

# Recent Advancement in the Development of Vaccines Against *Y. pestis* – A Potential Agent of Bioterrorism

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## 1. Introduction

A bioterrorism attack- is the deliberate release of biological agents such as viruses, bacteria, or toxins used to cause illness or death in people, animals, or plants (CDC). These agents are typically found in nature, but it is possible that they could be changed to increase their ability to cause disease, make them resistant to current therapeutics, or to increase their ability to be spread into the environment. Biological agents can be spread through the air, through water, or in food. Terrorists may use biological agents because they can be extremely difficult to detect and may not cause illness for several hours to several days. Some bioterrorism agents, like *Variola major* and *Yersinia pestis*, can be spread from person to person, while others e.g. *Bacillus anthracis* are not (Bioterrorism review, 2009). Biological agents make attractive weapons because they are relatively easy to obtain and carry from place to place, can be easily dispersed, and can cause widespread fear and panic beyond the actual physical damage they can cause. Many of the agents that could be used for bioterrorism have been divided into three categories A, B, and C, for public health preparedness based on various characteristics of the microbes or the diseases they cause.

Category A includes the most "dangerous" and highest priority for public health preparedness. Some of these pathogens can be transmitted from person-to-person, cause diseases with a high mortality rate and are likely to cause public panic and social disruption. Category A agents include *B. anthracis* (anthrax), *Variola major* (smallpox), *Francisella tularensis* (tularemia), *Y. pestis* (plague), *Clostridium botulinum* neurotoxin (botulism), and Viral hemorrhagic fever viruses (e.g. arenaviruses, filoviruses, bunyaviruses, and flaviviruses).

This chapter will discuss the Category A agent *Y.pestis*,the disease it causes,and recent efforts to develop vaccines.

Plague, a zoonotic disease caused by the gram-negative bacillus *Y. pestis* is primarily a disease of rodents, with transmission occurring through infected fleas. Human disease is acquired through rodent flea vectors, as well as respiratory droplets from animal to humans and humans to humans.

## 2. History of plague

The first reported pandemic of plague has been referred to as the “Great Plague of Justinian” (Sticker, 1908; Hirst,1953). This pandemic began around 532 AD in Egypt and spread through the Middle East and the Mediterranean basin, reaching Turkey, Constantinople, Greece, Italy, and the territories of France and Germany. The second pandemic, which is also known as the Black Death, began in 1334 in China and then spread westward along the trade routes to the Black Sea and eventually to Constantinople. The disease, which spread slowly and inevitably from village to village by infected rats and humans, or more quickly from country to country by ships, eventually killed 20 to 30 million people in Europe (Gottfried,1983). The third pandemic probably originated in the Chinese province of Yunnan around 1855 and spread to the southern coast of China, causing several smaller outbreaks. Larger epidemics occurred when the disease finally reached Canton and Hong Kong in 1894, thus marking the beginning of the third pandemic. Plague spread rapidly throughout the world through all inhabited continents, except Australia. Rats aboard the faster steamships that had replaced slow-moving sailing vessels in merchant fleets carried the disease. Between 1894 and 1903, plague entered 77 ports on 5 continents. During the early years of the third pandemic, the death toll in India and China alone was 12 million. In United State, plague was introduced in 1900. Between 1900 and 1924, most plague cases in the U.S. occurred in port cities along the Pacific and Gulf coasts (Link, 1955). More recently, a plague epidemic caused the death of several hundred residents in the Surat city of India between September and October 1994 (Perry and Fetherstone 1997).

The recent increase in the number of human plague cases together with the reappearance of epidemics in countries such as Malawi, Mozambique, and India has led to its designation by WHO as a re-emerging infectious disease (World Health Organization 2002, World Health Organization 2003). As of 30 July 2010, the Ministry of Health in Peru confirmed a total of 17 cases of pneumonic plague in Ascope province of Department La Libertad. The onset of symptoms for the last reported case of pneumonic plague was on 11 July 2010. During the investigations, 10 strains of *Y. pestis* were isolated from humans, rodents and domestic cats. In 2009, the Chinese Ministry of Health, reported a cluster of pneumonic plague cases in the remote town of Ziketan, Qinghai province. The first case was a 32 year old male herdsman, who developed fever and haemoptysis. Between 1990 and 2005, a total of 107 cases of plague were reported in the U.S.(Centers for Disease Control and Prevention, 2006). Recently there have been reports of 14 deaths potentially due to pneumonic plague in Madagascar ([http:// www.promed mail.org](http://www.promed mail.org)).

### 2.2 Plague as a biological weapon

In 1346, during the siege of Kafa (now Feodosia, Ukraine) the attacking Tartar forces catapulted the bodies of warriors who had died of plague into the besieged city as a weapon. It has been speculated that this operation may have been responsible for the advent of the Black Death in Europe (Wheelis, 2002; Lederberg, 2001). In World War II, Unit 731 of the Japanese army, developed plague-infected fleas in China resulting in outbreaks of plague (Harris, 1994). Later, to eliminate dependency on the flea vector, the U.S. and the Soviet Union biological weapons programs developed methods to aerosolize *Y.pestis*. The World Health Organization (WHO) has estimated that, if 50 kg of *Y. pestis* were released as

an aerosol over a city of 5 million people, 150,000 would get pneumonic plague infection of which 36,000 would die (WHO 1970). The plague bacilli would remain viable as an aerosol for 1 hour for a distance of up to 10 km.

### 3. Clinical characteristics of plague

Plague can be broadly classified into three forms:

#### 3.1 Bubonic plague

This is the classic form of the disease, which is characterised by swollen lymph nodes called buboes. Common symptoms are fever, headache, and chills occurring within 2 to 6 days of exposure to the organism either by flea bite or by contamination of open wounds with infected material. Gastrointestinal complications such as nausea, vomiting, and diarrhoea are common (Iteman et al., 1993, von Reyn et al., 1977). Buboes typically occur in the inguinal and femoral regions but may also occur in other regions of the body (Butler, 1989; Conrad et al., 1968). Bacteraemia or secondary plague septicaemia is frequently seen in patients with bubonic plague (Gage et al., 1992). In humans, the mortality of untreated bubonic plague is approximately 60%, but this is reduced to less than 5% with prompt, effective therapy.

#### 3.2 Septicaemic plague

Primary septicaemic plague occurs mainly in patients with positive blood cultures but no palpable lymphadenopathy. Clinically, septicaemia caused by *Y.pestis* resembles that caused by other gram-negative bacteria. Common symptoms include chills, headache, malaise, and gastrointestinal disturbances. Patients with septicaemic plague are more likely to experience abdominal complications than compared those with bubonic plague. Even with treatment, the mortality of septicaemic plague may range from 30 to 50% (Crook and Tempest 1992, Hull et al., 1987, Poland and Barnes, 1979); untreated septicemic plague is virtually 100% fatal.

#### 3.3 Pneumonic plague

Pneumonic plague is an infection of the lungs due to either inhalation of the organism (i.e., primary pneumonic plague), or dissemination to the lungs via the blood stream (i.e., secondary pneumonic plague). Pneumonic plague is the only form of the disease which is readily spread from person to person via respiratory droplets through close contact (2 to 5 ft) with an infected individual. It progresses rapidly from a flu-like illness to pneumonia with coughing and the production of bloody sputum. The incubation period for primary pneumonic plague is between 1 and 3 days. The last case of pneumonic plague in the U.S., resulting from person-to-person transmission occurred during the 1924 - 1925 epidemic in Los Angeles (Meyer, 1961). Between 1970 and 1993, 12% of U.S. plague patients developed pneumonia secondary to either the bubonic or septicemic form of the disease (Doll, 1994); In recent decades, 28% of human plague cases resulting from exposure to infected domestic cats in the U.S. presented as primary pneumonic plague. The mortality rate for untreated pneumonic plague is nearly 100%. Recent data from Madagascar epidemic indicates that mortality associated with respiratory involvement was 57%.

## 4. Microbiology

The genus *Yersinia*, a member of the family Enterobacteriaceae, consists of eleven species, of which three are pathogenic for humans: *Y. pestis*; *Y. pseudotuberculosis* and *Y. enterocolitica*. *Y. pestis*, is a gram-negative, non-motile, non-spore forming coccobacillus measuring 0.5 to 0.8  $\mu\text{m}$  in diameter and 1 to 3  $\mu\text{m}$  in length. The organism grows at 4 to 40<sup>o</sup> C with optimum growth at 28 to 30<sup>o</sup> C; the optimum pH range is 7.2 to 7.6 but extreme pH (5 to 9.6) can be tolerated (Brubaker, 1972; Holt et al., 1994; Poland and Barnes, 1979; Poland et al., 1994). The lipopolysaccharide of *Y.pestis* is characterized as rough, possessing core components but lacking the extended O-group side chains. There is no true capsule; however, a carbohydrate-protein surface component, termed capsular antigen or fraction 1 (F1) is produced during growth above 33<sup>o</sup>C (Barnes and Quan, 1992; Brubaker et al., 1972; Poland et al., 1994). Three biotypes (or biovars) of *Y. pestis* can be differentiated based on the conversion of nitrate to nitrite and fermentation of glycerol: *Antiqua*, *Orientalis* and *Mediaevalis*. Biotype *Antiqua* is positive for both characteristics, biotype *Orientalis* converts nitrate to nitrite but does not ferment glycerol, and biotype *Mediaevalis* ferments glycerol but does not form nitrite. Strains of the three biotypes exhibit no difference in their virulence or pathology in animals or humans (Brubaker et al., 1972; Poland et al., 1979). It is thought that *Antiqua* was responsible for the First Pandemic, *Mediaevalis* for the Second Pandemic, and *Orientalis* for the Third Pandemic. The majority of *Y. pestis* strains contain three virulence plasmids of 9.5, 70 to 75, and 100 to 110 kb (Ben-Gurion and Shafferman, 1981; Ferber and Brubaker, 1981; Filippov et al., 1990). In strain KIM and its derivatives, these plasmids are referred to as pPCP1 (pesticin, coagulase, plasminogen activator), pCD1 (calcium dependence), and pMT1 (murine toxin).

### 4.1 Life cycle

Plague primarily affects rodents. Transmission between rodents is associated with their fleas. While infection can occur by direct contact or ingestion, these routes do not normally play a role in the maintenance of *Y. pestis* in animal reservoirs. The rat flea (*Xenopsylla cheopis*), the classic vector for plague, ingests blood from an infected rodent (Hinnebusch and Schwan 1993). A bacterial load of 10<sup>4</sup> CFU/ml of rodent blood would ensure ingestion of at least 300 *Y. pestis* organisms. *Y.pestis* is cleared by some fleas but multiplies in the midgut (stomach) of others. Two days after an infected blood meal, the stomach exhibits clusters of brown specks containing *Y. pestis*. These develop into cohesive dark brown masses containing bacilli, a fibrinoid-like material, and probably hemin which extend throughout the stomach and into the proventriculus and esophagus. By 3 to 9 days after the infected blood meal, the bacterial masses may completely block the proventriculus, extend into the esophagus, and prevent newly ingested blood from reaching the stomach. As the hungry flea repeatedly attempts to feed, the blood sucked from the mammal mixes with bacilli and is regurgitated into the mammalian host (Bacot, 1914; Bacot, 1915; Bahmanyar, 1976; Bibikova 1977; Cavanaugh 1971, 1956). At higher environmental temperatures (> 28 to 30<sup>o</sup> C), blockage of fleas decreases and clearance of the infection increases, possibly due to the temperature regulation of hemin storage and/or Pla protease. (Burroughs, 1947; Cavanaugh, 1971; Cavanaugh, 1980; Kartman, 1969). The normal digestive process of fleas involves maintaining the blood meal as a liquid, which is subsequently degraded primarily by proteolytic enzymes (Wigglesworth, 1984). The fate of the blocked flea is death from starvation and dehydration (Bacot, 1914; Bibikova, 1977).

*Y. pestis* spreads from the site of the flea bite to the regional lymph nodes and multiplies, resulting in the formation of primary and sometimes secondary buboes (swollen lymph node). The bacilli can spread into the bloodstream (bacteremia), where they are preferentially removed by the spleen and liver. Growth of the organisms continues in the blood, liver, and spleen and eventually spreads to other organs (Pollitzer, 1954). Development of a bacteremia of sufficient degree and duration is essential for effective transmission in nature. Infection of the flea via the blood from a bacteremic rodent completes the cycle. If bubonic plague progresses to the pneumonic form in humans, the potential for respiratory droplet spread and a primary pneumonic plague epidemic occurs (Poland and Barnes, 1979; Poland et al., 1994). This type of epidemic is currently uncommon due to the advent of effective antibiotics and modern public health measures.

## 5. Diagnosis

Clinical diagnosis of disease is based on patient symptoms and exposure history. Bubonic plague is characterized by painful, swollen lymph node(s), fever, and a history of exposure to fleas, rodents, or other animals. It is very difficult to diagnose septicemic plague without a blood culture because of its resemblance to other gram-negative septicemias. Likewise, pneumonic plague has been mistaken for other pulmonary syndromes (Centers for Disease Control and Prevention, 1994). Recent data indicate that pneumonic plague should be suspected in symptomatic persons with a history of exposure to infected pets, especially cats (Craven et al., 1993; Doll et al., 1994; Gage et al., 1992).

Specimens for laboratory diagnosis include blood, bubo aspirates, and sputum, which can be stained with Gram, Giemsa, Wright, or Wayson stain (Poland et al., 1979). A positive fluorescent-antibody assay directed against purified F1, a capsular antigen expressed predominantly at 37°C can be used as presumptive evidence of a *Y. pestis* infection (Du and Forsberg, 1995; Poland et al., 1979). To confirm a diagnosis of plague by bacteriological means, it is necessary to isolate the organism. Other methods for diagnosing plague include: enzyme linked immunosorbent assays (ELISA) (Cavanaugh et al., 1979; Williams et al., 1984), polymerase chain reaction (PCR) assays (Norkina OV 1994), and DNA hybridization (McDonough et al., 1988). ELISAs have been used to measure levels of either F1 antigen or antibodies to F1 in serum (Williams et al., 1984). PCR-gel electrophoresis based methods have been developed for detecting *Y. pestis* in fleas and other specimens (Hinnebusch and Schwan, 1993; Norkina et al., 1994; Tsukano et al., 1996). Real-time PCR assays in various formats have also been developed for detecting and identifying *Y. pestis* (Higgins et al., 1998; Iqbal et al., 2000; Lindler and Tall, 2001; Loiez et al., 2003; Tomaso et al., 2003; Chase et al., 2005; Woron et al., 2006). Real-time PCR based methods are more specific, and require less time and labor than conventional PCR assays. Real-time PCR methods include SYBR Green (Saikaly et al., 2007), molecular beacons (Varma-Basil et al., 2004), TaqMan probes (Loiez et al., 2003; Chase et al., 2005) and minor groove binding (MGB) probes (Skottman et al., 2007), and target specific sequences on the chromosome and (or) plasmids. However, PCR based diagnosis is expensive compared to immunoassays, which may be useful for mass screening during epidemics.

## 6. Treatment

Patients suspected of having bubonic plague should be placed in isolation until two days after starting antibiotic treatment to prevent the potential spread of the disease. Antibiotics

such as streptomycin, gentamicin, oxytetracycline, tetracycline and chloramphenicol have been used to treat primary infection (Meyer, 1950). Due to the toxicity associated with streptomycin, patients are not usually maintained on this antibiotic for the full 10-day course but shifted to one of the other antibiotics, usually tetracycline. The tetracyclines are also commonly used for prophylaxis, while chloramphenicol is recommended for the treatment of plague meningitis (Becker et al., 1987). Newer antibiotics have been used to successfully treat experimental plague infections in mice (Bonacorsi et al., 1994). Recently, the quinolone levofloxacin was found to be effective against *Y. pestis*, *B. anthracis*, and *F. tularensis* (Peterson et al., 2010).

## 7. Immunology of *Y. pestis*

*Yersinia* spp like many other gram-negative bacterial pathogens, employ a specialized secretory apparatus called the type III secretion system (TTSS) to interact with host cells (Cornelis et al., 1998; Cornelis, 2000). The TTSS is a multicomponent secretion apparatus that injects specialized proteins (effectors) into the cytosol of the host cell where they interact with a variety of host proteins to manipulate cellular functions to ultimately benefit the pathogen (Galan and Collmer, 1999). The *Yersinia* effector proteins called Yops- (Yersinia Outer membrane Proteins) and other proteins involved in the TTSS are encoded on a 70-kb plasmid (Cornelis et al., 1998). The functions of the Yops are currently under intense study and fall into two general categories: proteins facilitating the translocation of Yops into the host cells, and those actually secreted into the cytosol. Notably, YopD, YopB, and LcrV (low calcium response protein V) appear to function in the translocation of other Yops into the cytosol whereas YopE, YopH, YopJ (Yop P in *Y. enterocolitica*), YopM, YopO, and YopT function within the host cell. Yops are virulence factors that can interfere with phagocytosis, inhibit the antimicrobial oxidative burst, inhibit the production of inflammatory cytokines (e.g. TNF- $\alpha$ ), and promote apoptosis in macrophages and neutrophils (Cornelis et al., 1998, 2000). Like TTSS effectors of other bacterial pathogens, Yops function by mimicking activities of host cellular proteins and either activate or inhibit cellular processes to promote the pathogen's survival and replication (Staskawicz et al., 2001).

LcrV, is an important virulence factor (Sing et al., 2002; Fields et al., 1999; Lee et al., 2000). It forms the tip of TTSS and helps to translocate effector proteins to host cells. LcrV can also be secreted into the environment (Fields et al., 1999) where it has been shown to down regulate host protective immune responses in an IL-10 mediated manner (Sing et al., 2002). Thus, pretreatment of wildtype peritoneal macrophages with recombinant LcrV (rLcrV) inhibited zymosan induced TNF- $\alpha$  production. The anti-inflammatory effect of macrophages was found to be IL-10 dependent because it could be reversed by neutralizing antibodies specific to IL-10. There was no effect of neutralizing antibodies against IL-4 or TGF- $\beta$ , two other cytokines known to inhibit inflammatory responses of macrophages (Sing et al., 2002). The cell receptors responsible for LcrV-induced IL-10 production were identified as CD14 and TLR2 (Sing et al., 2002). IL-10 secretion in response to rLcrV was abrogated in CD14- and TLR2- deficient macrophages. Furthermore, the TLR2 stimulating region of LcrV mapped to a short N-terminal 19 amino acid sequence (Sing et al., 2002). CD14 / TLR2 mediated production of IL-10 by LcrV was further established by the observation that TLR2-deficient mice were more resistant to *Y. enterocolitica* infection than their wild-type parents (Sing et al., 2002).

*Y. pestis* replicates extracellularly; whether its virulence relies upon intracellular replication remains a question of debate. *Y. pestis* replicates within macrophages/ dendritic cells as well as

*in vitro* (Cavanaugh et al., 1959; Janssen et al., 1969; Straley et al.; 1984; Pujal et al., 2005). Nevertheless, detailed kinetic studies of mice infected intranasally (Lathem et al., 2005) and rats infected intradermally (Sebbane et al., 2005), failed to observe significant numbers of intracellular organisms *in vivo*. However, *Y.pestis* bacilli were detected in spleen cells and in CD11b-expressing macrophages when mice were infected subcutaneously (Lukaszewski et al., 2005). In addition, studies of pneumonic plague in nonhuman primates have documented the presence of intact *Y. pestis* within alveolar macrophages (Finegold et al., 1969; Davis et al., 1996). Electron microscopy confirmed the presence of alveolar macrophages containing intact bacilli in the lungs of aerosol-infected macaques (Finegold et al., 1969).

Although, the growth of *Y. pestis* within phagocytes, plays an important pathogenic role, extracellular bacilli predominate during the late stages of infection although intracellular organisms have also been detected at that time (Finegold et al., 1969; Davis et al., 1996; Lukaszewski et al., 2005). These findings suggested that cells of the monocyte/macrophage lineage offer *Y. pestis* a protected intracellular niche that provides sufficient time for the pathogen to grow within mammals by upregulating expression of capsular F1 protein, LcrV and Yops (Cavanaugh et al., 1959).

One to 4 hours after infection of macrophages, *Y. pestis* rapidly expresses virulence markers such as Yops, F1 antigen, and V antigen. By 1 to 2 days postinfection, the virulence-associated proteins begin to paralyse host immune mechanisms by inducing apoptosis, suppressing the production of proinflammatory cytokines (e.g. TNF- $\alpha$ ), inhibiting Fc receptor-mediated phagocytosis, and preventing neutrophil chemotaxis (Perry and Fetherston, 1997). Inside macrophages, *Y. pestis* F1 protein (fraction 1 antigen) forms a capsule around the bacterium. This capsule enhances resistance to engulfment by both macrophages and neutrophils, probably by preventing interactions of receptors that could facilitate uptake of the pathogen (Du et al., 2002). It was also observed that *Y. pestis* produces a less-acylated (tetra-acylated) lipid A at 37 °C, which results in poor induction of host toll-like receptor (TLR) 4-mediated innate immune responses and ultimately poor activation of human macrophages (Kawahara et al., 2002; Kolodziejek et al., 2010). When *Y. pestis* KIM1001, which expresses a poorly TLR4-stimulating LPS, was modified to strongly induce TLR4, it became avirulent (Montminy et al., 2006).

A fimbrial structure in *Y. pestis*, PsaA (pH 6 antigen) is induced at 37°C in acidic media, an environment similar to that of the macrophage phagolysosome (Lindler and Tal, 1993; Price et al., 1995). PsaA selectively binds to apolipoprotein B (*apoB*)-containing lipoproteins (LDL) in human plasma (Makoveichuk et al., 2003), which may prevent recognition by the host immune system (Huang XZ 2004, Makoveichuk et al., 2003).

Infection by *Y.pestis* leads to a global depletion of NK cells and decreased secretion of IFN- $\gamma$ , resulting in reduced macrophage function. These immunomodulatory effects depend on the effector YopM (Kerschen et al.,2004). Phagocytes (macrophages and neutrophils) are the main target cells of the Yops. YopH, YopE, YopT, and YopO inhibit the phagocytosis of *Yersinia*, either by interfering with the host cell actin regulation of Rho GTPases (YopE, YopT, and YopO) or specifically and rapidly inactivating host proteins associated with signalling from the receptor to actin (YopH) (Aepfelbacher and Heesemann, 2001; Aepfelbacher et al., 2005; Andersson et al., 1996; Iriarte et al., 1998; Rosqvist et al., 1990). YopH can suppress the production of reactive oxygen intermediates by macrophages and PMNs (Green et al., 1995). Moreover, Yops also inhibit the proinflammatory responses

elicited by infected cells. YopP inhibits TNF- $\alpha$  and IL-8 release by macrophages, and epithelial, and endothelial cells, respectively (Boland and Cornelis 1998). TNF- $\alpha$  is a potent proinflammatory cytokine, released by activated macrophages and plays a crucial role in limiting the severity of the bacterial infection. In addition to YopP, YopM interacts with protein kinase C-like 2 and ribosomal protein S6 kinase, which are also involved in proinflammatory signalling (McDonald et al., 2003). The suppression of the production of proinflammatory factors not only reduces the activation of NK cells and phagocytes, but also destroys the inflammatory environment needed for adaptive immunity.

### 7.1 Current vaccine strategy

There is a need for a safe and effective plague vaccine to counter the threat of bioterrorism. Researchers have been trying for more than 100 years to develop such a vaccine (Titball and Williamson, 2004). The first vaccine consisting of a heat-killed broth of densely grown, fully virulent *Y. pestis* was developed in 1897 (Haffkine, 1897; Taylor, 1933). This formulation was found to be effective against bubonic plague but had undesirable side effects, such as high grade fever, in the majority of human recipients and severe adverse reactions limited its acceptance (Taylor, 1933). This vaccine was not effective against the pneumonic form of disease (Taylor, 1933; Lien-Teh, 1926). Later, Meyer and colleagues (1974, 1970) developed a more refined whole-cell plague vaccine comprised of formalin-killed *Y. pestis* organisms suspended in a saline solution. Ultimately, a vaccine of this type was licensed and sold as Plague Vaccine, USP, and was used to protect U.S. military personnel against bubonic plague during the Vietnam War (Meyer, 1970; Cavanaugh et al., 1974). However, these vaccines also caused significant adverse effects, including fever, headache, malaise, lymphadenopathy, erythema and induration at the site of injection (Meyer KF et al., 1974). In addition, they generally failed to protect mice and nonhuman primates against pulmonary *Y. pestis* challenge (Titball and Williamson, 2004; Meyer et al., 1974; Meyer et al., 1970; Kollé and Otto 1904).

In 1904, Kollé and Otto showed that relatively small quantities of live-attenuated *Y. pestis* were sufficient to protect rodents. Later, Strong (1906, 1908) reported that live-attenuated vaccines protected humans from bubonic plague. In subsequent years, this formulation was used to immunize millions of people in Indonesia, Madagascar and Vietnam (Girard, 1963). The results suggested that these vaccines were fully protective in humans against both the bubonic and pneumonic form of plague (Titball and Williamson, 2004; Meyer et al., 1970; Girard, 1963). Unfortunately, the live attenuated vaccines were found to be unstable, sometimes killing experimental animals (i.e., nonhuman primates) due to the retention of significant virulence (Welkos et al., 2002; Meyer et al., 1970; Meyer et al., 1974; Russell et al., 1995). In addition, they also produced frequent side effects in humans such as, debilitating fever, malaise and lymphadenopathy (Meyer et al., 1974). These safety concerns have limited the use of live-attenuated plague vaccines in the U.S. and Europe.

Current vaccines are based on variants of a pigmentation-negative *Y. pestis* strain EV76. Strain EV76 produces a robust T-cell response that contributes to protection against pneumonic plague in a murine model (Sha et al., 2008). Despite safety concerns and a high degree of immune variability among vaccine recipients, the N11EG line of strain EV 76 is still in use today (Zilinskas, 2006). However, uncertainty about the reversal of virulence makes the EV76 live attenuated option much less appealing than the development of new vaccines.



To overcome the problems associated with the EV76 strain, researchers are trying to find non-pathogenic substitutes by replacing it with a plasmid-expressed gene that could engender protection. In that context, an *Escherichia coli*-derived plasmid encoding the lipopolysaccharide LpxL, which was over-expressed in the EV76 strain, was chosen because of its immunogenicity and ability to activate TLR-4 (Szaba et al., 2008).

The *E.coli lpxL* gene was introduced into the *Y. pestis* chromosome, which encodes a hexa-acylated lipid A. LpxL is a potent TLR-4 agonist, capable of inducing a strong innate immune response. Immunization with this strain resulted in 100 % protection from subsequent subcutaneous and intranasal challenges (Sun et al., 2011). Genes for additional virulence proteins such as, Ail (attachment invasion locus, also designated as OmpX), plasminogen activator protease (Pla), and pH 6 antigen (Psa) have been deleted in an effort to generate effective live attenuated vaccine strains (Felek et al., 2010). In a pneumonic plague model, animals infected with a *ompX* mutant of *Y. pestis* CO92 survived for two days longer than those infected with the parent strain (Kolodziejek et al., 2010). Moreover,  $\Delta$ *caf1* mutants and  $\Delta$ *psaA* mutants exhibited decreased virulence in a murine infection model (Weening et al., 2011). In a recent study, a  $\Delta$ *caf1* mutant of *Y. pestis* CO92 was attenuated for virulence in a mouse model of bubonic plague but not in a pneumonic plague mouse model when compared to the WT CO92 strain (Sha et al., 2011).

## 7.2 Subunit vaccines based on the F1, LcrV and YscF proteins

The F1 antigen plays important role in preventing phagocytosis by macrophages. In 1952, Baker and colleagues purified the capsular F1 protein. F1 specific antibodies produced in rabbits, agglutinated plague bacilli and passively protected mice and rats following subcutaneous challenge with virulent *Y. pestis* (Baker et al., 1952). Passive transfer of F1-specific antibodies also protected macaques against pneumonic plague (Ehrenkranz and Meyer, 1955). Subsequently, vaccination with recombinant F1 was shown to protect mice against aerosolized *Y. pestis* (Andrews et al., 1996). Despite this apparent success, it is now well established that virulent F1-negative *Y. pestis* strains exist (Winter et al., 1960; Friedlander et al., 1995; Welkos et al., 1995; Davis et al., 1996; Worsham et al., 1995). Thus, vaccines based solely upon F1 antigen will likely fail to protect against all strains of *Y.pestis*.

The multifunctional LcrV protein is important for the virulence of *Y.pestis* (Brubaker et al., 2003; Une and Brubaker, 1984; Viboud and Biliska, 2005; Heesemann et al., 2006; Bacon et al., 1956; Janssen et al., 1963; Lawton et al., 1963, Une et al., 1984). Immunization with purified LcrV protected mice against subcutaneous challenge; protection was also observed following the passive transfer of LcrV-specific antibodies (Une T et al., 1984, Lawton et al., 1963, Une et al., 1984, Sato et al., 1991; Nakajima and Brubaker, 1993; Motin et al., 1994). Immunization with recombinant LcrV was shown to protect mice against aerosol infection with both F1-positive and F1-negative strains of *Y. pestis* (Motin et al., 1994; Price et al., 1989; Leary et al., 1995; Anderson et al., 1996; Anderson et al., 1998). In spite of these important findings, a vaccine based on LcrV alone did not fully protect against pneumonic plague, perhaps due to lack of cross-protective immunity against LcrV variants (Roggenkamp et al., 1997).

YscF, a recently identified vaccine candidate, is located on the cell surface and forms the TTSS channel, which is required for the secretion of Yops and toxins (Allaoui et al., 1995, Haddix and Straley, 1992; Hoiczuk et al., 2001; Marenne et al., 2003). Immunization of mice with YscF resulted in a high anti-YscF titer and provided partial protection against intravenous (i.v.) challenge with *Y. pestis* (Matson et al., 2005; Swietnicki et al., 2005).

Vaccines based on recombinant F1 and LcrV provided better protection than vaccines comprised of either subunit alone (Williamson et al., 1995,1996 ). F1 and LcrV formulations administered with the adjuvant alum provided protection in mice against pulmonary *Y. pestis* challenge (Williamson et al., 1997; Jones et al., 2000). In a similar study, investigators at the US Army Medical Research Institute of Infectious Diseases (USAMRIID) demonstrated that a formulation consisting of a recombinant F1-LcrV fusion protein (rF1V) and alum protected mice against pulmonary challenge with either F1-positive or negative strains of *Y. pestis* (Anderson et al., 1998, Heath et al., 1998).

Yops have also been investigated as protective antigens. Immunization of mice with recombinant Yops ( H, E, N, K, or M) engendered no significant protection against *Y. pestis* infection (Andrews et al., 1999; Leary et al., 1999; Nemeth et al., 1997). However, mice immunized with complexes of YopB, YopD, and YopE (BDE) produced high-titers of antibodies specific for Yop B, D, and E, and were protected against lethal intravenous challenge with F1- but not F1+ *Y. pestis*. Furthermore, mice passively immunized with anti-BDE serum were also protected from lethal challenge with F1- *Y. pestis* (Ivanov et al., 2008).

Huang et al. (2009) evaluated a vaccine consisting of a spray-freeze dried powder form of a recombinant F1-V fusion protein in a mouse model. The vaccine engendered an antibody response and provided 70-90% protection against lethal subcutaneous challenge with *Y. pestis*. Ren et al. (2009) developed a vaccine consisting of recombinant F1, and V from *Y.pestis*, and the Protective Antigen from *B. anthracis* (rF1 + rV+ rPA). This formulation protected mice from subcutaneous challenge with  $10^7$  colony-forming units (CFU) of a virulent *Y. pestis* strain, and fully protected rabbits against subcutaneous challenge with  $1.2 \times 10^5$  spores of virulent *B. anthracis*.

CpG oligodeoxynucleotide (ODN) has been used as an adjuvant together with F1-V antigen to enhance its immune response in mice. CpG ODNs significantly augmented the antibody response even up to 5 months and increased the efficacy of the vaccine in murine model of bubonic and pneumonic plague (Amemiya et al., 2009).

Immunization with flagellin and with F1-V elicited a robust humoral immune response in mice and two species of nonhuman primates. The flagellin-F1-V formulation fully protected mice against intranasal challenge with *Y. pestis* CO92 (Mizel et al., 2009). Oral immunization with cationic liposome-nucleic acid complexes (CLDC) combined with F1 antigen elicited protective immunity against lethal pneumonic plague in C57BL/6J. This formulation protected mice up to 18 weeks post vaccination. Protection mediated by oral CLDC with F1 antigen depends primarily on CD4+ T cells, with a partial contribution from CD8+ T cells (Jones et al., 2010).

Ramirez and Alejandra (2009) constructed an attenuated *Salmonella* Typhi strain that expressed the F1 antigen of *Y. pestis* (*S. Typhi* (F1)), and evaluated its immunogenicity. Newborn mice primed intranasally with a single dose of *S. Typhi* (F1) exhibited a mucosal and cellular immune response one week post immunization. *S. Typhi*(F1) enhanced the activation and maturation of neonatal CD11c+ dendritic cells, and MHC-II cell surface markers and the production of proinflammatory cytokines. The *S. Typhi*(F1)- based formulation improved the capacity of DC for antigen presentation and T cell stimulation in vitro.

The protective efficacy of the F1 + rV270 (an LcrV variant lacking amino acid residues 271–300) vaccines compared to that of EV76 was evaluated. The F1 + rV270 formulation was

tested in both guinea pigs and New Zealand White rabbits by determining the antibody response and protection against subcutaneous challenge with the virulent *Y. pestis* 141 strain (Qi Z et al., 2010).

Xiao et al. (2010) developed an anti-F1-specific human monoclonal antibody (mAb) (m252) and anti-V-specific human mAbs (m253, m254) against the F1 and V antigens, respectively. These monoclonal antibodies were found to be more effective than the corresponding mouse antibodies. Neutralization of TNF- $\alpha$  and IFN- $\gamma$  interfered with the protective efficacy of F1- or LcrV- specific antibodies against the fully virulent *pgm*-positive *Y. pestis* strain CO92 (Lin et al., 2010). Recently, a recombinant rF1+rV vaccine provided protection in *Cynomolgus macaques* against pneumonic plague following inhalational challenge with a clinical isolate of *Y. pestis* (CO92) (Williamson et al., 2011).

### 7.3 Plant based vaccines

The use of plant-based oral recombinant vaccines could be an alternative approach for plague immunoprophylaxis. However, F1 and LcrV genes expressed in recombinant plant tissue were relatively less immunogenic due to the lack of signals recognized by the innate immune system through Toll Like receptors. In one such study, Swiss-Webster mice exhibited significant protection following subcutaneous immunization with *Nicotiana tabacum* leaves that expressed a LcrV-F1 (F1-V) translational fusion protein on its surface (Arlen 2008). In a separate study, guinea pigs immunized with a transgenic *Nicotiana benthamiana* tobacco plant expressing the F1-V fusion protein were protected against a subsequent pneumonic plague infection (Del Prete, 2009). However, the amount of recombinant protein produced in plant-based vectors was generally poor. To overcome this problem, the N-terminal of the  $\gamma$ -Zein protein (produced in maize and induces protein body formation) was fused with an F1-V fusion construct, which resulted in up to three times higher accumulation of protein in *Nicotiana tabacum* driven tissues than the F1-V fusion protein alone (Alvarez et al., 2010). Plant-based vaccines have also been evaluated for other Category A agents such as Variola major virus and *B. anthracis* (Rigano et al., 2009). Recently, an F1-V fusion protein expressed in carrot tap roots and lettuce was found to be stable and immunogenic for mice. (Rosales-Mendoza et al., 2010a; Rosales-Mendoza et al., 2010b).

### 7.4 DNA vaccine strategies

DNA vaccines have been developed as an alternative to protein-based vaccines. LcrV- and F1-based DNA vaccines have been developed that contain either all or part of the open reading frames encoding either LcrV, F1, or both. One vaccine containing a portion of *LcrV* that encoded a 127-amino acid peptide, was found to elicit a strong humoral immune response. Furthermore, mice immunized with this vaccine exhibited a 60% survival rate following challenge with *Y.pestis* (Vernazza. et al., 2009). A vaccine consisting of the IL-12 coding sequence and the genes for F1 or LcrV was used to immunize mice intranasally. This formulation enhanced IgA production in the mucosa and showed 80% protection from a subsequent inhalational challenge with *Y.pestis* (Yamanaka et al., 2008)

Recently, a DNA vaccine based on F1 and YscF was constructed by fusion of the gene encoding YscF to the downstream sequence of F1. This strategy enhanced protection resulting from F1 or YscF DNA vaccines alone. This approach suggested a number of ways

to develop protective DNA vaccines (Wang et al., 2010). Immunization with the F1-V based DNA vaccine and the adjuvant, lymphotactin (LTN) resulted in high levels of serum IgG and mucosal IgA antibodies (Yamanaka et al., 2010). The LcrV based DNA vaccine elicited a CD8+ immune response against specific epitopes of this antigen (Wang et al., 2011). Immunization of mice with a DNA vaccine consisting of F1 and V and the gene encoding the heat-labile enterotoxin (LT) of *E. coli* as an adjuvant resulted in 40% protection (Rosenzweig et al., 2011).

### 7.5 Virus vector based vaccines

Live avirulent or attenuated recombinant viruses expressing genes encoding virulence antigens offer several advantages over their bacterial counterparts. Non-enveloped/ naked viruses may be a better vehicle for vaccine development as these viruses can be stored for a long time without losing their infectivity. In one such case, a recombinant vaccinia virus vector was used to express an F1-V fusion protein. The vaccine was orally administered to C57BL/6J mice and was found to protect against an inhalational challenge of ten times the lethal dose of *Y. pestis* KIM/D27. It provided 100% protection up to 45 weeks post-immunization (Bhattacharya et al., 2010). Moreover, a recombinant raccoon pox virus producing F1 antigen elicited significant protection in orally immunized prairie dogs (*Cynomys spp.*) (Rocke et al., 2008). More recently, two recombinant raccoon pox viruses producing the F1 antigen and a 307-amino-acid truncated form of LcrV engendered a better humoral response and protection in both mice and prairie dogs following subcutaneous challenge with virulent *Y. pestis* CO92 (Rocke et al., 2010a, b).

The route of immunization and booster plays an important role in the immune response and subsequent protection. A recombinant Vaccinia virus Ankara vector producing either the full-length F1 or the truncated 307 amino acid peptide form of LcrV was administered intramuscularly (IM). Vaccines consisting of truncated V antigen and full length F1 antigen provided 85% and 50% protection, respectively, against both intranasal and intraperitoneal challenges with *Y. pestis* CO92 (Brewoo et al., 2010). Recently, modified, non-replicating adenovirus vectors were evaluated for the development of antibodies against both the heavy and light chains of a previously identified anti-LcrV protective antibody. Surprisingly, immunized C57BL/6J male mice showed significant levels of IgG that persisted for up to 12 weeks and exhibited 80% protection in mice after intranasal challenge with a  $2 \times 10^4$ -cfu of fully virulent *Y. pestis* CO92 (Sofer-Podesta et al., 2009).

Human vesicular stomatitis virus (VSV) has been evaluated as an effective vector for the development of a novel plague vaccine. VSV was engineered to express LcrV. Immunized female BALB/c mice showed strong humoral responses with an IgG2a bias dichotomy and exhibited 90% protection from an intranasal challenge with *Y. pestis* CO92 (Chattopadhyaya et al., 2008). These findings highlight the importance of the choice of viral-vectors in the development of plague vaccines.

### 7.6 Synthetic vaccines based on defined B and T cell epitopes

The concept of synthetic peptide vaccines was laid by the pioneering work of Anderer who demonstrated that a peptide from tobacco mosaic virus (TMV) showed immunoreactivity with antiserum against TMV. In addition, a peptide coupled to a carrier induced specific virus

precipitating and neutralizing antibodies (Deber et al., 1985). The first step in developing a synthetic peptide vaccine for plague is to identify the relevant antigen(s) determine their amino acid sequence, and identify protective B and T cell epitopes. Sabhnani and Rao (2003) identified the immunodominant epitopes of F1 antigen. The immunogenicity of the B cell (B1, B2, and B3) and T cell (T1, T2) peptides was studied in mice using alhydrogel and liposomes as delivery vehicles. B-T constructs of F1 antigen engendered protection in mice. PLGA (poly (DL-lactide-co-glycolide) microsphere delivery of B-T constructs enhanced protection (Tripathi and Rao, 2006). Later, several B and T cell epitopes of V antigen were identified by direct binding, competitive, and T cell proliferation approaches. V antigen peptides a, g and j were found to be pure B cell epitopes and peptides d and k pure T cell epitopes, whereas other peptides b, f and i showed both B and T cell properties (Khan and Rao, 2008). Furthermore, mice immunized intranasally with B-T conjugates of V antigen peptides entrapped in microspheres resulted in high titers of serum and mucosal IgG and IgA upto 120 day postimmunization. Interestingly, some of the conjugates showed enhanced protection in mice challenged with live bacteria (Uppada and Rao, 2009). Gupta et al. (2009) demonstrated the cell mediated immune response of some of the best B-T conjugates in different strains of mice. Surprisingly, some of the B-T conjugates of F1 and V antigen resulted in good lymphocyte proliferation and cytokine production *in vitro* as determined by ELISPOT assay. FACS analysis of some conjugates showed the presence of IFN- $\gamma$  and perforin secreting CD4+ cells as compared to CD8+ T cells (Gupta et al. 2011), which demonstrated the importance of CD4+ T cells in conferring immunity in the host.

## 8. Future perspectives

The development of a fully protective vaccine against plague remains a challenge. A perfect vaccine must protect humans against all three biotypes of *Y.pestis*. None of the formulations of F1 and V based vaccines were fully protective against experimental infections. The ideal vaccine would stimulate robust antibody and cell mediated immune response with respect to serum IgG, IgG sub classes and mucosal IgA along with Th1/ Th2 /Th17 cytokines correlation. These parameters could be exploited for protection studies in humans. Standardized procedures will facilitate human clinical trials to determine vaccine formulations, dosages and schedules that best prime protective responses. Incorporating additional antigens such as YscF into F1/LcrV-based vaccines and modifying existing formulations, on both the DNA and protein level, will be more effective and could lead to fully protective vaccine against all strains of *Y.pestis*. Furthermore, using different ways of immunization with novel delivery vehicles and adjuvants could enhance the immune response and efficacy of different formulations. Currently, we have extended our study by designing MAP (Multiple Antigen Peptide) incorporating the relevant protective epitopes of F1, V and YscF antigen in PLGA nanoparticles using CpG, as an adjuvant to activate Toll Like Receptor 9 (TLR-9) of the innate immune system (Uppada et al. 2011). This preparation gave a better immunogenicity profile than that of single epitope based immunogens.

A better understanding of virulence mechanisms, host pathogen interactions that operate within the body and especially the lungs during infection, could provide some new alternative targets for vaccines and therapeutics. The focus should be on the pneumonic form of disease rather than the bubonic and septicemic forms. Identifying agonists of TLR-2 or TLR-4 is also an important area of research for plague vaccine. Synthetic microbial

products that activate the Th1 and Th17 pathways are also beneficial to host immunity. However, given present concerns for bioterrorism, which may involve the release of aerosolized *Y. pestis*, there is now a greater need to explicitly characterize virulence factors that impact pulmonary disease.

## 9. References

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This book consists of nine chapters, written by international authorities, discussing various aspects of bioterrorism preparedness and response. Five of the chapters are agent-specific and highlight the pathogenesis, prevention and treatment, and the potential of specific organisms (*Rickettsia* and *Yersinia pestis*) or toxins (ricin, botulinum neurotoxins, and staphylococcal enterotoxins) to be used for nefarious purposes. Four chapters discuss different aspects of detecting and responding to a bioterrorism attack. These include methods for spatio-temporal disease surveillance, international laboratory response strategies, detection of botulinum neurotoxins in food and other matrices, and the use of physical methods (ie Raman spectroscopy) to detect spores.

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