Review Article

Salmonella-based plague vaccines for bioterrorism

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Yersinia pestis, the causative agent of plague, is an emerging threat as a means of bioterrorism. Accordingly, the Working Group on Civilian Biodefense, as well as the Centers for Disease Control and Prevention, has specified *Y. pestis* as a prime candidate for use in bioterrorism. As the threat of bioterrorism increases, so does the need for an effective vaccine against this potential agent. Experts agree that a stable, non-invasive vaccine would be necessary for the rapid large-scale immunization of a population following a bioterrorism attack. Thus far, live *Salmonella*-based oral vaccines show the most potential for this purpose. When delivered via a mucosal route, *Salmonella*-based plague vaccines show the ability to protect against the deadly pneumonic form of plague. Also, mass production, distribution, and administration are easier and less costly for attenuated *Salmonella*-based plague vaccines than for plague vaccines consisting of purified proteins. Most attenuated *Salmonella*-based plague vaccines have utilized a plasmid-based expression system to deliver plague antigen(s) to the mucosa. However, these systems are frequently associated with plasmid instability, an increased metabolic burden upon the vaccine strain, and highly undesirable antibiotic resistance genes. The future of *Salmonella*-based plague vaccines seems to lie in the use of chromosomally encoded plague antigens and the use of in vivo inducible promoters to drive their expression. This method of vaccine development has been proven to greatly increase the retention of foreign genes, and also eliminates the need for antibiotic resistance genes within *Salmonella*-based vaccines.

Key words: Bioterrorism, plague vaccine, *Salmonella*, *Yersinia pestis*

Bioterrorism

Bioterrorism is defined as the intentional use of a biological organism or one or more of its components or products to cause disease, social disruption, and ultimately death [1]. Terrorists who employ these methods seek to cause a considerable amount of morbidity and mortality within target populations. The recent escalation of terrorism in particular countries (such as the 2001 World Trade Center attack in New York City and the 2004 train bombings in Madrid) has resulted in a heightened awareness of terrorism in general and has increased fears of an attack around the world. The introduction of *Bacillus anthracis* (the causative agent of Anthrax) into the United States postal system in October 2001 alerted the public to the fact that bioterrorism is also possible, if not imminent.

Acts of bioterrorism are extremely devastating due to the fact that they threaten the protective immunity of a population by introducing agents to which that population is immunologically naïve [2]. After this protective immunity has been breeched by a large-scale biological attack, high levels of morbidity will most likely overwhelm public hospitals and clinics with most victims requiring special medications, critical therapies (such as ventilators), and other treatments for critical care [3]. The intense fear of bioterrorism is also likely to cause mass psychogenic illness in a given population, which can ultimately lead to socioeconomic disruption [1].

The Centers for Disease Control and Prevention (CDC) has developed a hierarchical classification system for potential agents of bioterrorism. Certain characteristics were used in order to designate each pathogen as a Class A, B, or C agent. These classes are organized based on the overall public health impact of the agent (disease and mortality rates), public perception of the agent, dissemination potential, the need and/or

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Table 1. Class A biological agents (as classified by the Centers for Disease Control and Prevention) and their associated diseases

Class A agent	Disease
Variola major	Smallpox
Bacillus anthracis	Anthrax
Yersinia pestis	Plague
Clostridium botulinum (botullinum toxin)	Botulism
Francisella tularensis	Tularemia
Viral hemorrhagic fever viruses[a]	Viral hemorrhagic fevers

[a]Viral hemorrhagic fever viruses include the Ebola, Marburg, Lassa, Machupo, Junin, and Guanarito viruses.

availability for special health preparations, and the ability of the agent to be converted into a weapon [2,3]. Class A agents include some of the deadliest organisms known to man and are those with the highest priority with respect to national security [3]. These agents are listed in Table 1 in no specific order. Class A agents are easily transmitted from person to person and have the potential to cause widespread panic if released within a population [2]. Most importantly, the agents in this class have the ability to cause a high level of mortality, with the greatest impact being on the public health system and civilian psyche [3]. In 1998, the Working Group on Civilian Biodefense (an expert panel of academic and government leaders) determined that if Class A agents were used to intentionally infect a population, they would cause the maximum amount of illnesses and deaths [4]. Therefore, these agents (as opposed to Class B and C agents) were identified as those with critical importance and it is believed that they require immediate attention [5].

Given that there is no adequate defense to protect against most agents of bioterrorism (including Class A agents), public health organizations are under pressure to develop protective therapies, such as vaccines [2]. Most naturally-acquired infections with probable bioweapons can be successfully controlled following treatment with a single antimicrobial agent or a combination of antimicrobials. However, antimicrobial treatments would prove to be highly ineffective during a catastrophic bioterrorism event. According to Greenfield and Bronze, all possible agents share the potential for naturally occurring or genetically engineered resistance to currently available antimicrobial therapy [6]. Therefore, it is suggested that the first line of defense against a terroristic attack with a bioweapon be vaccination [6].

Plague As a Biological Weapon

Of the agents listed in Table 1, *Yersinia pestis* (the plague bacillus) has emerged as a major threat. Plague is ubiquitously recognized as a highly contagious and extremely deadly disease. Its killing power is undisputable and unmistakable. Accordingly, *Y. pestis* has been responsible for 3 major plague pandemics, ultimately resulting in millions of deaths worldwide. Although advances in medicine, public health, and sanitation efforts have made pandemics of such an immense magnitude and significance improbable, deadly epidemics caused by a bioterrorism event remain a distinct possibility. The CDC has expressed concern about the use of plague as a biological weapon because *Y. pestis* is not only an organism that is found in nature, but it can also be found in numerous supply houses around the world [4]. As a result, *Y. pestis* has emerged as a potential bioweapon with the ability to cause death and destruction on a massive scale. *Y. pestis* has the ability to cause 3 forms of plague in humans. Bubonic plague is the result of an infection of the lymph nodes. Septicemic plague is *Y. pestis*-mediated bacteremia, while primary pneumonic plague is an intense infection of the lungs that develops after direct inhalation of the bacillus. Of these 3 forms, primary pneumonic plague is by far the deadliest. This is also the form of plague most likely to develop following a plague-mediated biological attack [5].

The realization that *Y. pestis* would be most effective as a bioweapon in an aerosolized form led biological weapon programs (especially those of the United States and the Soviet Union) to develop techniques to aerosolize the plague bacillus directly in the years following World War II [5]. Studies during this time demonstrated that *Y. pestis* could remain viable as an aerosol for at least 60 minutes and (with sufficient wind currents) could travel a distance of up to 10 km [5]. The World Health Organization (WHO) reported in 1970 that a worst case scenario would be the equivalent of releasing 50 kg of *Y. pestis* over a densely populated area containing 5 million people [5]. In this case, it is projected that inhaled aerosolized *Y. pestis* would lead to the development of primary pneumonic plague in as many as 150,000 persons; of which 36,000 would not be expected to survive the infection [5]. The situation would be exacerbated as inhabitants of an infected area attempted to flee in order to escape the disease, thereby furthering its spread via respiratory droplets and direct person-to-person contact [5]. Under these circumstances,

the use of an effective plague vaccine could help limit the number of casualties and deaths resulting from an acute *Y. pestis* infection [2]. Therefore, development of such a vaccine has been deemed crucial. Although it has been suggested that the most promising vaccine thus far is an injected protein subunit vaccine consisting of 2 highly immunogenic plague antigens (the F1 and V antigens), experts believe that this type of vaccine would be highly ineffective in the event of a plague epidemic resulting from bioterrorism [7]. Rapid, large-scale immunization would most likely require a non-invasive method of vaccination. (A non-invasive method would allow self-administration of the vaccine in most cases, therefore greatly reducing the time needed to vaccinate an entire population). Also, a substantial amount of the vaccine would need to be produced in a relatively short period of time.

Although protection against the pneumonic form of plague is paramount, candidate vaccines should demonstrate the ability to protect against bubonic and septicemic plague as well. An apparent breakthrough in the development of a successful plague vaccine has arisen in the form of *Salmonella*-based vaccines. In addition to being able to induce mucosal and systemic immunity capable of protecting against all 3 forms of plague, orally delivered *Salmonella enterica* serovars carrying *Y. pestis* antigens offer the advantage of being easier and safer to administer than a vaccine requiring a needle-based delivery [7].

Live attenuated *S. enterica* serovars as carriers of *Y. Pestis* antigens

In the event that *Y. pestis* is used as an agent of bioterrorism, it will most likely be in an aerosolized form [3]. Victims will undoubtedly become infected after direct inhalation of the bacillus. In this case, the mucous membranes within the respiratory tract represent an important portal of entry for the agent, as pneumonic plague is sure to develop after subsequent colonization of the lung mucosa. In order to effectively combat the development of primary pneumonic plague, it is necessary to employ vaccination strategies that initiate intense mucosal immune responses within the lungs. Because live attenuated *Salmonella*-based vaccines are proven to be effective as mucosally delivered vaccines against *Y. pestis*, they represent a promising strategy for an efficient plague vaccine to combat bioterrorism. The ability of a live attenuated *Salmonella*-based vaccine to propagate and colonize the gut of vaccinees allows adequate persistence of the vaccine and ample

opportunity for immunostimulation within the gut [8]. More specifically, when live salmonellae carrying plague antigens reach the Peyer's patches of the small intestine, they are able to present these antigens directly to the T and B lymphocytes of the gut-associated lymphoid tissues [9]. Intense humoral, T-helper, and cytotoxic immune responses within the mucosa that are specific for *Y. pestis* antigens are ultimately stimulated [9]. Protective immunity against *Y. pestis* is very likely to develop in the lung mucosa and prevent the development of the deadly pneumonic form of plague as a consequence. Furthermore, oral immunization with a *Salmonella*-based plague vaccine via the gut can stimulate the production of secretory antibodies specific for *Y. pestis* on all mucosal surfaces throughout the body, as well as a systemic immune response capable of protecting against the milder bubonic and systemic forms of plague [7-9]. These vaccines also offer the advantage of being easier and safer to administer than a purified protein vaccine, which requires a needle-based delivery [7]. This particular advantage makes live attenuated *Salmonella*-based vaccines expressing *Y. pestis* antigens suitable for the rapid large-scale immunizations necessary following a bioterrorism attack.

Live oral vaccines based on attenuated *S. enterica* serovars, such as Typhi and Typhimurium, are gaining popularity as vehicles for the delivery of heterologous antigens to the mucosal surfaces of vaccinees. These *Salmonella*-based vaccines are fully capable of eliciting humoral, cell-mediated, and mucosal immune responses after successful vaccination [6,10]. Not only do such vaccines elicit vigorous immune responses against their heterologous antigens, protective immunity is also stimulated against the *Salmonella* carrier itself. Attenuated strains of *S. enterica* serovar Typhi (such as the *aroA, aroD, htrA, and phoP* mutants) are likely to be used as a delivery system for plague antigens in humans because they are capable of establishing a limited innocuous infection within their hosts [10,11]. Small-scale clinical trials have demonstrated that these mutants are avirulent and safe for human use [11]. In any case, before a candidate vaccine can be subjected to randomized clinical trials, it must first be tested in an appropriate animal model. With respect to plague vaccine research, the murine model is used due to its ability to provide meaningful indication of the efficacy of candidate plague vaccines in humans [12]. However, the *S. typhi* typhoid fever which develops in man is difficult to mimic in the murine model. In contrast to *S. typhi, S. enterica* serovar Typhimurium does indeed

reproduce a human-typhoid-like illness in mice. For this reason, *S. typhimurium* mutants (particularly strains harboring mutations in the aromatic amino acid biosynthetic pathways) are customarily employed in the early developmental stages of *Salmonella*-based vaccines against plague [11]. Once protective efficacy has been demonstrated in mice using *S. typhimurium*, the same vaccine strain may be recreated in an *S. typhi* background and tested for its ability to prevent or ameliorate plague in higher primates, and eventually humans.

Delivery of plague antigens using chromosomal intergration and in vivo inducible promoters

Although this approach to vaccine development has demonstrated great potential, the theory of using *S. enterica* serovars as vehicles to stimulate mucosal immunity against foreign antigens is not without its limitations. Immunizing bacteria must present their antigens in an effective manner in order for this approach to result in immunologically relevant responses within vaccinees. Also, the antigen must be present in sufficient quantity in order to stimulate immune responses that will ultimately lead to protection [6]. The most widely used method to accomplish such tasks in *Salmonella*-based vaccines is the constitutive expression of heterologous antigens from multicopy plasmids, which results in the constant production of the foreign antigen. However, constitutive synthesis of antigens from high-copy number plasmids results in a so-called 'metabolic burden' on bacterial vector strains. Galen et al has established that this metabolic burden drains the "portion of the host cell's resources (either in the form of energy or raw materials) that is required to maintain and express foreign DNA, as either RNA or protein in the cell" [13]. This assault on the carrier's resources in turn leads to a reduction of fitness [6,10]. Additionally, when plasmid-based expression systems are utilized, the associated metabolic burden can actually result in the loss of the plasmid encoding the heterologous antigens [10]. As the metabolic burden of the *Salmonella* vector increases, the rate of plasmid loss also increases [10]. Reduced growth rate is an inescapable result of an increased metabolic burden as well, and is also considered to be a selective pressure for the loss of resident plasmids encoding heterologous antigens in the absence of selection [10]. In fact, plasmid retention has proven to be a hindrance during the development of attenuated *Salmonella*-based vaccines. According to Everest et al, plasmid loss frequently occurs during vaccine

preparation once the selective pressures applied in vitro (such as those used to maintain the plasmid within the vector species on laboratory medium) are removed, or once the *Salmonella* vector itself has entered the host [14]. The loss of expression plasmids would ultimately have a negative affect on the immunogenicity and the protective efficacy of the vaccine [6]. Furthermore, traditional plasmid-based expression systems require antibiotic resistance genes for plasmid retention and stability. The mere presence of these resistance genes raises serious regulatory and safety issues if the vaccine is intended for human use [15]. Issues regarding the stability and expression of heterologous antigens from *Salmonella*-based vaccines, as well as the use of antibiotic markers in their construction, have seriously limited the potential of *Salmonella*-based oral vaccines in general [16].

Several methods have been investigated in an effort to avoid dangerously high levels of antigenic production from expression plasmids, while reducing plasmid instability in *Salmonella*-based vaccines [13-17]. One such approach relies on the insertion of genes encoding heterologous antigens into the chromosome of an *S. enterica* vaccine strain and using in vivo inducible promoters to drive their expression. In this instance, in vivo inducible promoters are used to effectively control the expression of heterologous antigens from the chromosome of an attenuated *S. enterica* carrier. After vaccination, genes encoding foreign antigens will only be expressed after the *Salmonella* vector reaches the proper environmental niche within the vaccinated host, thereby signaling the transcription and synthesis of foreign antigens [6]. Not only does this combined approach eliminate the issue of plasmid retention and stability, it also negates the need for antibiotic resistance genes within the vaccine construct. Such a vaccine would therefore be suitable for human use.

The notion of delivering foreign antigens using chromosomal integration and in vivo inducible promoters in the development of *Salmonella*-based vaccines has been well established using classic models of pathogenesis, such as *Plasmodium falciparum*, the causative agent of malaria. When the immunodominant malaria antigen circumsporozoite was expressed chromosomally using an in vivo induced promoter, strong immune responses against this antigen were elicited [6]. In addition, when a PagC-alkaline phosphatase fusion protein was expressed from the chromosome of *S. enterica*, relevant anti-malaria immune responses were also stimulated [16]. With

such promising results, it is now suggested that this approach be applied to the development of a *Salmonella*-based plague vaccine. Not only would genes encoding plague antigens be stably retained within the *Salmonella* vector, the vaccine strain would be void of antibiotic resistance genes that could potentially be transferred to other unrelated bacteria residing within vaccinees. Ideally, promoters chosen to drive the expression of plague antigens should be induced subsequent to the *Salmonella* vector entering specific antigen presenting cells (such as the macrophage) in order to initiate protective immune responses [16]. The *ssaG* promoter, an in vivo inducible promoter associated with the *Salmonella* pathogenicity island 2, has been studied extensively as a means for the controlled expression of heterologous antigens in *Salmonella*-based vaccines [10,16]. This promoter has shown an ability to be up-regulated at least 400-fold within macrophages [16]. In addition, Stratford et al used the *ssaG* promoter to effectively control the expression of the hepatitis B virus core antigen and the B subunit of the *Escherichia coli* heat-labile toxin after the integration of these antigens into the chromosome of *S. enterica* serovar Typhi ZH9 [10].

An innovative idea for the insertion of plague antigens into the chromosome of *Salmonella* vector strains and for the deletion of antibiotic resistance genes within the vaccine construct is the use of the Red recombinase system initially described in bacteriophage lambda (λ). Specifically, a 'knock-in' approach utilizing the Red recombinase system has been proposed to insert a functional expression cassette (also known as a targeting construct) into the chromosome of an acceptable *S. typhimurium* vector strain in a single step [17]. This method for creating recombinant *Salmonella*-based vaccines would also allow for the simultaneous generation of deletions in the chromosome. These deletions could then be used to further attenuate *Salmonella* vector strains. Expression cassettes inserted in a chromosomal target gene would be stably maintained in the absence of selective pressure [17]. The integration of an expression cassette (containing an in vivo inducible promoter fused to a gene encoding a highly immunogenic plague antigen) and the excision of a functional gene present on the chromosome of the vector strain (such as *aroD*) would occur in a single recombinational event. In essence, the two entities would be exchanged. In this 'knock-in' system, recombination would be mediated by the phage λ Red recombinase [18]. This enzyme is synthesized under the control of the arabinose inducible promoter (*araC*) on an easily curable, low copy number plasmid. The Red recombinase enzyme would allow the uptake of the linear targeting construct and facilitate its integration into the chromosome of the *Salmonella* vector strain. Any antibiotic marker present within the construct may be subsequently removed by flippase (FLP) recombinase-mediated excision. Initially, antibiotic resistance genes must be present within the expression cassette for in vitro plasmid maintenance and to identify cells that have undergone successful recombination. However, such genes must be removed before the vaccine can be approved for human use. In order to take advantage of the FLP recombinase system, resistance genes must be situated between 2 identical stretches of DNA called FLP recombinase target (FRT) sequences. Once the FLP recombinase has located 2 identical FRT sequences, it facilitates the removal of DNA located between the 2 sites [17]. In this case, the intervening sequence that is removed would be one that codes for antibiotic resistance. This Red recombinase knock-in approach has been perfected in *S. enterica* Typhimurium and shows great promise in the development of an *S. typhimurium*-based plague vaccine.

Conclusions

According to the CDC and the WHO, in a bioterrorism attack utilizing *Y. pestis*, the bacillus will almost certainly be released upon a target population in an aerosolized form. Undoubtedly, a pneumonic plague epidemic will be the result of such an event. Therefore, it is imperative that a plague vaccine developed to combat bioterrorism have the ability to protect against the deadly pneumonic form of the disease, in addition to septicemic and bubonic plague. This can best be achieved by stimulating an immune response within the mucosa of the vaccinee that is capable of protecting against subsequent aerosolized plague challenge. Recombinant *Salmonella*-based plague vaccines have been linked to intense humoral, T-helper, and cytotoxic immune responses against the plague antigens they carry [19]. Such responses have proven to be effective in protecting against subsequent challenge with virulent *Y. pestis* [20-24]. However, no such plague vaccine has been approved for human use due to the reliance on antibiotic resistance genes and the instability of expression plasmids within these constructs. Therefore, the creation of a successful *Salmonella*-based plague vaccine will most likely require the chromosomal expression of

highly immunogenic plague antigens using in vivo inducible promoters. The chromosomal insertion of plague antigens would increase their retention within the vaccine construct and eliminate the need for antibiotic resistance genes. Furthermore, the increased metabolic burden placed upon the *Salmonella* vector strain would be drastically reduced by driving the expression of plague antigens with an in vivo inducible *Salmonella*-derived promoter.

Acknowledgments

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