Anthrax vaccines: past, present and future

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Most livestock vaccines in use throughout the world today for immunization against anthrax are derivatives of the live spore vaccine formulated by Sterne in 1937 and still use descendants of his strain 34F$_2$. Credit belongs to this formulation for effective control in many countries with considerable reduction, sometimes complete elimination, of the disease in animals and, since man generally acquires it from livestock, in man also. However, there are some contraindications of its use and situations in which it cannot be easily administered, and room for development of a successor is discussed. The human vaccines, formulated for at-risk occupations and situations, date from the 1950s (UK vaccine) and 1960s (US vaccine). The rather greater need for improvement of these as compared with the veterinary vaccine stimulated valuable research during the 1980s which has led to a number of promising candidate alternatives for the future.

EARLY LIVESTOCK VACCINE: PASTEUR, CARBOZOO AND STERNE

Some 80 years after Jenner's celebrated vaccination and publication of *An Inquiry Into the Cause and Effects of Variolae Vaccinae* (Sampson Low, London, 1798), microbiology's founding fathers had begun the first systematic studies on protection afforded by vaccination against a number of the most troublesome animal diseases of the day. Most noted of these were Pasteur's demonstrations of protective immunizations against fowl cholera in 1880[1], anthrax in 1881[2] *(Figure 1)* and rabies in 1885[3]. In fact, in the case of anthrax, close examination of the records[4] has revealed that credit for the first recorded demonstration of protection induced by attenuated strains of *Bacillus anthracis* really belonged to W.S. Greenfield at the Brown Animal Sanatory Institution in London[5,6].

It was, however, Pasteur's vaccine schedule that became adopted for use. This involved two inoculations 2 weeks apart. The first dose consisted of *B. anthracis* cells from cultures which had been incubated at 42-43°C for 15-20 days (Pasteur I vaccine) and which was pathogenic only for mice and young guinea pigs. The second dose consisted of cells from cultures incubated at 42-43°C for only 10-12 days and which were rather less attenuated (Pasteur II vaccine).

The Pasteur duplex vaccine became widely used for cattle and sheep in Europe and South America over the next 50 years. In the 1920s and 1930s, the procedure was modified[7]. First, in the 1920s suspension of spores in 50-60% glycerine was found to increase longevity and improve the immunizing efficiency of the spores and the double Pasteurian vaccine was replaced by single vaccines consisting of spores suspended in 50% glycerol. The strains were attenuated to such an extent as to be non-virulent for rabbits but virulent for guinea pigs and the intricate manipulations needed to meet this requirement rendered these vaccines impractical in the long run. In the 1930s, the practice of adding saponin (1-10%) to the Pasteur II or other virulent or slightly attenuated strains was introduced. Saponin at these concentrations provoked a violent inflammatory response at the inoculation site which limited generalization of the anthrax infection.

Figure 1 From an original monograph on anthrax vaccination entitled 'Vaccinations Preventives contre le Charbon du Betail' (Compagnie de Vulgarisation du Vaccin Charbonneux Pasteur) 1886. The operator is probably Louis Pasteur himself. (Photograph kindly supplied by Dr J. Ezzell.)

As reviewed by Sterne *et al.* (1939)[8], there was an epidemic of reports between 1929 and 1937 on the application and merits of vaccines consisting of spores from isolates designated as having various levels of virulence suspended in 4-10% saponin which was reported to neutralize the virulence. Particularly popular for a while, it seems, was

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Originally Published in: *Vaccine* 1991 Volume 9 Pages 533-539
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'Carbozoo', initially produced at the Istituto Sieroterapico Milanese and later in the USA by Lederle and alleged to consist of a fully virulent strain of *B. anthracis* (but which Sterne *et al.*[8] considered to be somewhat attenuated) suspended in 10% saponin. Apparently no fuller details were made public as to its constitution or manufacture although it was claimed to be almost innocuous for guinea pigs and harmless for rabbits.

Carbozoo was evidently quite effective in terms of protection but the high saponin content appears to have resulted in unacceptable adverse reactions and presumably it was this that was predominantly responsible for its disappearance from the market. In detailed studies on this vaccine and on the influence of saponin on protection, Sterne *et al.*[8] concluded that the value of the saponin lay in the significantly enhanced protective immunity rather than the only slight reduction in virulence it produced and they suggested that saponin should be used with mild strains *(meaning more attenuated)* to improve immunity, rather than with strong strains to reduce virulence. They also demonstrated that the high concentrations (10%) of saponin considered necessary by the protagonists of Carbozoo were in fact unnecessary and that 0.5% saponin was effective while being non-reactogenic.

Although effective and serving their purpose well for many years, the heat-attenuated vaccines suffered from declining potencies[9] and troublesome variations in virulence resulting in occasional untoward losses among vaccinated animals and they could not be administered safely to certain particularly susceptible species[10].

It was, in fact, Sterne's own live spore vaccine[8-12] which in the long term became the world's most potent weapon against anthrax. The strain used was a rough avirulent dissociant derived from subculture of an isolate from a case of bovine anthrax on 50% horse serum nutrient agar with incubation under a 30% CO_2 atmosphere for 24 h[10]. His final formulation consisted of 600,000-1,200,000 spores ml^{-1} suspended in 0.5% saponin in 50% glycerine-saline[12] and apart from the spore content of today's products being ± 10^7 ml^{-1}, the formulation of livestock vaccines used in most countries of the world today remains essentially as specified by Sterne and still uses his strain $34F_2$.

RESIDUAL VIRULENCE IN LIVE VACCINES

Vaccine protection studies, quality control tests and reports from the field all bear witness to a residual virulence in attenuated live spore vaccines. The dose required to give a protection level against virulent challenge approaching 100% in guinea pigs frequently results in a proportion of deaths from the immunizations[13-15] and LD_{50} values of as low as 10^3 spores were found in certain inbred strains of mice[16]. In the field, special care has to be taken when vaccinating certain species such as goats and llamas, which appear to be more susceptible to ill effects from the vaccine than other domestic herbivores.

HUMAN VACCINES

In the USSR, a live spore vaccine, reported to be a Sterne strain vaccine[17], is produced for human use by the Tblisi Scientific Research Institute of Vaccines and Serums, 3 Gotu Street, 380042, Tblisi, USSR. The vaccine is administered by scarification of the skin of the shoulder through a 10-20 μl drop of vaccine containing ±4 x 10^8 spores. There are, however, numerous patient conditions in which it is recognized in the manufacturer's recommendations that the use of this vaccine is contraindicated.

Table 1 PA, LF and EF contents of existing licensed UK and US human vaccines[a]

Direct measurement – UK vaccine (alum precipitated)
PA = ±5 μg/0.5 ml dose[32]
LF = ±2.5 μg/0.5 ml dose[a]
EF = trace[b]

Indirect measurements – antibody titres in human vaccinees and immunized guinea pigs (mean values of accumulated readings detailed in references 14, 20 and 39)

		Titres		
Vaccine[c]		Anti-PA	Anti-LF	Anti-EF
Humans	UK	1024	1024	w[d]
	US	8192	Neg.	Neg.
Guinea pigs	UK	16400	8192	512
	US	32800	16	Neg.

[a]With the UK vaccine, direct measurement was possible since the alum could be dissolved out with 0.05 M citric acid and the values verified by comparison with pre-precipitated vaccine (the UK vaccine is made in the PHLS Centre for Applied Microbiology and Research for the Department of Health). With the US vaccine (aluminum hydroxide adsorbed), no way was found of dissolving off the aluminum hydroxide or of otherwise separating it from the antigen; consequently, the PA, LF and EF content can only be inferred indirectly by comparison of antibody titres in immunized humans or animals.
[b]Turnbull, unpublished data
[c]All 2 weeks after a course of three bi-weekly doses (0.5 ml in humans, 0.25 ml in guinea pigs).
[d]Weak reaction; below level at which a titre could be assigned

In the western world, the live spore vaccine is considered unsuitable for administration to humans and vaccines developed for this purpose in the UK in the 1950s and in the US in the 1960s remain in use today. The UK vaccine (licence no. 1511/0037, Department of Health, 10 Russell Square, London WC1B 5EB) consists of an alum-precipitated cell-free filtrate of Sterne strain cultures grown so as to maximize the protective antigen (PA) content. The US vaccine (product licence no. 99, Bureau of Laboratories, Michigan Department of Public Health, Lansing, MI, USA) is an aluminum hydroxide-adsorbed cell-free filtrate of cultures of a non-capsulating, non-proteolytic derivative of strain V770 from a case of bovine anthrax in Florida in 1951[18,19].

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This strain is alleged to produce PA in the relative absence of other anthrax toxin components, the lethal factor (LF) and edema factor (EF), and culture conditions are aimed at further reduction of any LF and EF. Recent serological studies have supplied evidence that the PA content is higher and the LF and EF content is much lower in the US vaccine than in the UK vaccine[14,20] *(Table 1*; also see below).

EFFECTIVENESS AND IMPACT OF ANIMAL AND HUMAN VACCINES

As reviewed by Sterne *et al.*[8] the many trials on Carbozoo and other saponin-suspended live spore vaccines in herds, many of them large, of cattle, sheep and horses demonstrated almost dramatically the protective efficacy of these vaccines; marked reduction or complete absence of cases was reported in herds that hitherto had suffered major losses from anthrax. Trials with his own vaccine[9,12] on the impact of the spore vaccines in South Africa over the period 1925 to 1941 highlighted the effectiveness of his vaccine. The reduction in catastrophic outbreaks of earlier years brought about by the forerunners to Sterne's vaccine made farmers more critical of the reactions caused by the vaccines. Attempts to reduce these led to a loss of potency and the resulting upsurge of outbreaks in 1934-36. Following replacement of the old vaccines with his new $34F_2$ vaccine, the downward trend commenced again. Now, as then, farmers and veterinarians will testify that the vaccine will effectively cut short outbreaks.

It is much harder to produce data demonstrating and quantifying the effectiveness of the human vaccines. In this country notifications of human anthrax were already on the decline at the time of introduction of the vaccine *(Figure 2*), presumably due to improved factory hygiene and monitoring with, where appropriate, sterilization of imported animal products.

At a time when the US vaccine was being introduced into factories concerned with processing animal products, Brachman *et al*[21] found it possible to compare case rates among workers at risk in four mills who had received the vaccine and those that had not. Of 26 cases, occurring over a 4-year period, only one occurred in the group that had received the full course of the vaccine: four cases occurred among those who had received only part of the vaccine course and the remainder in those that had received no vaccine. The data indicated a 92.5% degree of effectiveness.

The study of Brachman *et al.*[21] remains the only one supplying hard data on the effectiveness of the vaccines in humans. However, with all the usual cautions that must be applied when extrapolating data from animals to humans, tests in animals have indicated that the protective efficacies of both the UK and the US vaccines are less than ideal *(Table 2*)[13,20,22,23]. The differences observed in the different studies summarized in *Table 2* are attributable to challenge strain differences, the use of different batches of US vaccine and differences in the ages of the guinea pigs used, but all serve to underscore the need for improved performances.

RESEARCH INTO NEW VACCINES

Overall satisfaction with the performance of the veterinary live spore vaccine has meant that recently the predominant emphasis has been on improving the human vaccines. In addition to the uncertain performance outlined above, the injection into human beings of crude and undefined preparations is increasingly regarded as unsatisfactory, particularly, as in the case of the anthrax vaccines, when they are associated with frequent complaints of unpleasant side-reactions.

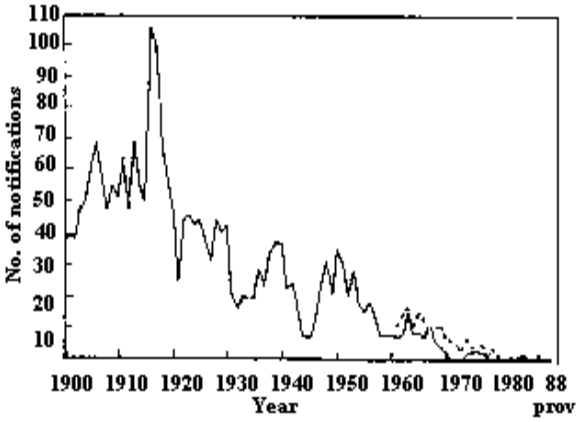

Figure 2 Notifications of human anthrax under the Factories Acts and Public Health Act United Kingdom, 1900-1988 (reproduced from Turnbull *et al*[32], kindly updated by the PHLS Communicable Diseases Surveillance Centre —, Factories Acts; ----, Public Health Act)

It is for these reasons that, over the past few years, a significant amount of work has gone into developing a second generation anthrax vaccine for administration to humans which, as well as being fully defined (containing only essential ingredients and producing effective levels of protection with a single or, at worst, two doses), produces no side-reactions.

Anthrax having become a rare human disease in the west since the 1950s and 1960s, demand for the vaccines had become minimal and for a long period there was little incentive to produce alternatives to those available. Ironically, the incentive that arose was the supposed threat suggested by the belief of western intelligence that a substantial human epidemic of anthrax in 1979 in Sverdlovsk, an industrial city in the Urals, was due to accidental escape of *B. anthracis* from a military biological installation[24]. The real extent and cause of the outbreak in Sverdlovsk remain unrevealed but the result was that the 1980s were a decade of relative opulence for anthrax research, out of which arose major new levels of understanding of the biochemical, molecular and genetic basis of the disease and the host's defences against it.

As far as improved vaccines were concerned, the

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first of these advances was the ability fully to purify and define the anthrax toxin components, PA, LF and EF, which had first been separated and partially characterized in the l950s and l960s[25,26]. The improved purifications[27-31] allowed the development of enzyme immunoassays by which it became possible for the first time to monitor the response to the vaccines in humans and guinea pigs and to relate the response in the guinea pigs to protective immunity[14,15,20,22,23,32].

The second major advance with significant bearing on development of improved vaccines was the discovery[33-36] that the genes encoding the toxin lay on a large plasmid subsequently designated pXO1 and that curing *B. anthracis* of this plasmid resulted in non-toxigenic avirulent derivatives.

During the course of vaccine-related studies in the l980s, these two developments - a sensitive and specific immunoassay for the toxin components and the ability to produce non-toxigenic pXO1$^-$ strains of *B. anthracis* - led to the clarification of a number of matters.

1. It was readily demonstrated that the toxin, or some part of it, was needed in a vaccine for induction of protective immunity. Live non-toxigenic strains conferred no protection while toxigenic non-capsulated (i.e. avirulent) strains, e.g. the Sterne strain, induced good protection[22].
2. The belief, first formulated in 1963[26], that PA (then called Factor II) was the main immunogen has been verified. PA in the absence of EF and LF has now been shown to be capable of producing effective protection both as a purified entity[23,32,37] and when produced free of EF and LF in appropriately cloned *B. subtilis*[15,38]. Consequently, protection has been noted in vaccinated animals with high levels of anti-PA in the absence or near absence of anti-EF and anti-LF[14,20,32]. EF and LF appear capable of inducing only relatively low degrees of protection[23].
3. Although it became apparent that the presence of PA in a vaccine is essential to protection, it is also clear that the relationship between PA and protection is not straightforward *(Table* 2). One or two doses of live spore vaccine may result in better protection but markedly lower anti-PA titres than three doses of human vaccine or even purified PA[20,22,23,39]. At first it was thought that this indicated that one or more other unidentified antigens were necessary for effective protection but further trials and analysis suggested that the phenomenon could be attributed to the involvement of cell-mediated immunity (CMI). The addition of totally non-specific cellular entities such as killed *Corynebacterium ovis*, *Bordetella pertussis* or Freund's complete adjuvant to either the human vaccines or purified PA enhanced their protective effects[20,32]. This stimulation of CMI is more than simply the effect of inciting the coincidental inflammatory response observed by Sterne[40] on combining the live spore vaccine with saponin; the performance of purified PA in inducing protective immunity was not found to be enhanced by irritants such as saponin[20].

These progressive discoveries, then, pointed the way to a number of approaches aimed at second generation improved human vaccines. The experimental vaccines resulting have taken three forms:

1. Purified PA vaccines in which additions aimed at promoting the necessary cellular immune response have been combined with purified PA[20,32,37].
2. Recombinant vaccines, the chief example of which to date has been *B. subtilis* cloned with the PA gene[15,38]. The PA gene has also been cloned into and expressed by baculovirus and vaccinia virus[41], but the results of protection tests with the recombinant viruses have not been published.
3. Mutant vaccines; Ivins *et al.*[42] derived two *Aro*$^-$ mutants by Tn916 mutagenesis of a Sterne strain descendant of *B. anthracis.* Unable to synthesize aromatic amino acids not available in the mammalian host, these would be expected to lack even the residual virulence of the parent pXO1$^+$/pXO2$^-$ Sterne strain and would, therefore, be theoretically safe for use in a live spore human vaccine. Following the discovery by Leppla *et al*[28,30] that an essential prerequisite for toxic action by anthrax toxin is binding of LF or EF (competitively) to PA before reaching or at the eukaryotic cell surface and that the binding site for LF and EF on PA must first be exposed through cleavage by a trypsin-like protease[43-45], Singh *et al.*[46] identified and deleted the enzyme cleavage site thereby rendering the PA + LF and PA + EF combinations non-toxic (because LF and EF could no longer bind to the PA) without altering the immunogenicity. A Sterne-type strain of *B. anthracis* in which the gene for PA carried the deletion could again be expected to be entirely avirulent.

Table 3 summarizes the prototype vaccines for the immediate future. A combination of PA with adjuvants 'DeTox' or 'Tri-Mix' (Ribi Immunochem Research, USA) perhaps shows most promise for human vaccine purposes among those within these alternatives that have been subjected to protection tests. DeTox is a mixture of monophosphoryl lipid A and cell wall skeleton from the BCG strain of the tubercle bacillus; TriMix is DeTox + trehalose dimycolate. As well as being a chemically defined formulation lacking whole cell bacteria, it has now been shown that considerably better protection can be induced with single doses of these formulations than with even three doses of the conventional UK or US vaccines (see Ref. 38, also Turnbull, unpublished results). This vaccine is essentially ready for clinical trials although a source of funding to cover this complex process[47] has not been identified yet.

The *B. subtilis* recombinant vaccines of Ivins *et al.*[15,38] were able to produce levels of protection in guinea pigs broadly equivalent to those induced by the conventional Sterne strain live spore vaccine but only when administered in 10- to 100-fold higher doses than the Sterne strain vaccine[15]. The high doses required and the fact that *B. subtilis* produces a host

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of unspecified enzymes makes this particular vaccine one of simply academic interest, but the real appeal of the recombinant approach is that, with more appropriate bacterial vectors such as *Salmonella*

Table 2 Protection induced by three doses[a] of the UK and US human vaccines and by one to three doses of live spore vaccines (LSV and STI) in guinea pig protection tests

Reference	Vaccine	Challenge strains[b]	Survival (%)		Mean[c] anti-PA titre
			Vaccinated	Unvaccinated controls	
13	US	Vollum/Vollum 1B100	16		10000
	LSV-3		100		3000
	US	9 other strains	28	3	(10000)
	LSV-3		97		(3000)
15	US	Ames	75		20535
	LSV-1		73	0	392
	LSV-2		88		10392
20	UK	Vollum	100		16400
	US		100	0	32800
	LSV-1		100		4096
	STI		100		4096
	UK	Ames/NH/Pen Res	33		(16400)
	US		17	0	(32800)
	LSV-1		65		(4096)
	STI		72		(4096)
	UK	Ames/NH/Pen Res	12[d]	0	(16400)
	US		4[d]		(32800)
22	US	Vollum 1B	71	0	64508
	LSV-2		100		16124
23	US	Vollum 1B	67	0	58310
	LSV-3		87		14404
	US		67	10	31623
	LSV-3		90		2512

[a]The standard vaccination schedule with the human vaccines begins with a short course of three doses 2-3 weeks apart. Full designations of the UK and US human vaccines are in the text. LSV-1 = live spore (Sterne strain $34F_2$, livestock) vaccine, one dose (1-5 x 10^6 spores/dose). LSV-2 = two doses 2-3 weeks apart, etc. STI = live spore vaccine made from the Russian vaccine strain. Braces indicate tests carried out simultaneously.

[b]Vollum used to be termed 'vaccine sensitive' and strains such as Ames, NH (New Hampshire) and Pen Res (a naturally penicillin-resistant isolate) were termed 'vaccine resistant' according to the results of the challenge tests in vaccinated animals. Tests with newer vaccine formulations have shown that the latter are not truly resistant (see also *Table 3).*

[c]Note the poor correlation between anti-PA titre and protection. PA is essential to protection but it is not possible to determine the protected status of the individual from that individual's anti-PA titre. Parentheses indicate these figures are a repeat of those above.

[d]Accumulated results of several experiments

species, it offers the potential for development of oral vaccines for both humans and animals. Furthermore, the recombinant strains could carry immunizing antigens for simultaneous vaccination against several diseases. Prototype bivalent *Salmonella typhi* Ty2la vaccines expressing *S. typhi* O and *Shigella sonnei* O antigens[48,49], *S. typhi* O and *Escherichia coli* LT-B[50] or *E. coli* CFA I[51], and *S. typhi* O and *Vibrio cholerae* O[52] antigens have been designed already. Similarly, bivalent vaccine strains of *Salmonella enteritidis* carrying *E. coil* LTB[53] and *Salmonella typhimurium* carrying the gene for an immunizing fraction of tetanus toxin[54] have been constructed. Theoretically, the carrier could code for protective antigens from more than one pathogen in addition to the *S. typhi* itself.

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The auxotrophic mutant vaccines of Ivins *et al.*[42] also proved themselves able to confer protection at levels equivalent to their Sterne strain parent[37] and were shown in Sterne strain-susceptible mice to have lost their virulence. However, they suffered the drawbacks of possessing a self-transmitting tetracycline resistance factor and an ability to revert, albeit at a low rate, to the *Aro*$^+$ parent type. A live vaccine in which the *B. anthracis* produces a form of

Table 3 Protection conferred by experimental alternative anthrax vaccines

Vaccine					
Type	Details	No. of doses	Challenge strain	Protection (% of survival)	Reference
Subunit	PA (70 μg) + TriMix[a]	1 or 2	Ames	100	37
	PA (12.5-50 μg) + TriMix or DeTox	1	New Hampshire	75-100	[b]
	as above	2	New Hampshire	100	[b]
Recombinant	10^8 *B. subtilis* cloned with the PA gene	1	Ames	0-6	37
	10^8 as above	2	Ames	6-71	37
	10^9 as above	2	Ames	95	37
	10^{10} as above	2	Ames	100	37
Auxotrophic mutants	10^8 Transposon Tn916 mutagenized *Aro*$^-$	1	Ames	87-100	37
	10^8 Sterne strain vegetative cells	2	Ames	100	37

[a]Ribi Immunochem Research, Montana, USA. DeTox is a mixture of monophosphoryl lipid A (detoxified endotoxin) and cell wall skeleton from the BCG strain of the tubercle bacillus; TriMix is DeTox + trehalose dimycolate (purified cord factor from mycobacteria)
[b]Turnbull, unpublished results

PA which cannot be enzymatically cleaved into active form[46] may become acceptable for human use should particular advantages over a fully defined chemical (PA) vaccine become apparent. It may, for example, prove able to confer stronger protective immunity with a single dose and, even more probable, longer lasting protection.

NEW ANIMAL VACCINES

The excellent performance of the Sterne live spore vaccine over the half-century since it was first produced has meant that there has been little motivation to develop a new successor. However, the residual virulence it retains for certain animal species, the limited duration of protection conferred and the fact that it must be administered by injection make it less than ideal in certain situations. In developing countries, for example, where syringes and needles are in short supply for human use, let alone for animal purposes, this mode of administration places an immediate impedance on anthrax control. This has obvious consequences for any programmes aimed at global control or eradication of the disease.

Similarly in African wildlife, where anthrax competes with poaching and other man-made threats for eliminating the increasingly precious and dwindling herbivore species, annual intramuscular administration of the vaccine is clearly an impractical approach to control.

Interest in new developments in anthrax vaccines is, therefore, arising in the veterinary context also, particularly with respect to the development of a fully avirulent formulation that can be administered by some simple oral route procedure. *B. anthracis* not being an invasive organism, it is unlikely that simple oral route administration of the existing vaccine would lead to development of immunity, although even this has not been examined yet. Either some means of ensuring the establishment of its microinfection in the gastrointestinal tract must be designed or a genetically engineered invasive organism such as *S. typhimurium* carrying and expressing the anthrax protective antigen gene must be constructed and a way of safely administering this without loss of potency, operator hazard or environmental damage must be found. An important challenge, therefore, still lies ahead in the field of veterinary anthrax vaccine and there are some moves now to address this.

FURTHER READING

A more comprehensive account of anthrax vaccine development can be found in reference 56.

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Originally Published in: *Vaccine* 1991 Volume 9 Pages 533-539