vivo, toxicity, and the need for multiple administrations. Nanocarrier-based delivery systems facilitate uptake of nanovaccines by phagocytic cells, the gut-associated lymphoid tissue, and the mucosa-associated lymphoid tissue, leading to efficient antigen recognition and presentation and offering an opportunity to enhance the humoral and cellular immune responses [215]. In addition, modifying the surfaces of nanocarriers with a variety of targeting moieties allows the delivery of antigens to specific cell surface receptors, thereby stimulating specific and selective immune responses [216].

Zeng et al firstly demonstrated that intranasal mucosal vaccination of mice with nano-structural and single-molecule force bases of *Y. pestis* V antigen fused with protein anchor (V-PA) loaded on gram positive enhancer matrix (GEM) vaccine particles elicited robust antigen-specific immune response. This study indicated that high-density, high-stability, specific, and immunological pH-responsive loading of immunogen nanoclusters on vaccine particles could readily be presented to the immune system for induction of strong antigen-specific immune responses [217]. Hoeprich's group tried to immobilize hexa-His-tagged LsrB, a *Y. pestis* transport protein onto Nickel-chelating nanolipoprotein particles (NiNLPs) [218]. Then, they employed a nanolipoprotein particle (NLP)-based vaccine delivery platform to co-deliver both subunit antigens and amphipathic adjuvants such as monophosphoryl lipid A and cholesterol-modified CpG oligodeoxynucleotides, which can bind His-tagged protein antigens. Immunization with this co-delivery platform primed 5–10 times higher antibody titers against His-tagged influenza hemagglutinin 5 and *Y. pestis* LcrV antigens in mice than with coadministration formulations and nonadjuvanted NiNLPs. This study indicated that colocalized delivery of adjuvant and antigen could induce significantly greater immune response in mice than coadministered formulations [219].

Narasimhan's group developed a novel biodegradable polyanhydride nanoparticle-encapsulated with F1-V vaccine. Immunization with the nanoparticle-based vaccine induced higher titer and higher avidity anti-F1-V IgG1antibody that persisted for at least 23 weeks post-vaccination in mice than immunization with the recombinant protein F1-V alone and MPLA-adjuvanted F1-V. The single-dose intranasal immunization with nanoparticle-based

F1-V vaccine induced long-lived protective immunity against pneumonic plague. After intranasal challenge, no *Y. pestis* were recovered from the lungs, livers, or spleens of mice vaccinated with the nanoparticle-based F1-V vaccine [220, 221]. They also compared the deposition within the lung and internalization by phagocytic cells of F1-V encapsulated polyanhydride nanoparticles with that of soluble F1-V alone or F1-V adjuvanted with monophosphoryl lipid A (MPLA). Results demonstrated that encapsulation of F1-V into polyanhydride nanoparticles prolonged its presence, while MPLA-adjuvanted F1-V is undetectable within 48 h. Moreover, the inflammation induced by the nanovaccine is mild compared with the marked inflammation induced by the MPLA-adjuvanted F1-V [222]. Further, they investigated the effect of nanoparticle chemistry and its attributes on the kinetics and maturation of the antigen-specific serum antibody response. Results demonstrated that decoration of polyanhydride nanoparticle chemistry facilitated improving antibody titers, avidity, and epitope specificity. Their studies indicated that immunization with nanoparticle encapsulated with subunit vaccine formulations could induce long-lasting and mature antibody responses, which can be used for the rational design of effective vaccine [223].

Gregory et al decorated the 15 nm gold nanoparticles (AuNP) with *Y. pestis* F1-antigen using N-hydroxysuccinimide and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride coupling chemistry. Compared with mice vaccinated with AuNP-F1 in PBS or unconjugated F1-antigen in PBS or alhydrogel, mice vaccinated with AuNP-F1 in alhydrogel generated the highest IgG antibody response to F1-antigen [224].

Rao's group employed the bacteriophage T4 DNA nanoparticles carrying reporter genes, vaccine candidates, functional enzymes, and targeting ligands that were efficiently delivered into cells or targeted to antigen-presenting dendritic cells. Mice vaccinated with a single dose of F1-V plague vaccine containing both gene and protein in the T4 head elicited robust antibody and cellular immune responses [225]. Based on this work, they delivered F1mut-V fusion protein by phage T4 nanoparticle, in which the F1 was eliminated polymerization by transplanting the NH_2-terminal β-strand of F1 to the COOH-terminus, but the T cell epitopes of F1 were retained. The F1mut-V was displayed on phage T4 nanoparticle via the small outer capsid protein, Soc. The immunization with purified F1mut-V monomer adjuvated alhydrogel or the T4-decorated F1mut-V without any adjuvant induced robustly immunogenic responses in mice. Vaccination with either the purified F1mut-V mixed with alhydrogel or T4 decorated F1mut-V without adjuvant provided complete protection to mice and rats against intransal challenge with high doses of *Y. pestis* CO92. This novel delivery platform might generate new-type vaccines and genetic therapies [226].

New adjuvanted plague vaccines.

Adjuvants are compounds that enhance the specific immune response against co-inoculated antigens. Thus, antigens mixed with adjuvants are required to achieve the generation of a strong immune response [227]. Jones et al indicated that intranasal immunization with F1-V formulated with a Proteosome-based adjuvant (Protollin) elicited high titers of anti-F1-V IgA in lungs of mice whereas intranasal immunization with F1-V alone or intramuscular immunization with Alhydrogel adjuvanted F1-V did not, and also induced higher serum

titers of anti-F1-V IgG than those induced by intramuscular Alhydrogel adjuvanted F1-V, which provided 100% and 80% protection against aerosol challenge with 170 LD_{50} and against 255 LD_{50} of Y. pestis respectively [228]. This study suggested that Protollin might be more effective adjuvant than Alhydrogel to induce potent immune responses.

Several studies demonstrated that interleukin-12 (IL-12) could be used as a highly effective vaccine adjuvant against bacterial and viral infections [229–236]. Kumar et al showed that intranasal vaccination with inactivated *Y. pestis* CO92 (iYp) adjuvanted with IL-12 provided complete protection for mice against an i.n. challenge with a lethal dose of *Y. pestis* CO92. Survival of the vaccinated mice correlated with levels of systemic and lung antibodies, and immunization with iYp adjuvanted with IL-12 reduced pulmonary pathology, proinflammatory cytokines, and the presence of lung lymphoid cell aggregates after *Y. pestis* challenge. Protection against pneumonic plague was partially dependent upon Fc receptors and could be transferred to naïve mice with immune mouse serum and was not dependent upon complement. Interestingly, depletion of CD4 and/or CD8 T cells from vaccinated mice before challenge did not affect their survival. This study suggested the safety, immunogenicity, and protective efficacy of i.n. administered iYp plus IL-12 in a mouse model of pneumonic plague [237].

Do et al investigated a novel approach based on targeting of dendritic cells using the DEC-205/CD205 receptor (DEC) via the intranasal route as way to improve mucosal cellular immunity to the vaccine. Intranasal administration of *Y. pestis* LcrV (V) protein fused to anti-DEC antibody together with poly IC as an adjuvant induced high frequencies of IFN-γ secreting $CD4^+$ T cells in the airway and lung as well as pulmonary IgG and IgA antibodies. Anti-DEC:LcrV was more efficient to induce IFN-γ/TNF-α/IL-2 secreting polyfunctional $CD4^+$ T cells when compared to non-targeted soluble protein vaccine. In addition, the intranasal route of immunization with anti-DEC:LcrV was associated with improved survival upon pulmonary challenge with the virulent CO92 *Y. pestis*. Taken together, these data indicate that targeting dendritic cells via the mucosal route is a potential new avenue for the development of a mucosal vaccine against pneumonic plague [238].

Dinc et al evaluated the efficacy of a novel SA-4–1BBL costimulatory molecule as a Th1 adjuvant to improve cellular responses generated by the rF1-V vaccine. They found that rF1-V recombinant antigen adjuvanted with SA-4–1BBL had better efficacy than with alum in generating CD4+ and CD8+ T cells producing TNF-α and IFN-γ for Th1 responses. However, SA-4–1BBL as a single adjuvant did not generate a significant antibody response against rF1-V. SA-4–1BBL in combination with alum did not increase antibody titers to F1 and LcrV, but significantly increased the ratio of Th1 regulated IgG2c to the Th2 regulated IgG1 in C57BL/6 mice. Protective experiment indicated that a single vaccination with rF1-V adjuvanted with SA-4–1BBL+alum provided better protection against bubonic challenge with *Y. pestis* CO92 than vaccines containing individual adjuvants [239]. The results suggested that SA-4–1BBL as an adjuvant generated a more balanced Th1 cellular and humoral immune response and might be employed to deal with other pathogens.

Plant-based plague vaccine.

Plants are emerging as an economical alternative to fermentation-based expression systems for producing vaccine antigens from bacteria, viruses, parasites. Several vaccines in Phase I human clinical trials accomplished with plant-made technology have been reviewed in recent paper [240]. The tobacco cell derived vaccine against the Newcastle disease virus was the first licensed plant-derived vaccine [241]. ZMapp used to fight for Ebolavirus infection was a plant-made antibody as a hot spot recently reported by scientific news [242].

Santi et al used *Nicotiana benthamiana* by using a deconstructed tobacco mosaic virus-based system to rapidly synthesize high levels of the plague antigens F1, V, and fusion protein F1-V. Subcutaneous immunization with these plant-derived purified antigens F1, V, and fusion protein F1-V to guinea pigs generated systemic immune responses and provided protection against an aerosol challenge of virulent *Y. pestis* CO92 [243]. Alvarez et al employed tomato to deliver *Y. pestis* F1-V antigen as oral vaccine to facilitate antigen delivery and induce mucosal immune response [244]. Mice were primed subcutaneously with bacterially-produced F1-V and boosted orally with transgenic tomato fruit. Analysis of antibody responses indicated that the F1- and V-specific IgG1 concentrations were significantly higher in mice boosted with the transgenic tomato fruit than in mice boosted with W.T. (non-transgenic tomato fruit) and F1- and V-specific mucosal IgA was elicited only in mice boosted with oral transgenic F1-V tomato [244]. Also they found that genetically modified tomato with the highest P19 protein levels was correlated with the highest F1-V antigen accumulation [245]. On the other hand, they tried to use Zera technology to induce protein body formation in non-seed tissues. Zera (gamma-Zein ER-accumulating domain) is the N-terminal proline-rich domain of gamma-zein that is sufficient to induce the assembly of protein bodies (PB) formation in the rough endoplasmic reticulum (ER)-derived organelles. Their studies demonstrated that Zera-F1-V protein accumulation was at least 3× higher than F1-V without Zera fusion in three different host plant systems: *N. benthamiana*, *Medicago sativa* (alfalfa) and *Nicotiana tabacum* NT1 cells [246].

Yusibov' group reported that the F1, LcrV and F1-LcrV antigens of *Y. pestis* fused with a thermostable carrier molecule, lichenase (LicKM), from *Clostridium thermocellum* were synthesized in *N. benthamiana*. Subcutaneous immunization with the purified antigens as vaccines from *N. benthamiana* in Cynomolgus Macaques induced high titers of serum IgG, mainly of the IgG1 isotype, against both F1 and LcrV. Challenge study indicated that the LcrV-F1 plant-produced vaccine conferred complete protection against aerosolized *Y. pestis* [247, 248]. This study clearly demonstrates the efficacy of a plant-produced plague vaccine candidate in a primate model. Additionally, Del Prete et al. showed that F1, V and F1-V fusion protein produced in *N. benthamiana* administered to guinea pigs resulted in immunity and protection against an aerosol challenge of virulent *Y. pestis*. The plant-derived F1, V and F1-V antigens could engage in TLR2 signalling and activated IL-6 and CXCL-8 production in monocytes, however did not affect the production of IL-12, IL-10, IL-1β, and CXCL10. Native F1 antigen and recombinant plant-derived F1 (rF1) and rF1-V all induced similar specific T-cell responses [249].

The engineering of chloroplasts for the production of vaccines and biopharmaceuticals has ushered in a new era in biotechnology [250]. Arlen et al. firstly reported high expression of

the plague F1-V fusion antigen in chloroplasts as an oral vaccine that provided protection against aerosol challenge of *Y. pestis* CO92 [251]. Rosales-Mendoza et al. synthesized F1-V fusion antigen as an oral vaccine in lettuce [252] or carrot [253] via Agrobacterium-mediated transformation. An ELISA analysis confirmed that the expected antigenic F1-V protein was successfully expressed in transgenic lines. Mice immunized subcutaneously with lettuce or carrot expressing the F1-V antigen developed systemic humoral responses [252, 253].

6. Perspectives

Y. pestis began to be used as a biological weapon at least 700 years ago and is today considered one of the more likely bioweapons, owing to its extreme virulence, its low infectious dose, and the ease of its transmission. Pneumonic plague caused by inhaling *Y. pestis* has a short incubation period and progresses rapidly to a fatal infection, and victims often become sources of secondary infections as indicated by historical plague epidemics [2, 254]. All these factors stimulate studies on development of different plague vaccines including live recombinant, purified subunit, recombinant subunit, DNA, chemical fractions, plant-based vaccine.

Currently, two subunit vaccines based on rF1 and rV antigens have passed through Phase I and II clinical trials and into the licensing process. Although direct determination of efficacy is not possible due to ethical considerations, human immune responses to subunit plague vaccine have shown good correlation with macaque and mouse immune responses [91]. The third one, rV10 vaccine is currently undergoing US Food and Drug Administration (FDA) pre-Investigational New Drug (pre-IND) authorization review for a future phase I trial [86]. The rV10 vaccine provided complete protection for guinea pigs against intranasal challenge with 1,000 MLD *Y. pestis* CAC1 (*caf1A*::IS*1541*) [104]. Passive transfer of rLcrV- or rV10-specific antibodies to BALB/c mice provided protection for them intravenously challenged with *Y. pestis* or *Y. pestis* strains expressing polymorphic *lcrV* KIM D27 (KIMD27) or *lcrV* WA-314 (NCM4) [255]. Also, plague molecular microencapsulated vaccine based on rF1 and rV antigens in Russian have passed through Phase I clinical trials (http://www.niigpk.ru/science/klinicheskie-issledovaniya/zavershennye-klinicheskie-issledovaniya). Thus, subunit vaccines are the most promising prospects for human use in current situations.

The live attenuated *Y. pestis* vaccines, EV76 derivatives, can induce great protection against bubonic and pneumonic plague, but these vaccines are not favored in the United States and Europe due to safety concerns [15, 118]. However, live, attenuated *Y. pestis* strains can be altered rationally to become safe vaccines eliciting both humoral and cellular immune responses providing stronger protection against *Y. pestis* than vaccines based on only one or two antigens. Thus, development of new, improved, live attenuated *Y. pestis* vaccines should be encouraged. In addition, since nearly thirty years ago, live attenuated mutants of *Salmonella* have attracted considerable attention as vectors for the delivery of a variety of heterologous vaccine antigens [256–261]. Much progress was achieved in developing safe efficacious live attenuated *Salmonella* vaccines for poultry [262], swine [263], cattle [264] and humans [265–270]. The vaccines for poultry and swine are licensed and used internationally to prevent infection with broad host range and host-species adapted

S*almonella* serotypes. *Salmonella*-based vaccine administration is needle-free and easier and less expensive to manufacture (~10 cents/dose for human vaccines) than subunit vaccines. Several groups are endeavored to develop a vastly improved array of means to enhance the safety, efficacy and utility of *Salmonella* antigen delivery technologies [178, 271, 272]. Use of the *Salmonella* vector system for delivery of multiple *Yersinia* antigens also has good prospects against plague.

Y. pestis is now endemic in rodent populations in many regions around the world making it difficult to control [273]. Bubonic plague is primarily a disease of rodents that is spread by fleas in nature, humans are occasionally infected either by flea bite or by inhalational exposure, usually through a secondary host, for example, a wild rabbit or prairie dog or domestic cat or, rarely, through another infected person [2]. Plague sero-prevalence also indicated that wild animals involved in the persistence and transmission of *Y. pestis* [274–279]. A viable alternative strategy is to immunize targeted wild rodent populations against plague, especially those living in close contact with humans. This approach would directly address the source of *Y. pestis* and prevent its spread into humans. Importantly, it would also provide a means to potentially reduce plague epidemic in rodent populations at treated areas, which is not possible today with existing tools. As enteric pathogens, *S. typhimurium* or *Y. pseudotuberculosis* based vaccines can be oral administration, and less expensive to manufacture than subunit vaccines. Therefore, palatable baits containing live *S. typhimurium* or *Y. pseudotuberculosis* based vaccines for herd immunization might reduce plague epidemics.

Acknowledgements

This work was supported by National Institutes of Health grants R21AI095872 to WS.

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Table 1.

Immunogenicity and protective efficacy of sub-unit antigens of *Y. pestis*

Antigen	Function	Immunization route	Protective efficacy	Reference
LPS	Lipopolysaccharide	s.c.	Bubonic - not protective	[35]
pH 6 antigen	Fimbrial adhesion	s.c.	Provided a significant protection (70%) against a intranasal infection with *Y. pestis* KIM5 (Pgm-) in the iron dextran-treated mouse model and no any protection against s.c. challenge with fully virulent strains 231 and I-1996.	[44] [183]
YopD	Type III system - translocation Yop	s.c. prime and s.c. and i.p. booster	No protection against encapsulated CO92 by s.c. challenge; Partial protection against non-encapsulated CO92 by s.c. challenge.	[46]
YopE	Type III system - cytotoxin effector Yop	s.c. prime and s.c. and i.p. booster	No protection against encapsulated and non-encapsulated CO92 by s.c. challenge.	[46, 280]
YopH	Type III system-PTPase effector Yop	s.c. prime and s.c. and i.p. booster	No protection against encapsulated and non-encapsulated CO92 by s.c. challenge.	[46]
YopK	Type III system - regulates Yop release	s.c. prime and s.c. and i.p. booster	No protection against encapsulated and non-encapsulated CO92 by s.c. challenge.	[46, 280]
YopM	Type III system - effector Yop	s.c. prime and s.c. and i.p. booster	No protection against encapsulated and non-encapsulated CO92 by s.c. challenge.	[46, 281]
YopN	Type III system - regulates Yop release	s.c. prime and s.c. and i.p. booster	No protection against encapsulated and non-encapsulated CO92 by s.c. challenge.	[46, 280]
YpkA	Type III system - Ser/Thr kinase effector Yop	s.c. prime and s.c. and i.p. booster	No protection against encapsulated and non-encapsulated CO92 by s.c. challenge, but significantly prolonged mean survival time.	[46]
YadC	A member of the oligomeric coiled-coil adhesins	s.c.	Partial protection against F1$^-$ *Y. pestis* challenge in mice	[51]
YscF	*Yersinia* secretory factor F	i.p.	Partial protection against intravenous challenge with *Y. pestis* KIM5	[57]
YPO0606	Autotransporter protein YapF	i.m.	Partial protection against s.c. challenge with *Y. pestis* 201strain	[43]
YPO1914	Inner membrane ABC transporter YbtQ	i.m.	Partial protection against s.c. challenge with *Y. pestis* 201strain	[43]
YPO0612	Putative sugar binding protein	i.m.	Partial protection against s.c. challenge with *Y. pestis* 201strain	[43]
YPO3119	Heat shock protein HtpG	i.m.	Partial protection against s.c. challenge with *Y. pestis* 201strain	[43]
YPO3047	Putative sulfatase YdeN	i.m.	Partial protection against s.c. challenge with *Y. pestis* 201strain	[43]
YPO1377	Putative outer membrane lipoprotein carrier protein	i.m.	Partial protection against s.c. challenge with *Y. pestis* 201strain	[43]
YPCD1.05c	YopE chaperone SycE/YerA; YopE-targeting protein	i.m.	Partial protection against s.c. challenge with *Y. pestis* 201strain	[43]
YPO0420	Putative lipoprotein	i.m.	Partial protection against s.c. challenge with *Y. pestis* 201strain	[43]
YPO3720	Hemolysin activator protein	i.m.	Partial protection against s.c. challenge with *Y. pestis* 201strain	[43]
Ail/OmpX	Outer membrane protein X	i.m.	Partial protection against bubonic plague, no protection against pneumonic plague in mice model	[58]

Antigen	Function	Immunization route	Protective efficacy	Reference
OmpA	Outer membrane protein A	i.m.	Partial protection against bubonic plague, no protection against pneumonic plague in mice model	[58]
Pla	The plasminogen activator	i.m.	Pla-immunized mice were 60% protected against i.n. challenge of Δ*caf1 Y. pestis* CO92 at a 15 LD_{50}, but no protection against s.c. challenge of Δ*caf1 Y. pestis* CO92 at a 500 LD_{50}.	[58]
F1	Surface capsule	s.c.	Bubonic & pneumonic - protective	[58, 63–66, 88]
LcrV	Type III system-part of the injectosome	i.m.	Bubonic & pneumonic - protective	[11, 18, 19, 71, 81–86]
F1-V		i.m.	Great protection against bubonic and pneumonic plague	[58, 104]
V10		i.m.	Great protection against bubonic and pneumonic plague	[104]
V10–2		i.m.	Great protection against bubonic and pneumonic plague	[104]
F1 + rV270		i.m.	Great protection against bubonic and pneumonic plague in mice, Guinea pigs, rabbits and rhesus macaques	

Table 2.

live attenuated *Y. pestis* strains as vaccines against plague

Y. pestis mutant	LD_{50}	Immunization	Protective efficacy	Reference
Y. pestis Kimberley53 Δ*pcm*	$>10^7$ CFU in female OF1 outbred mice by s.c.	s.c. vaccination with 10^6 CFU	complete protection against s.c. challenge with 10^5 CFU of *Y. pestis* Kimberley53	[134]
Y. pestis Kimberley53 Δ*nlpD*	$>10^7$ CFU for s.c and airway routes of infection in Female OF1 mice	s.c. immunization with 10^7 CFU of mutant strain	Provides complete protection against s.c. challenge with 10^5 LD_{50} of *Y. pestis* Kimberley53 and 82% protection against i.n. challenge with 5500 CFU of *Y. pestis* Kimberley53	[135]
Y. pestis 231 Δ*nlpD*, *Y. pestis* I-3455 Δ*nlpD*	$>10^7$ CFU in BALB/c mice by s.c. and $>1.5\times10^{10}$ CFU in guinea pigs	s.c. vaccination with 10^4 CFU for mice and 5×10^3 CFU for guinea pigs	Δ*nlpD* mutants induces immunity 10^5 times more potent than the one induced by the administration of the EV vaccine strain. At the same time, $NlpD^-$ bacteria failed to protect guinea pigs in the case of a subcutaneous challenge with *Y. pestis*, inducing a 10^6 times less potent protection compared with that conferred by immunization with the EV vaccine strain.	[136]
Y. pestis GB Δ*dam*	2.3×10^3 CFU in female BALB/c mice by s.c.	s.c. vaccination with 1.5×10^3 CFU	complete protection against s.c. challenge with 7500 CFU of *Y. pestis* GB	[282]
Y. pestis CO92 Δ*yopH*	$>10^7$ CFU in female outbred CD1mice by i.n. and s.c. respectively	s.c. and i.n. vaccination	s.c. vaccination with ~10^7 provides 70% protection against s.c. challenge with ~10^5 CFU of *Y. pestis* CO92. i.n. vaccination with ~10^7 CFU of CO92 Δ*yopH* provides 100% protection against s.c. challenge and 61.5% protection against i.n. challenge with ~10^5 CFU CO92, respectively.	[133]
Y. pestis EV Δ*lpxM*	$>2.5\times10^4$ CFU in female BALB/c mice by s.c.	s.c. vaccination with 2.5×10^4 CFU of mutant strain	Provides ~40% protection against s.c. challenge with virulent strain *Y. pestis* 231	[283]
χ10004 (pCD1Ap) Δ*relA* Δ*spoT*	5.8×10^5 CFU for s.c. infection in Female Swiss Webster mice	s.c. immunization with 2.5×10^4 CFU of mutant strain	A single s.c. vaccination could provide complete protection against s.c. challenge with 1.5×10^5 CFU of virulent strain and 60% protection against i.n. challenge with 2.0×10^4 CFU of *Y. pestis* KIM5+	[122]
Y. pestis CO92 Δ*pgm* Δ*smpB-ssrA*	$>10^6$ CFU by i.v. infection and $>10^8$ CFU by i.n. infection in C57BL/6 mice	i.v. and i.n. immunization with 10^4 CFU of mutant strain	Provides complete protection against i.n. challenge with 2×10^5 CFU of *Y. pestis* CO92Δ*pgm*	[147]
χ10010 (pCD1Ap) Δ*crp*	$>3\times10^7$ CFU by s.c. infection and $>10^5$ CFU by i.n. infection Female Swiss Webster mice	s.c. immunization with 3.8×10^7 CFU of mutant strain	Provides 80% protection against s.c. challenge with 10^7 CFU of virulent strain and no protection against i.n. challenge with 1×10^4 CFU of *Y. pestis* KIM5+	[123]
χ10017 (pCD1Ap) ΔP_{crp}::*araC* P_{BAD} *crp*	$>4.3\times10^5$ CFU by s.c. infection and ~10^5 CFU by i.n. infection in female Swiss Webster mice	s.c. immunization with 3.0×10^4 CFU of mutant strain	Provides complete protection against s.c. challenge with 10^5 CFU of virulent strain and 70% protection against i.n. challenge with 1×10^4 CFU of *Y. pestis* KIM5+	[123]
Y. pestis GB Δ*guaBA*	$>7\times10^4$ CFU by s.c. and i.v. infection in female BALB/c mice	s.c. and i.n. immunization with 7×10^4 CFU of mutant strain	Provides 100% protection against s.c. challenge with ~ 66 LD_{50} of the wild-type *Y. pestis* strain GB.	[284]
χ10015 (pCD1Ap) Δ*lpxP*::P_{lpxL} *lpxL*	~10^7 CFU by s.c. infection and 2.7×10^3 CFU by i.n. infection in female Swiss Webster mice	s.c. immunization with 2.2×10^6 CFU of χ10015(pCD1Ap)	Provides complete protection against s.c. challenge with 3.57×10^7 CFU of virulent strain and 90% protection against i.n. challenge with 1.24×10^4 CFU of *Y. pestis* KIM5+	[132]

Y. pestis mutant	LD_{50}	Immunization	Protective efficacy	Reference
χ10027 (pCD1Ap) Δ*lpxP32*::P_{lpxL} *lpxL* Δ*lacI23*::P_{lpp} *lpxE*	LD_{50}s: 2.3 × 10^4 CFU for s.c. infection and 7.8 × 10^4 CFU for i.n. infection	ND	ND	[131]
χ10028 (pCD1Ap) *pgm*⁻ Δ*lpxP*::P_{lpxL} *lpxL*	~10^8 CFU by s.c. infection and 5×10^3 CFU by i.n. infection in female Swiss Webster mice	s.c. immunization1.4 × 10^7 CFU of χ10028(pCD1Ap)	Provides 80% protection against s.c. challenge with 3.57 × 10^7 CFU of virulent strain and 60% protection against i.n. challenge with 1.24 ×10^4 CFU of *Y. pestis* KIM5+	[132]
χ10030 (pCD1Ap) ΔP_{crp} TT *araC* P_{BAD} *crp* Δ*lpxP*::P_{lpxL} *lpxL*	>10^8 CFU by s.c. infection and >10^6 CFU by i.n. infection in female Swiss Webster mice	s.c. immunization 1.4×10^7 CFU of χ10030(pCD1Ap)	Provides complete protection against s.c. challenge with 3.57 × 10^7 CFU of virulent strain and 80% protection against i.n. challenge with 1.24 × 10^4 CFU of *Y. pestis* KIM5+	[132]
ΔyscN *Y. pestis* CO92	>4.44 × 10^6 CFU by s.c. infection in female Swiss Webster mice	s.c. immunization with 4.98 × 10^7 CFU of ΔyscN *Y. pestis* CO92 and s.c. booster at 28 days with 3.22 × 10^7 CFU	Provides 90% protection against s.c. challenge with 180 CFU of virulent *Y. pestis* CO92	[137]
Δ*lpp* Δ*msbB Y. pestis* CO92	LD50s: >30 CFU by s.c. infection and ~ 10^3 CFU by i.n. infection in female Swiss Webster mice	Survival animals re-challenged by *Y. pestis* CO92	Provide partial protection against s.c. and i.n. challenge	[138]
Δ*yscB Y. pestis* biovar Microtus strain 201	LD_{50} of the Δ*yscB* mutant in BALB/c mice is estimated to be more than 10^6 CFU, which is more than 400,000-fold higher than 3 CFU of the wild-type strain.	s.c. immunization with 1.63 × 10^4 CFU of Δ*yscB* mutant	The Δ*yscB* mutant provided 87.5% or complete protection by the subcutaneous or intranasal challenge with 1.24 × 10^6 CFU of virulent *Y. pestis* strain 141.	[139]
Y. pestis biovar Microtus strain 201	**Avirulent to humans or primates**	s.c. immunization with 1.4 × 10^{10} CFU of *Y. pestis* strain 201	The immunization provided 83.3% protection by the subcutaneous challenge with 1.74 × 10^9 CFU of virulent *Y. pestis* strain 141 for rhesus macaques.	[140]
Δ*lpp*Δ*msbB* Δ*rbsA Y. pestis* CO92	20–50 LD_{50} in a mouse model of pneumonic plague	Survival animals re-challenged by *Y. pestis* CO92	The triple mutant-infected mice at 50 LD_{50} were 90% protected upon subsequent challenge with 12 LD_{50} of *Y. pestis* CO92	[141]
Δ*ail* Δ*lpp* Δ*msbB Y. pestis* CO92	LD_{50} of the triple mutant (Δ*ail* Δ*lpp* Δ*msbB*) was equal to 6,800 LD_{50} of *Y. pestis* CO92	Survival animals re-challenged by *Y. pestis* CO92	Partially protection against intranasal challenge with the bioluminescent *Y. pestis* CO92 strain (20 to 28 LD_{50}s)	[142]
V674pF1	LD_{50} of the V674pF1 strain in OF1 female mice is more than 10^9 CFU	Oral immunization with 10^8 or 10^9 CFU of V674pF1 strain	A very high challenge dose of 10^7 CFU CO92 (3,300×LD_{50}) was used to mimic a severe contamination, mice vaccinated with 10^8 CFU of V674pF1 showed 80% protection and this protection reached 94% when a vaccine dose of 10^9 CFU was administered.	[148]
χ10057 Δ*asd-206* Δ*msbB868*::P_{msbB} *msbB(EC)* ΔP_{crp21}::TT *araC* P_{BAD} *crp*	LD_{50} of the χ10057 strain in Swiss Webster mice is more than 10^9 CFU	Oral immunization with 10^9 CFU of χ10057(pYA5199) strain	Provide 83% protection against i.n. challenge with 1. 3 × 10^4 CFU of virulent *Y. pestis* KIM6+ (pCD1Ap) strain	[149]

Note: *Y. pestis* GB, LD_{50} (s.c.) = 1CFU [285]; *Y. pestis* KIM5+, LD_{50} (s.c.) < 10 CFU, LD_{50} (i.n.) ~100CFU [132]; *Y. pestis* Kimberley53, LD_{50} (s.c.) 1~3 CFU, LD_{50} (i.n.) = 550 CFU [135]; *Y. pestis* 231, LD_{50} (s.c.) < 10 CFU [286]; *Y. pestis* CO92, LD_{50} (s.c.) =1.9 CFU, LD_{50} (i.n.) ~250 CFU, LD_{50} (aerosol) ~2100 CFU [65, 287, 288]; *Y. pestis* CO92Δ*pgm* strain, LD_{50} (i.n.) =2 × 10^4 CFU [147].
CFU: Colony forming units; LD_{50}: 50% lethal dose; s.c.: Subcutaneous; i.n.: Intranasal; i.p.: Intraperitoneal.

Table 3.

Evaluation of *Salmonella* vaccine strains expressing antigen(s) of *Y. pestis*

Strain	Genotype	Antigen(s)	Immunization route	Protective efficacy	Reference
S. Typhimurium					
SL3261	*aroA*	F1	i.v. or oral	i.v. immunization provides complete protection/oral immunization provides 90% protection against 50 LD_{50}s of *Y. pestis* GB strain s.c. challenge	[289]
SL3261	*aroA*	F1/V fusion	i.v.	provides 85% protection against 50 LD_{50}s of *Y. pestis* GB strain s.c. challenge	[170]
SL3261	*aroA*	F1 expressed under different promoters	oral	ND (serum antibody responses to F1 reported)	[290]
SL3261	*aroA*	LcrV	oral	provides 30% protection against 97 LD_{50}s of *Y. pestis* GB strain s.c. challenge	[171]
SL3261 SLDAPD	*aroA*	F1	oral	oral immunization provides complete protection against 8.8×10^4 LD_{50}s of *Y. pestis* GB strain s.c. challenge	[291]
H683	*asd aroA*	F1 and/or LcrV	oral	90% protection against ~1000LD_{50} *Y. pestis* 195/P s.c. challenge and 90% protection against ~100LD_{50} *Y. pestis* Madagascar	[119]
χ8501	*hisG* Δ*crp-28* Δ*asdA16*	F1 or LcrV	i.n. or oral	3 doses of strain synthesizing F1 or LcrV provided 80% or 60% protection against 2×10^3 CFU of *Y. pestis* (Yokohama-R strain) i.p. challenge, respectively	[292]
χ8501	*hisG* Δ*crp-28* Δ*asdA16*	Truncated LcrV (aa131-aa327) and Psn	oral	Truncated LcrV and Psn immunization provide 80% protection against 3000 CFU of *Y. pestis* CO92 and 75% protection against 1300 CFU of *Y. pestis* CO92 by s.c. challenge	[174]
χ9447	Complex genotype[a] Strain displays "regulated in vivo lysis"	LcrV196 on "runaway-like replication plasmid"	oral	Oral immunization provides 87.5% survival post-s.c. challenge with 4.49×10^2 CFU or 5.63×10^3 CFU of *Y. pestis* CO92. Oral immunization shows great efficacy with 75% and 50% survival post-i.n. challenge with 4.1×10^3 CFU or 4.4×10^4 CFU of *Y. pestis* CO92, respectively.	[293]
χ9558	Complex genotype[b]	PsaA	oral	No protection against s.c. challenge with 4.49×10^2 CFU of *Y. pestis* CO92 and 25% protection against i.n. challenge with 4.1×10^3 CFU of *Y. pestis* CO92	[184]
χ8501	*hisG* Δ*crp-28* Δ*asdA16*	Psn, HmuR, LcrV196 LcrV5214	oral	Immunization with the strain expressing Psn or LcrV196 afforded nearly full protection against s.c challenge and partial protection against i.n. challenge. While immunization with the strain expressing LcrV5214 or HmuR no protection against s.c. or i.n challenge.	[175]
χ9558	Complex genotype[b]				
S. Typhi					
BRD1116	*aroA aroC htrA*	F1	i.n.	intranasal immunization provides 65% protection against 113 CFU of *Y. pestis* GB strain s.c. challenge	[176]
ACAM948CVD (CVD 9080)	*htrA*, *aroC*, *aroD*	F1	i.n.	~80% protection against i.v. challenge with 10,000 CFU of *Y. pestis* EV76 in 0.2 ml of sterile PBS. $FeCl_2$ (40 μg/mouse) was administered i.p. immediately.	[177]
CVD 910FV	*guaBA* and *htrA*	F1, LcrV	i.n.	mice receiving the optimized bivalent vaccine were fully protected against lethal pulmonary challenge	[294]

Note: *Y. pestis* GB, LD_{50} (s.c.) = 1CFU [285]; *Y. pestis* 195/P, LD_{50} (s.c.) = 1CFU [295]; *Y. pestis* Madagascar, LD_{50} (i.n.) ~100 CFU [295]; *Y. pestis* (Yokohama-R strain), LD_{50} (i.p.) = 15 CFU [296]; *Y. pestis* EV76, LD_{50} (i.v.) =56 CFU [177]; *Y. pestis* CO92, LD_{50} (s.c.) =1.9 CFU, LD_{50} (i.n.) ~250 CFU, LD_{50} (aerosol) ~2100 CFU [65, 287, 288];

CFU: Colony forming units; LD_{50}: 50% lethal dose; s.c.: Subcutaneous; i.n.: Intranasal; i.v. Intravenous; i.p.: Intraperitoneal; ND: no detected.

[a] Δ*pmi-2426* Δ(*gmd-fcl*)*-26* ΔP_{fur81}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA21*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT ΔP_{murA7}::TT *araC* P_{BAD} *murA* Δ*endA2311*

[b] Δ*pmi-2426* Δ(*gmd-fcl*)*-26* ΔP_{fur81}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA27*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT Δ*sopB1925* Δ*agfBAC811*