Review Article

Developing live vaccines against *Yersinia pestis*

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Abstract

Three great plague pandemics caused by the gram-negative bacterium *Yersinia pestis* have killed nearly 200 million people and it has been linked to biowarfare in the past. Plague is endemic in many parts of the world. In addition, the risk of plague as a bioweapon has prompted increased research to develop plague vaccines against this disease. Injectable subunit vaccines are being developed in the United States and United Kingdom. However, the live attenuated *Y. pestis*-EV NIIEG strain has been used as a vaccine for more than 70 years in the former Soviet Union and in some parts of Asia and provides a high degree of efficacy against plague. This vaccine has not gained general acceptance because of safety concerns. In recent years, modern molecular biological techniques have been applied to *Y. pestis* to construct strains with specific defined mutations designed to create safe, immunogenic vaccines with potential for use in humans and as bait vaccines to reduce the load of *Y. pestis* in the environment. In addition, a number of live, vectored vaccines have been reported using attenuated viral vectors or attenuated *Salmonella* strains to deliver plague antigens. Here we summarize the progress of live attenuated vaccines against plague.

Introduction

Y*ersinia pestis*, the causative agent of plague, is an aerobic, non-motile, gram-negative bacillus belonging to the family *Enterobacteriaceae*. Plague is a zoonotic infection transmitted to humans most commonly via the bite of an infected flea, typically *Xenopsylla cheopis* [1]. Natural reservoirs of *Y. pestis* include rodents, squirrels, and prairie dogs. Large reservoirs of *Y. pestis* still exist on all major inhabited continents, except Australia [2] and it still remains a serious public health threat in those regions [2,3]. Plague was responsible for at least 3 great pandemics and killed nearly 200 million people [2] at times when the global human population was likely far less than one billion. Current epidemiological records suggest 4,000 human plague cases annually worldwide [4]. Three clinical forms of human plague exist: bubonic, septicemic, and pneumonic [5]. *Y. pestis* cells spread from the site of the infected flea bite to the regional lymph nodes, grow to high numbers causing the formation of a bubo, and spill into the blood-stream where bacteria are removed in the liver and spleen. Growth continues in the liver and spleen, spreads to other organs, and causes a septicemia. Fleas feeding on septicemic animals complete the infection cycle. Humans, highly susceptible to plague, are accidental hosts through close contact with animal reservoirs. In humans bubonic plague can develop into an infection of the lung (secondary pneumonic plague); this can lead to aerosol transmission (primary pneumonic plague) [2, 6].

In addition to the potential for natural infections, *Y. pestis* is considered to be among the top five potential biological weapons [7]. One of the earliest recorded biological warfare attempts using plague was by Tartar forces, laying siege to 14th-century Kaffar (now called Feodosia, Ukraine), who catapulted their plague victims into the city in an attempt to start an epidemic among the defending forces. During World War II, Japanese forces released plague-infected fleas from aircraft over Chinese cities. More recently, an Ohio man with extremist connections tried to obtain *Y. pestis* from the American Type Culture Collection [7,8,9,10,11]. Other evidence suggests that *Y. pestis* was being developed for potential biological warfare use in the former Soviet Union [7,8,9,10,11] as well as in US and in Great Britain. Plague remains an important bioterrorism

Table 1. 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	[131]
SL3261	aroA	F1/V fusion	i.v.	provides 85% protection against 50 LD_{50}s of *Y. pestis* GB strain s.c. challenge	[65]
SL3261	aroA	F1 expressed under different promoters	oral	ND (serum antibody responses to F1 reported)	[132]
SL3261	aroA	LcrV	oral	provides 30% protection against 97 LD_{50}s of *Y. pestis* GB strain s.c. challenge	[66]
SL3261	aroA	F1	oral	oral immunization provides complete protection against 8.8×10^4 LD_{50}s of *Y. pestis* GB strain s.c. challenge	[133]
SLDAPD 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	[68]
χ8501	hisG Δcrp-28 ΔasdA16	F1 or LcrV; Strain displays "regulated in vivo lysis"	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.	[134]
χ8501	hisG Δcrp-28 ΔasdA16	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	[69]
χ9447	Complex genotype[a]	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.	[135]
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	[79]
χ8501	hisG Δcrp-28 ΔasdA16	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.	[70]
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	[71]
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.	[72]

Note: *Y. pestis* GB, LD_{50} (s.c.) = 1CFU (136); *Y. pestis* 195/P, LD_{50} (s.c.) = 1CFU (68); *Y. pestis* Madagascar, LD_{50} (i.n.) ~100 CFU (68); *Y. pestis* (Yokohama-R strain), LD_{50} (i.p.) = 15 CFU (137; *Y. pestis* EV76, LD_{50} (i.v.) ≈56 CFU (722); *Y. pestis* CO92, LD_{50} (s.c.) =1.9 CFU, LD_{50} (i.n.) ~250 CFU, LD_{50} (aerosol) ~2100 CFU (138, 139, 140);
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] Δpmi-2426 Δ(gmd-fcl)-26 ΔP$_{fur81}$::TT araC P$_{BAD}$ fur ΔP$_{crp527}$::TT araC P$_{BAD}$ crp c2 ΔaraE25 ΔaraBAD23 ΔrelA198::araC P$_{BAD}$ lacI TT ΔP$_{murA7}$::TT araC P$_{BAD}$ murA ΔrelA ΔendA2311
[b] Δpmi-2426 Δ(gmd-fcl)-26 ΔP$_{fur81}$::TT araC P$_{BAD}$ fur ΔP$_{crp527}$::TT araC P$_{BAD}$ crp ΔasdA27::TT araC P$_{BAD}$ c2 ΔaraE25 ΔaraBAD23 ΔrelA198::araC P$_{BAD}$ lacI TT ΔsopB125 ΔxigBAC81l

threat because the organism can be easily obtained from any of the numerous and widely dispersed animal reservoirs of plague [2]. Additionally, *Y. pestis* is easily genetically manipulated to create strains with specific engineered traits, such as constructing *Y. pestis* strains resistant to multiple antibiotics often used to treat plague patients.

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, offer 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 [12,13]. For longer-term protection and to counter drug resistance, vaccination is believed to be crucial [14,15]. There is currently no licensed vaccine for use in the United States and the lack of a safe, effective vaccine for human use puts both military personnel and the general public at risk. Here, we briefly summarize recent progress in the development of injectable vaccines, which has been recently described in more detail elsewhere [14,15,16,17,18]. Live vaccines have a number of advantages over injectable vaccines including mucosal delivery (needle-free); stimulation of cellular, humoral and mucosal immunity; and low cost [19]. Furthermore, they can be formulated to preclude the need for refrigeration (e.g. cold chain) [20,21]. Our primary focus here is therefore to describe progress in the development of live vaccines for plague.

Killed whole-cell vaccine and subunit vaccines

Plague Vaccine (USP), which was licensed for human use in the United States and the United Kingdom, has not been available in the US since 1999. However, USP vaccine is still used for research in the UK [22,23]. 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 [24,25]. However, these vaccines cause significant adverse reactions, particularly after booster injections, which are needed to maintain protection [5]. 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 [17,24,26,27]. Thus killed whole cell vaccines are probably not suitable for defense against weaponized pneumonic plague.

Recent efforts to create a safe and effective plague vaccine have focused on the development of recombinant subunit vaccines that elicit antibodies against two well-characterized *Y. pestis* antigens, the F1 capsule and the virulence protein LcrV [28,29,30, 31]. While there has been some controversy surrounding the efficacy of subunit vaccines in some non-human primates [18], several candidates are currently moving toward licensure. The usefulness of F1 as a protective antigen is not clear, since F1$^-$ strains can cause plague (see below). Currently, the recombinant F1V (rF1V) being developed at DynPort Vaccine Company is in a Phase 2b clinical trial (www.clinicaltrials.gov). In addition, the rV10 vaccine (truncated recombinant LcrV protein) is currently undergoing US Food and Drug Administration pre-Investigational New Drug authorization review for a future phase I trial [16].

Live 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 [32]. There are many examples of successful live attenuated vaccines delivered by injection, including the current bacterial vaccine for tuberculosis (BCG) [33] and viral vaccines for measles, mumps, rubella, chicken pox and yellow fever [34]. Rabies vaccines are now available in two different attenuated forms, one for use in humans, and one for animals [35]. There are also a number of mucosally delivered live vaccines. These include oral vaccines against poliovirus [36], cholera [37], rotavirus [38] and typhoid fever [37,39] and the nasally delivered vaccines against influenza [40,41,42].

Most pathogens gain entry to the host via mucosal surfaces [43,44]. 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 [45]. Therefore, live attenuated vaccines have advantages

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	[115]
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	[141]
Y. pestis CO92 Δ*yopH*	>10^7 CFU in female outbred CD1mice by i.n. and s.c., respectively	s.c. 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.	[114]
Y. pestis EV Δ*lpxM*	>2.5×10^4 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	[142]
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	[116]
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+	[104]
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*	[121]

Strain	Immunization		Result	Ref
χ10010 (pCD1Ap) Δcrp	>3×10^7 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+	[105]
χ10017 (pCD1Ap) ΔP_crp::araC P_BAD crp	>4.3×10^5 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+	[105]
Y. pestis GB ΔguaBA	> 7 × 10^4 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 *Y. pestis* strain	Provides 100% protection against s.c. challenge with ~ 66 LD_50 of the wild-type *Y. pestis* strain GB.	[143]
χ10015 (pCD1Ap) ΔlpxP::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 challenge strain and 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 x 10^4 CFU of *Y. pestis* KIM5+	[112]
χ10028 (pCD1Ap) pgm⁻ ΔlpxP::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. immunization 1.4 × 10^7 CFU of χ10028(pCD1Ap) KIM5+	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+	[112]

Note: *Y. pestis* GB, LD_50 (s.c.) = 1CFU (136); *Y. pestis* KIM5+, LD_50 (s.c.) < 10 CFU, LD_50 (i.n.) ~100CFU (112); *Y. pestis* Kimberley53, LD_50 (s.c.) 1~3 CFU, LD_50 (i.n.) = 550 CFU (116); *Y. pestis* 231, LD_50 (s.c.) < 10 CFU (144); *Y. pestis* CO92, LD_50 (s.c.) =1.9 CFU, LD_50 (i.n.) ~250 CFU (aerosol) ~2100 CFU (138, 139, 140); *Y. pestis* CO92Δpgm strain, LD_50 (i.n.) =2 × 10^7 CFU (121); CFU: Colony forming units; LD_50: 50% lethal dose; s.c.: Subcutaneous; i.n.: Intranasal; i.p.: Intraperitoneal.

over subunit vaccines as they are typically taken orally, still inducing strong mucosal and durable immunity [44,46]. 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.

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 [47,48,49]. 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 [50,51]. In a subsequent study, they fused either F1 or LcrV to the adenovirus capsid protein, pIX [50,51]. Both constructs elicited strong humoral immunity in mice immunized intramuscularly with greater efficacy than an injection of adjuvanted purified V or F1.

Rose's group devised a vaccine utilizing recombinant vesicular stomatitis virus (VSV) vectors expressing the *Y. pestis lcrV* gene [52,53]. 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 [52,53]. 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 than the previous vaccine that encoded the non-secreted form. Furthermore, 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 [52,53]. Another group demonstrated the suitability of a vaccinia viral vector expressing either *lcrV* or *caf1* (gene for F1) and found it to be highly immunogenic in BALB/c mice and safe in immunocompromised SCID mice [54,55].

In a provocative study, 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* [56]. 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 [56]. While it is not clear whether this observation can be translated into a proactive approach to provide immunity against plague, it is an interesting observation that deserves further thought and consideration when evaluating any vaccine.

There have been a number of recent reports describing studies to develop viral-vectored bait vaccines to be used to control environmental sources of plague. One group has constructed a recombinant vaccinia virus to direct synthesis of an F1-V fusion protein with promising results [54,55]. 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*. Workers 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 [57] and black-tailed prairie dogs (*Cynomys ludovicianus*) vaccinated intramuscularly with RCN-F1 survived subcutaneous challenge with virulent *Y. pestis* [58]. 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 [58,59], demonstrating that oral bait immunization of prairie dogs can provide protection against plague.

Live bacterially vectored plague vaccines

The commensal, non-pathogenic bacterium *Lactococcus lactis* has been used to deliver LcrV [60,61] 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 [62,63,64]. This colonization pathway results in the induction of mucosal and systemic immune responses. Table 1 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 [65], LcrV [66] and F1 capsule on the surface of the cell [67]. 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 [68]. 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 [67,69,70], indicating that the use of *Salmonella* as a plague vaccine may provide an additional benefit.

A few 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 [71]. A similar vaccine strain was administered intranasally to 7-day old mice [72]. 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.

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 [73,74]. 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 [75]. 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 [76]. Additionally, there are known polymorphisms of LcrV that may influence protective efficacy [77]. 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 [70], HmuR [70] and PsaA, also called pH 6 antigen [78], which forms a fibrillar structure on the *Y. pestis* cell surface [79]. Psn and HmuR are outer membrane proteins involved in iron acquisition [80,81]. The role of PsaA in virulence is not clear [82,83,84], but available data indicates it may serve as an adhesin [85] and an antiphagocytic factor [86]. We demonstrated that *Salmonella* delivering Psn elicited significant protective immunity against subcutaneous challenge [70]. 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 [79]. When delivered by our *Salmonella* strains, HmuR was poorly immunogenic and did not confer protection against either challenge route [70].

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, Republic of 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 [87]. 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 [88]. 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 [88]. This strain was employed for the development of the live vaccine designated as EV NIIEG, which was manufactured in the USSR from 1940 [89]. 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 [17,89]. 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 to 12 months against bubonic and, to some extent, pneumonic plague [15,89].

However, EV76 vaccine strain can cause disease in some non-human primates, raising questions about its suitability as a human vaccine [90]. This live Pgm$^-$ strain conferred greater protection against bubonic and pneumonic plague than killed vaccines in animals, but it sometimes caused local and systemic reactions [23,26,90,91]. In addition, a live Pgm$^-$ strain retains virulence when administered by the intranasal (i.n.) and intravenous (i.v.) routes [18,90,92]. 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 [24,93]. 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. In *Salmonella enterica*, some attenuated mutants have proven to be highly effective vaccines [94,95,96]. Therefore, mutations that effectively attenuate *Salmonella*, such as *aroA*, *phoP*, *htrA* and *lpp* genes, were introduced into *Y. pestis* but those mutations had only a limited effect on *Y. pestis* virulence [97,98,99,100]. In addition, a deletion of the *Y. pestis* global regulator gene *rovA* was tested and its impact on virulence was also inadequate for

use as a vaccine [84]. Table 2 lists recent developments of live, rationally attenuated *Y. pestis* mutants as vaccines against plague.

In *Salmonella,* Δ*relA* Δ*spoT* mutants are attenuated [101] and *crp* mutants are attenuated and immunogenic [102]. It has also been established that *Y. pestis crp* mutants are attenuated for virulence [103]. In our laboratory, we examined the vaccine potential of *Y. pestis* Δ*relA* Δ*spoT* [104] and Δ*crp* mutants [105]. The Δ*relA* Δ*spoT* mutant was partially attenuated (subcutaneous $LD_{50} = 5 \times 10^5$ CFU, parent strain $LD_{50} = 10$) and protective against bubonic plague (subcutaneous challenge [s.c.]), but poorly protective against pneumonic plague (intranasal challenge). The Δ*crp* mutant was completely attenuated (s.c. $LD_{50} > 10^7$ CFU) and partially protective against both bubonic and pneumonic plague [105].

We have developed a system in *Salmonella* termed regulated delayed attenuation, in which the bacterium is modified such that virulence gene expression is dependent on the presence of arabinose and/or mannose. When cells are grown in the presence of arabinose, 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 [106]. We applied this strategy to *Y. pestis*, constructing a strain with *crp* under transcriptional control of the *araC* P_{BAD} promoter [105]. The resulting strain was partially attenuated ($LD_{50} = 4.3 \times 10^5$ CFU) and protective against both bubonic and pneumonic plague.

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*, which results 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, which is not recognized by TLR4 [107] that preferentially recognizes hexa-acylated lipid A [108,109,110]. 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 [111]. We exploited this observation to reduce the residual virulence of our arabinose-regulated *crp* strain by constructing a strain that expresses *E. coli lpxL* from the chromosome, providing greater genetic stability than plasmid

expression. The resulting strain, χ10030(pCD1Ap), produces hexa-acylated lipid A at 37°C and carries the arabinose-regulated *crp* gene [112]. Our results demonstrated an increase in the LD_{50} of χ10030(pCD1Ap) by s.c. and i.n. inoculation of more than 1.5 x10^7 and 3.4 x 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 [113]. 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* Δ*yopH* mutant is attenuated and provides a high level of protection against bubonic and pneumonic plague in mice [114]. Flashner *et al.* conducted a study to identify *Y. pestis* mutants as vaccine candidates and identified *pcm* and Δ*nlpD* mutants that were attenuated and elicited protective immunity [115,116]. Identification of other attenuating mutations that target unique *Y. pestis* virulence genes will be of significant interest for developing safe attenuated *Y. pestis* vaccines.

Y. pseudotuberculosis, a recent ancestor of *Y. pestis* [117], 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 more than 95% genetic identity and a common virulence plasmid with a conserved co-linear backbone [118]. 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* [119,120,121]. While protection was not robust it was significant,

demonstrating the feasibility of using this approach. As an enteric pathogen, a live attenuated *Y. pseudotuberculosis*-based vaccine can be given orally, making it a suitable choice to be made into bait for administration to wild animals to reduce naturally occurring plague reservoirs.

Summary

Subunit vaccines based on rF1 and rV antigens are the most promising prospects and 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 [122]. Since *Y. pestis* is easily genetically manipulated to create strains with specific engineered traits which lack highly immunogenic traits irrelevant to human pneumonic disease (e.g., the F1 capsule) or by reengineering some major virulent factors with known functional polymorphisms, such as LcrV, subunit vaccines might fail to protect against cleverly engineered *Y. pestis* strains. Previously tested killed whole-cell preparations or live-attenuated plague vaccines are currently not favored in the United States because of safety and efficacy concerns, but live, rationally attenuated strains of *Y. pestis* have been shown in animal models to provide strong protection against both bubonic and pneumonic plague. Modern live *Y. pestis* vaccines should elicit humoral and cellular immune responses against a variety of relevant antigens, providing stronger protection against weaponized *Y. pestis* than vaccines based on only one or two antigens. Therefore, we recognize the importance of continuing research toward the development of new, improved live-attenuated vaccines [14,123].

Plague is a zoonotic disease and its reservoirs exist in wild rodent populations on the continents of Africa, Asia, South America, and North America [124]. While 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 are involved in the persistence and transmission of *Y. pestis* [125,126,127,128,129,130] so lowering the incidence of plague infections in wild animals would likely reduce zoonotic transmission of

the disease to humans. Therefore, palatable baits containing live vaccines for oral immunization to reduce infection of wild animals are alternative method to control plague epidemics.

Memorial addendum

We dedicate our review to the memory of our dear friend and colleague, Professor Gianfranco Del Prete, who made many notable contributions to our understanding of host immune responses to bacterial and parasite pathogens and who frequently contributed to our discussions on those topics and in regard to our mutual interest in vaccines against *Yersinia pestis*.

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