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Pandemic and Vaccines – The Case of Deadly Anthrax Infection, Vaccine Development and Evolution

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ABSTRACT

Introduction: Anthrax is a highly infectious, zoonotic disease occurring worldwide in domestic as well as wild herbivores. It is caused by gram-positive, rod-shaped, anaerobic and sporulating bacteria *Bacillus anthracis*. Humans and other secondary consumers are infected when they come in contact with contaminated animals or animal products and consume anthrax contaminated meat. The symptoms are manifested in the three forms namely cutaneous, gastrointestinal and inhalational, and result in high mortality.

Objective: Timely detection and treatment of anthrax remain a big challenge for scientists and clinicians. The only way to prevent this disease is to vaccinate people living in maximum threat areas. The present study is an attempt to compile available information on conventional anthrax vaccines, vaccines under development, and prospects of developing new anthrax vaccines along with their advantages and disadvantages.

Conclusion: This review gives an insight into the cause and mechanism of anthrax infection, the current status of anthrax vaccines available in the market, vaccines under process and the need and future prospects of anthrax vaccine development. The need of the hour is to put in more concerted efforts towards development, testing and use of the new generation vaccines.

Key Words: Anthrax, Vaccine, Anthrax toxins, Recombinant Vaccine, New generation Vaccine, *Bacillus anthracis*

INTRODUCTION

Anthrax is a highly infectious, zoonotic disease occurring worldwide in domestic as well as wild herbivores. The causal organism of this disease is anaerobic and spore-forming bacteria known as *Bacillus anthracis*. Miscellaneous outbreaks of anthrax have occurred from time to time in animals and humans calling for enhanced and concerted efforts towards eradication of this disease. Based on the route of infection in humans and other susceptible animals the disease is categorized into three categories.

(i) Cutaneous infection

More than 95% of anthrax cases are cutaneous and occur when the spores come in contact with the injured part of the body, especially skin. People consuming contaminated meat or handling wool, animal hides etc. are more prone to this infection. The incubation period of cutaneous anthrax is about 0.15 to 12 days^{1,2} (Fig 1³). The primary symptoms are painless lesion at the injection site, nodes swelling, headache, fever and malaise.

It has a 20% fatality rate without antibiotic and <1% with antibiotic treatment^{1,4,5}.

(ii) Gastrointestinal infection

This type of anthrax generally occurs after consuming contaminated uncooked meat and the symptoms appear in the form of acute inflammation in the intestinal tract. Gastrointestinal anthrax has 1-7 days of incubation period⁶. Preliminary signs are fever, nausea, appetite loss, vomiting of blood, abdominal pain, and bloody diarrhoea⁷(Fig 2⁸). The fatality rate in this case is about 25- 60%¹.

(iii) Inhalational infection

Inhalational anthrax or pulmonary anthrax occurs due to the inspiration of *B. anthracis* spores. The incubation period is reported from 1 to 43 days⁹.

Mechanism of Anthrax Pathogenesis

There are two toxic enzymatic effectors proteins involved in the pathogenesis of anthrax which is Lethal Factor (LF)

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and Edema Factor (EF). The LF and EF both bind to a third protein Protective Antigen (PA). PA is cleaved by furin like cell surface proteases and bind to the cell surface receptors molecule, Tumor Endothelial marker 8 (TEM8) and Capillary Morphogenesis Gene 2 (CMG2)^{10, 11} and forms a ring-shaped heptamer complex. It enters into the host cell through endocytosis by acidification of the endocytic vacuole¹². The LF is a zinc metalloprotease which acts as an inhibitor of signal transduction through the mitogen-activated protein kinase (MAPK) cascade by cleaving most MAPK kinases (MAPKKs or MEKs) and preventing the phosphorylation of MAPKs, while EF is a calcium/calmodulin-dependent adenylate cyclase which increases cyclic AMP and leads to edema¹³ (Fig 3¹⁴).

Treatment

The only way to treat the deadly anthrax is by vaccination. People living in maximum threat areas have to be vaccinated and prevent the spread of this lethal disease. Described here-with are the available vaccination methods and upcoming vaccination strategies against anthrax.

Livespore based vaccines

Live spore vaccine, also known as the Sterne strain vaccine, has proven to be effective in the drastic decline of the anthrax incidences worldwide¹⁵. These are *B. anthracis* strains based vaccines and do not produce capsule¹⁶. In Russia, it was known as Zenkowsky strain or STI-1 while in China it was famous as A16R strain^{17, 18}. The disadvantage of live spore vaccine is that it causes necrosis at injection site leading to it being discontinued in most countries.

Protective Antigen (PA) Based Vaccines

B. anthracis strain (V770-NP1-R) in the USA was used as oxygenic, non-capsulated and non-proteolytic agent for the treatment of anthrax. Its culture supernatant was used for immunization. Its culture supernatant was adsorbed in aluminium hydroxide and was known as Anthrax vaccine adsorb or AVA and was registered as BioThrax®^{19, 20}. An improved version of the same is now available²¹. The advantages of this vaccine are long term immunity by providing multiple booster doses after 12 and 18 months (>5 years). Another PA-based vaccine, alum precipitated culture filtrate of the Sterne strain 34F2 is available in the UK which is registered as Anthrax Vaccine Precipitated (AVP)²⁰. It generated antibodies against PA, which have anthrax toxins neutralization activity and provide immunity against anthrax²²⁻²⁶. Though AVA and AVP are advantageous over live spore vaccines, their widespread use is hindered by the fact that they take long time to generate protective immunity that effects post-exposure anthrax prophylaxis. Besides, it is suitable for a limited animal model for potency testing^{27, 24}.

Consequently, the call of the hour was to bring about an improvement in the existing vaccination strategies along with additional immunogens, expansion of novel adjuvants, novel delivery methods and agents for a safer and more convenient/effective new generation anthrax vaccine. The new generation anthrax vaccines which are at different stages of development are described below.

Live bacterial vaccines

Many live bacterial vaccines are under process like sporogenic *Bacillus subtilis* transformants²⁸, Attenuated *B. anthracis* strains expressing rPA^{29, 30} or mutant PA or LF³¹, psoralen-killed but metabolically active (KBMA) *B. anthracis* Sterne vaccines³², aromatic amino acid-deficient *B. anthracis* Sterne mutants³³ and many other live attenuated strains of bacteria modified through recombinant DNA technology to express PA *in vivo* (e.g., *Salmonella*, *Lactobacillus*) and evaluated for their potency as oral vaccine³⁴. The oral vaccines based on *Salmonella*^{35, 36} and *Lactobacillus*^{37, 38} have potential advantages in providing protection in animal models against spore challenge. However, culturing and storage requirements of this vaccine offer a serious limitation to its usage³⁴.

Recombinant PA based vaccines

PA has been well established as the key component of anthrax vaccine^{39, 40, 41}. Protective antigen (PA) is the non toxic component of the *B. anthracis*, due to this non toxic property it could be an ideal candidate for anthrax vaccine. The single-dose of PA-based anthrax vaccine could elicit an effective immunity, safe and efficacious against *B. anthracis*. New generation vaccines propose the development of recombinant PA vaccines for anthrax treatment⁴². The expression system which is based on *Escherichia coli* for preparation of recombinant PA (rPA) served as the receptor for enzymatic moieties of anthrax toxins (ATs) and elicited toxin neutralizing antibodies (tested in white rabbits and rhesus macaques against an aerosol containing *Bacillus anthracis* spores (IVRI strain, tox+ cap+). In other hosts, the similar expression of RPA gene has been also evaluated like the attenuated strain of *B. anthracis*, *Saccharomyces cerevisiae*, *Baculovirus*, *Vaccinia virus* and tested for providing protection against *B. anthracis*^{43, 44}. Further, many rPA proteins purified from *B. anthracis*^{45, 46} and *E. coli* combined with aluminium-based adjuvants are under clinical trial as next-generation anthrax vaccine. Besides these, numerous antigen-based delivery methods like microspheres⁴⁷, nanoemulsions⁴⁸, nanofibres⁴⁹, nanoparticles^{50, 51} and liposomal and others with variable success have been applied to improve the immunogenicity. The PA-based liposomal formulation has been reported to induce high titers of Lethal Toxin (LeTx), neutralizing antibodies and confer complete protection against challenge⁵².

However, this system of vaccination exhibits variability in the extent of the immune response in different experimental model organisms. Overall the advancement in PA-based vaccines (i.e., recombinant, purified, modified), failed in providing complete protection as like AVA and other conventional vaccines.

Capsule vaccines

The capsule vaccines are polysaccharides which provide protection against bacterium phagocytosis by generating humoral immune response⁵³. *B. anthracis* poly-gamma-D-glutamic acid (γ DPGA) capsule covalently conjugated with membrane protein of *Neisseria meningitidis* serotype B and was reported to protect mice against parenteral anthrax challenge⁵⁴. The γ DPGA capsule based vaccine significantly protects monkeys, but not rabbits, against aerosolized *B. anthracis* spore challenge⁵⁵. However, it has been observed by ELISA and a macrophage opsono-adherence assay that the γ DPGA capsule-based vaccine induced robust anti-capsule antibody responses in both species. In another study, the complete protection against cutaneous anthrax were observed when γ DPGA peptidoglycan preparation (GluPG) is conjugated with PA^{56, 57} developed sortase-conjugation method (linking the γ DPGA capsule to the receptor binding domain (D4) of PA) for improving the earlier used random chemical cross-linking. The construct elicited robust antibody response against both the antigens and conferred complete protection against wild-type or *pagA* mutant *B. anthracis* Ames strain in guinea pigs⁵⁸ developed a dual vaccine by conjugating the γ DPGA capsule with PA, which elicited antibodies against both the bacilli and toxin component. The native PA was replaced with dominant-negative inhibitory PA mutant. This resulted in improved immunogenic response. In another study, intranasal immunization of mice with PA and PGA-BSA protein conjugate exhibited an immune response against both PA and PGA. The anti-PA antibodies have the capability to neutralize the LeTx while anti-PGA antibodies can kill PGA-producing bacteria by complement activation.⁵⁹ It is also anticipated that the addition of the capsule can further improve PA-based vaccines. Experiment

Epitope vaccines

The epitope is the part of an antigen molecule to which an antibody is attached- also known as the antigenic determinant. Multiple antigenic peptides (MAPs) displaying aa 304–319 or 305–319 from domain 2 of PA (loop domain) have been shown to elicit antibodies specific to a linear determinant in PA and mediated high-titer neutralization of LeTx *in vitro*^{60, 61}. Besides, immunogenicity and protective efficacy of MAP in conjunction with a heterologous T-cell epitope from *Plasmodium falciparum* was investigated in rabbits⁶¹. It was reported to elicit toxin neutralizing antibodies and provide protection from aerosolized spores of *B. anthracis*

Ames strain. In another development⁶², it was demonstrated that strong immunogenicity of ID-II epitope (aa 626–676 of PA) showing protection with whole PA. Many AT neutralization-associated humoral epitopes have been identified using solid-phase epitope mapping and confirmatory assays⁶³. The studies have established the feasibility of the use of epitopes for anthrax prophylaxis making them a prospective candidate for effective vaccination

Subunit vaccines

Evaluation of PA domain for subunit vaccines is also being envisioned as the next generation vaccines after a better understanding of molecular pathogenesis, immune mechanisms and structure of PA has been developed over the past several years⁶⁴. In a study, it was found that the conjugation of LF and EF in engineered strains expressing PA and EF/LF which resulted in keen antibody response and offered more protection as compared to the strains expressing only PA⁶⁵. Further developments in this direction resulted in the preparation of antibodies which are directed against anthrax PA domain 4 (PA-D4) which protects mice from *B. anthracis* infection⁶⁶. It was concluded that addition of other *B. anthracis* proteins or protein domains can improve the efficacy of PA-D4 based vaccine. In another study, it has been found that fusion products of PA, LF and EF on bacteriophage T4 capsid demonstrated a strong antigen-specific response and LeTx neutralizing antibodies as compared to the phage displaying PA alone⁶⁷. Yet another study states that immunization with a hybrid molecule composed of the key domains of PA and LF elicited PA and LF specific antibodies with LeTx neutralizing antibodies and protected rabbits against *B. anthracis*⁶⁸. This led to the conclusion that the fusion conferred robust protection against anthrax infection as compared to PA alone.

Plant vaccines (Oral Vaccine)

The vaccines which are based on the plant are environmental and eco-friendly, easy to use, economical, devoid of any animal or pathogens and least challenging for manufacture/process development. More importantly, they provide both humoral and cell-mediated immune response along with providing long-lasting immunity^{69, 70}. Several researchers have already reported the expression of PA in transgenic chloroplast^{71, 72}. The expressed PA in transgenic chloroplast has antibodies neutralization ability which reduces the cost of protein expression. In a study it was found that more than 360 million doses of PA-based anthrax vaccine yielded in one-acre transgenic tobacco plantation⁷³. Expression of rPA in tomato⁷⁴ was shown to be stable at higher temperature with no requirements of cold storage, transportation issues and PA purification. The transgenic chloroplast containing expressed PA-D4 has 5.3% of total soluble protein, which has the ability to protect the mice against *B. anthracis* spores

after oral immunization⁷⁵. In another study, a subunit vaccine comprising PA-D4 and domain 1 of LF as fusions to lysozyme (LicKM – a thermostable enzyme from *Clostridium thermocellum*), expressed in *Nicotiana benthamiana* – was shown to generate LeTx neutralizing antibodies⁷⁶. Consequently, edible vaccines can be looked up as an alternate source of anthrax vaccine with additional benefits of being economical and suitable for mass immunization.

DNA vaccines

DNA vaccines are expected to be advantageous because it is easy for construction, and offers the scope of combining different antigens, lowering the cost of production by eliminating the need for protein expression and antigen purification. A cationic lipid-based bivalent DNA vaccine encoding genetically detoxified PA and LF proteins was tested against *B. anthracis* spores. The mice models⁷⁷ have shown promise in nonhuman primates and passed the clinical trial evaluating immunogenicity and safety parameters⁷⁸. The evidence of immunogenicity and type of immune response generated by DNA vaccines can be further modified and enhanced by targeting the antigen to different cellular location.

Virus & virus-like particle vaccines

Virus-like particles (VLPs) are multi-protein structures which mimic the organization and conformation of native viruses but lack the viral genome⁷⁹. There are many live viral vaccines produced which have best protection capacity against anthrax spores like adenovirus-based⁸⁰, Venezuelan equine encephalitis virus-vectored vaccines^{81, 80}. Parvovirus B19 has been used for the expression of VLP conjugated PA-D4 which have robust neutralizing anti-PA antibody⁸². The Flock House virus have high affinity PA-binding domain at Anthrax toxin cell receptor which shows multiple copied of affinity⁸³. A group of researcher have identified that the chimeric VLP inhibit the toxicity of lethal toxin while binding with PA. Therefore, the non-infectious VLPs are considered as an effective method for neutralizing the anthrax toxins at cell surface receptors. However, only a little work has been done in this direction calling for more research inputs.

Nanotechnology based vaccines

Nanotechnology marks a new era in the field of medicines and having a wide range of applications in treatments, drug delivery, tissue engineering and diagnostics diagnostics⁸⁴. Recently the nano-particle delivery based micro-encapsulated in poly (lactide co- glycolide) (PLGA) forms of PA and Domain 4 were developed but their efficacy remains to be proven⁸⁵. For this reason, a new suitable alternative of next-generation anthrax vaccine is necessary. Nano-particle based approach using silver or gold nanoparticles as nano-conjugates of PA and PA-D4 seems to be a new possibility with numerous unexplored potentials.

Table 1 summarizes the different types of vaccines used for anthrax treatment.

CONCLUSION

Anthrax treatment has been a challenge to the clinicians across the globe resulting in constant efforts towards the development of efficient vaccination for the disease. While conventional vaccines are still being used, the requirement of multiple booster doses has accounted for high cost and shortage of conventional vaccines for all. It has, therefore, become imperative not only to identify alternative immunogens for immunization against anthrax toxins but also look out for alternative vaccination strategies. Several types of research have shown potential candidature in next-generation vaccines which may have single shot requirements, are cost-effective, eco-friendly, easy to handle and efficacious. The need of the hour is to put in more concerted efforts towards development, testing and use of these new generation vaccines,

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Table 1: Advantages and Limitations of available Anthrax vaccines

S.N.	Vaccine	Advantages	Limitations
1	Livespore based vaccines	No capsule formation.	Necrosis at injection site.
2	Protective Antigen (PA) Based Vaccines	Long term immunity.	Requires multiple booster doses.
3	Live bacterial vaccines	Protection in animal models against spore challenge.	Requirement of specific culturing and storage conditions
4	Recombinant PA based vaccines	Provides high titers of Lethal Toxin (LeTx), neutralizing antibodies.	Not better than conventional vaccines
5	Capsule vaccines	Significant protection to monkeys by γ DPGA capsule based vaccine.	Failure in protecting rabbits against infection
6	Epitope vaccines	Protection from aerosolized <i>B. anthracis</i> spores, toxin neutralization.	Restricted to Ames strain, not yet proven against other <i>B. anthracis</i> strains
7	Subunit vaccines	Strong antigenic response with fusion products of PA, LF and EF	No antigenic response against PA alone.
8	Plant vaccines (Oral Vaccine)	Eco-friendly, easy to use, economical, devoid of pathogens, ease of manufacture /process development	Varied response, stability issues.
9	DNA vaccines	Ease of construction, scope of antigen combinations, combining different antigens, no need of protein expression and antigen purification, cost effective	Failure in antigen targeting to different cellular locations.
10	Virus & virus-like particle vaccines	Effective for neutralizing the anthrax toxins at cell surface receptors	Limited work done, needs further validation.
11	Nano technology based vaccines	Environmental friendly, cost effective.	Efficacy yet to be validated



Figure 1: Cutaneous Infection.



Figure 2: Gastrointestinal Infection.

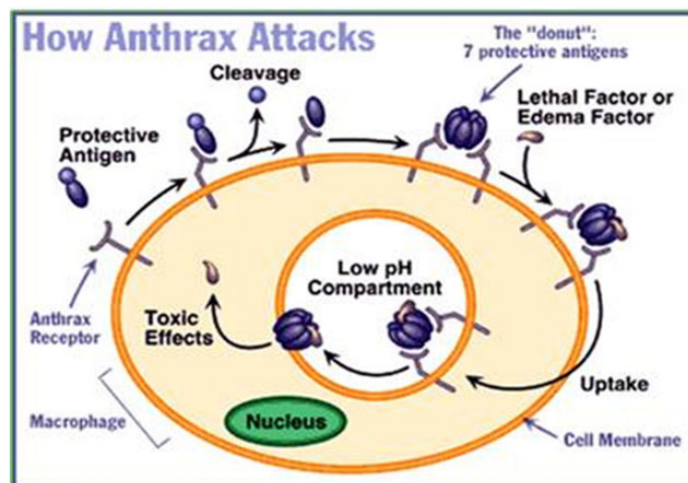


Figure 3: Mechanism of Anthrax infection.