Onderstepoort Journal of Veterinary Science and Animal Industry, Volume 12, Number 1, January, 1939.

Printed in the Union of South Africa by the Government Printer, Pretoria.

The Preparation of Anthrax Spore Vaccines (for Cattle and Sheep) in South Africa.

By MAX STERNE and E. M. ROBINSON, Section of Bacteriology, Onderstepoort.

Introduction.

Anthrax spore vaccines have been used in South Africa for the past seventeen years with, on the whole, very satisfactory results. In the course of time the original methods of preparing the vaccine have been somewhat modified, and we think the publishing of the modifications recently introduced may interest other workers in this field.

Viljoen, Curson and Fourie (1928), whose paper was concerned with the period 1922-1925, discussed the preparation and use of anthrax vaccine in great detail. A brief summary of their method of preparing vaccine is given below.

An attenuated strain (the well-known Boshoff strain) was grown on nutrient agar in Woodhead flasks until sporulation was well advanced. This took about a week. The flasks were left at room temperature for another week, and the growth then washed off with saline. Twice the amount, by weight, of glycerine was then added to the pooled suspensions from a number of Woodhead flasks. This concentrated suspension was tested as follows:

1	rabbit	received	0·1	c.c.	subcutaneously.
1	,,	,,	0·01	,,	,,
1	guinea-pig	,,	0·1	,,	,,
1	,,	,,	0·01	,,	,,
1	,,	,,	0·001	,,	,,

The rabbits were expected to live and the guinea-pigs to die.

Thereafter sheep and goats were inoculated as follows:—

2	sheep	each	received	20·0	c.c. of the concentrated suspension, subcutaneously.
2	,,	,,	,,	0·1	,, ,,
2	,,	,,	,,	0·02	,, ,,
2	,,	,,	,,	0·01	,, ,,
2	,,	,,	,,	0·005	,, ,,
1	goat		,,	10·0	,, ,,
1	,,		,,	0·1	,, ,,
1	,,		,,	0·02	,,
1	,,		,,	0·01	,, ,,
1	,,		,,	0·005	,, ,,

All these animals were expected to survive, including the sheep and goats which got 20·0 and 10·0 c.c. Three weeks later all these animals were inoculated with 1,000 M.L.D. of a virulent spore suspension, to test their immunity. The batch was considered good if all the animals survived, average if one or two died, and poor if more died. Poor batches were usually discarded.

Good vaccines caused a temperature reaction in 90 per cent. of the animals tested. The subsequent test with virulent spores, however, rarely caused any reaction. Good batches, that is those of which 0·005 c.c. of the undiluted vaccine protected sheep, were diluted 1:200 for issue; average batches 1:100, and poor batches, where 0·01 c.c. had failed to protect, were discarded.

We examined the protocols of the batches passed and issued at this time and extracted the following additional information.

The 0·1 to 0·001 c.c. amounts of the undiluted vaccine killed guinea-pigs in 24 to 72 hours.

The vaccine occasionally killed rabbits and goats, but was used if it did not kill rabbits when retested.

Summarizing these statements we find that the vaccine was expected to be avirulent for sheep, almost avirulent for goats and rabbits, and very virulent for guinea-pigs. At this time two million doses were issued per year and very few complaints received. As the demand for vaccine increased complaints were made about severe reactions and deaths following inoculation. A possible reason for this was that the popularity of the vaccine and the propaganda conducted by the Veterinary Department resulted in many farmers inoculating against anthrax even where the disease was not an imminent danger. Where anthrax was a serious menace, occasional severe reactions and deaths were disregarded; but sequelae to inoculation were not regarded with equanimity in areas where the danger from the natural disease was slight. It is possible therefore that the increasing volume of complaints had more to do with the changing nature of the inoculation than with any considerable change in the vaccine.

To obviate such reactions, batches were later (1926) issued at higher dilutions, such as 1:500, and this in turn adversely affected the immunity produced. A further trouble experienced at this time was irregular sporulation and the consequent rapid deterioraton of some batches. Quin (personal communication) stated that this irregularity was attributed to the presence of mucoid growth and that steps were taken to ensure cultures as free from mucoid growth and capsulated organisms as possible. (Growth at 32° C, selection of non-mucoid strains). At this time also peptone was omitted from the nutrient agar, in the hope that the poverty of the medium would induce better sporulation. This peptone-free medium was used until 1935. In 1930 to 1932 even greater stress was laid on the need for non-mucoid cultures by Coles (personal communication), who maintained that severe reactions in inoculated animals were usually associated with batches containing many capsulated bacilli. Between 1929 to 1935 the temperature of incubation was raised to 35° C. Owing to many complaints of lack of immunity in 1927 to 1929, the virulence of the strains used was increased again, in 1930; but the dilution at which the vaccine was issued was higher. Nevertheless, some severe reactions still occurred, and during this period there were reports both of undue virulence and of lack of immunizing power.

We have, in this introduction, rather stressed the untoward results of immunization. Actually, considering the vast issue, the vaccine was very satisfactory. There were enough unfortunate incidents, however, to cause perturbation amongst those responsible for preparing the vaccine.

As a result of research done between 1934 and 1938, much information was gained about the behaviour of attenuated strains, and we have been able to adopt a more standardized technique and to obtain far more consistent results than formerly. We therefore give below, briefly, the methods now used to prepare the ordinary anthrax single spore-vaccine.

Preparation of Vaccine.

Selection of Vaccine Strains.

The attenuation of anthrax strains by the Pasteurian method is a tedious and often unsatisfactory procedure; for although it is not difficult to cause a strain to lose virulence, it is difficult to find strains which maintain this new level of virulence. There are, however, enough stable, attenuated strains in existence to make the undertaking of new attenuations unnecessary. The Boshoff strain, attenuated by Kind (1922), has given good results in this and other countries. We have also found strains isolated from *Carbozoo Mailänder* and *Carbozoo Lederle* very suitable for vaccine production. The virulence of these two strains, which are very much the same in appearance and behaviour, can be easily exalted by guinea-pig passage, and reduced by ageing the cultures on nutrient agar.

Nevertheless, spore suspensions prepared from any of these strains remain remarkably stable at the level of virulence of the culture from which the spores arose. Thus, within limits, vaccines of different potencies can be prepared.

Characteristics of Vaccine Strains.

These must grow luxuriantly on nutrient agar. The edges of the growth should be slightly rough, and the surface glistening and shining, thus indicating the presence of many capsulated bacilli. Here our requirements differ radically from those of the earlier workers at Onderstepoort, who wanted growths containing as few capsulated bacilli as possible. Coles contended that the capsulated bacilli were responsible for severe reactions and in this he was probably correct; for it has been shown, Bail (1915), Schaefer (1936), Stamatin (1934), Sterne (1937), that the virulent phase of anthrax is capsulated. Nevertheless since virulence for guinea-pigs was, and is, a criterion used in assessing the value of a vaccine, there must be organisms in the capsulated phase in all the vaccines. The earlier workers, by reducing the number of mucoid bacilli, but retaining the virulence standard for guinea-pigs, were obviously using a culture containing relatively few, but rather virulent bacilli in the capsulated phase. It appears that such vaccines, if used on beasts more sensitive than ordinary, sometimes produced very marked reactions and occasional deaths. For this reason we, while retaining approximately the same virulence standard for guinea-pigs, attain this by using cultures with large numbers of capsulated bacilli which are, necessarily, more attenuated than formerly. This is, technically, an easier standard to maintain, seeing that attenuated strains tend naturally to a mucoid habit of growth.

Culture of Vaccine Strains.

Medium.—We have reverted to using nutrient agar containing peptone. The composition is as follows:—Meat 500 grm., peptone (Merck's) 10 grm., sodium chloride 5 grm., di-sodium-hydrogen phosphate (Na_2HPO_4) 2 grm., agar-agar 25 grm., water 1,000 c.c. The pH is adjusted to 7·4. Exhaustive tests showed that the omission of peptone did not favour sporulation, but merely slowed down the rate of growth and lessened the amount of culture ultimately harvested. Good sporulation appeared to be associated rather with a vigorous than with a retarded growth. Each batch of medium is tested for its ability to support a vigorous growth of the vaccine strain, before being used.

Inoculation.—A twenty-four-hour Mason's tube (Mason 1933) culture of the vaccine strain is scraped off into 500 c.c. nutrient broth. This fairly dense suspension is used, immediately, to inoculate about 50 Woodhead flasks, of one litre capacity, each containing 250 c.c. nutrient agar. Formerly, a twenty-four-hour broth culture was used as the inoculum, but this technique was discarded owing to the tendency of a liquid medium to select unwanted dissociants.

Growth.—The flasks are incubated at 37·5° to 38·5° C. This temperature must be rigidly adhered to, as we have found that lowering the temperature affects sporulation adversely. Strains which sporulate readily at 37·5° C. may show only masses of metachromatic granules at 35° C. or 36° C. We now realize that much of the earlier difficulty with sporulation was the result of lowering the temperature to inhibit capsulation.

After two days the surface of the growth should be raised and slimy, and smears should show large numbers of capsulated bacilli as well as a good proportion of spores. Later the mucoid appearance is gradually lost, while the proportion of spores increases rapidly. Sporulation ought to be complete by the fifth day, and the growth ready to wash off. If, as occasionally happens, sporulation at this time is poor, the batch is discarded, because such lots never make good vaccine.

An average-sized batch consists of 200 Woodhead flasks.

Testing of Vaccine Batches.

Testing of virulence in guinea-pigs and rabbits.—Each Woodhead flask yields 80-90 c.c. of glycerinized spore suspension.

2 rabbits each receive 0·1 c.c. subcutaneously, and must survive.

3 guinea-pigs each receive 0·1 c.c. subcutaneously, and should die.

3 guinea-pigs each receive 0·01 c.c. subcutaneously, and should die.

3 guinea-pigs each receive 0·001 c.c. subcutaneously, and should die.

The guinea-pigs are carefully selected and should weigh 500 grm. each. In interpreting these results, the survival time after inoculation must be taken into consideration. If all the guinea-pigs die between 48 and 72 hours, or if all, irrespective of the dose, die at about the same time, the vaccine will be too virulent. The differences in dosage, 0·1 to 0·001 c.c. of the mother suspension, must show a well-marked effect. Batches where 0·1 to 0·001 c.c. kill guinea-pigs in 24 to 72 hours are discarded, even if the rabbits survive. This interpretation differs radically from that of Viljoen and his co-workers. The deaths of the guinea-pigs should range from 3-7 days after inoculation. Occasionally a guinea-pig will survive; this is not considered a bad sign. If any rabbit dies of anthrax the batch is discarded. In practice rabbits do not die if the vaccine shows average virulence for guinea-pigs, so the latter test is regarded as the more important.

Safety and immunity test in sheep and goats.—The following table shows a typical and satisfactory test.

TABLE I.—*Safety and Immunity Test on Sheep and Goats. Batch No. 99 (for Cattle and Sheep).*

SAFETY TEST.			IMMUNITY TEST.	
Sheep.	Amount of Undiluted Vaccine Injected.	Result.	No. of *Certain Killing Doses* of Virulent Spores Injected 3 Weeks Later.	Result.
1	20·0 c.c.	L.	100	L.
2	20·0 c.c.	L.	,,	L.
3	0·1 c.c.	L.	,,	L.
4	0·1 c.c.	L.	,,	L.
5	0·1 c.c.	L.	,,	L.
6	0·1 c.c.	L.	,,	L.
7	0·01 c.c.	L.	,,	L.
8	0·01 c.c.	L.	,,	L.
9	0·01 c.c.	L.	,,	† (3)
10	0·01 c.c.	L.	,,	L.
Goats.				
1	10·0 c.c.	L.	,,	L.
2	10·0 c.c.	L.	,,	L.
Control Sheep.				
1	Nil.	—	1	† (4)
2	Nil.	—	10	† (2)

L = Lived. † (3) = Died on 3rd Day.

The immunity is tested with a dilution of a stock glycerine-saline suspension of the virulent Boshoff strain. This suspension was prepared two-and-a-half years ago and has not yet started to lose virulence. As it is impossible to establish the M.L.D. for sheep at a reasonable cost, we have adopted as our standard ten times an approximately determined M.L.D. This we call a Certain Killing Dose (C.K.D.).

A batch is never passed unless *all* the sheep and goats survive the safety test. It is passed, however, if one or two fail to withstand the virulent spores. We also deviate from the criteria formerly used in the following respects:—

(1) Not more than one or two of the eight sheep immunized with 0·1 and 0·01 c.c. should show temperature reactions.

(2) Most of these sheep *should* show temperature reactions to the virulent test dose.

These subsidiary criteria are important because judging on deaths and survivals only becomes a haphazard affair, when done on so few animals. Sometimes, for example, a very solid immunity produced in sheep is the only indication that a batch may be too virulent.

If the tests have been satisfactory, the concentrated suspension is diluted 1:25, 1:50 or 1:100 with glycerine-saline depending on whether two, one or no sheep died in the immunity test. Some latitude is allowed here. If the guinea-pigs had died fairly quickly, a batch would be issued at 1:100 even if one or two of the sheep died in the immunity test. The dose for cattle is 1·0 c.c. and for sheep 0·5 c.c. of the diluted suspension. No batch is ever issued at dilutions higher than 1:100. If a lot of vaccine is rather virulent, as judged by the laboratory test, it is discarded, not diluted. Thus most of these tests are designed to select vaccines of slightly lower virulence than those formerly chosen.

Only a fraction of the total amount of vaccine issued is used on sheep. As we have found these animals more susceptible than cattle, we select sheep vaccine from the milder cattle batches.

Comparison of tests on sheep done between 1926-1935 *and between* 1936-1938.—Table II summarizes the safety and immunity tests done on sheep prior to 1935 and after 1935 when the modifications mentioned above were introduced. In the period 1926-1935 dosages below 0·01 c.c. are not included, as tests with these smaller amounts have now been discontinued.

TABLE II.—*Summary of Tests on Vaccine Batches* (1926-1938).

No. of Sheep Immunized.	Period over which Tests were done.	No. of c.c. Concentrated Suspension received by each Sheep.	No. which died after Immunity Test.	Per cent. dying after Immunity Test.
139	1926–1935	20·0	1	0·7
139	,,	0·1	22	15·8
149	,,	0·01	61	40·1
CONTROLS NOT REGULARLY INCLUDED DURING THIS PERIOD.				
36	1936–1938	20·0	0	0·0
71	,,	0·1	8	11·2
78	,,	0·01	12	15·4
CONTROLS.				
68	1936–1938	Nil.	68	100·0

From 1926-1935 batches were issued at dilutions of 1:100 to 1:500 of the concentrated suspension depending on whether the vaccine was weak or strong. In spite of several batches in this series having immunized down to 0·001 c.c. there was, on the average, a big drop in immunizing power between the 0·1 and 0·01 c.c. level. From 1936-1938, although the batches were on the average less virulent than the earlier ones, there was an insignificant drop in

immunizing power between the 0·1 and 0·01 c.c. level; the latter being the level at which the vaccine is issued. We believe the main reason for this more even protection to be the increased effective dosage, which has been brought about as follows:—

(1) The presence of large numbers of capsulated bacilli.

(2) The increase in harvests brought about by using peptone in the medium.

The concentration of the vaccine is, relatively, still higher, because we now dilute 1:25 to 1:100 as compared with 1:100 to 1:500 in former years. A point not brought out in Table II is that there was little variability between batches after 1936.

Field tests.

(1) 500 doses, at double strength, are injected into cattle in the field.

(2) If there are no, or very slight, reactions to the preliminary test, a further 10,000 doses are issued in small lots to different areas.

(3) If no complaints are received, the routine issue of the batch may be commenced.

The yearly issue of vaccine is now six million doses. Each batch contains about a million, and twelve to fifteen such lots are prepared during the year. More batches are actually started, but get discarded at different stages of manufacture. From the twelve to fifteen successful batches a final selection is made, mainly on field tests and reports.

If at any time adverse reports about a batch are received *from different localities*, the issue of that batch is suspended. Experience has shown it wiser to assume that most untoward results are caused by the vaccine, than to blame the farmer, the needles, the condition of the cattle, the hot weather, the cold weather, the drought, the rain or the depression. We have stressed the statement that the reports should come from different areas. This avoids confusion with post-inoculation accidents consequent on infections, such as those caused by *Cl. chauvoei* or *Cl. septicum*.

Discussion.

Comparison of field and laboratory results.—It must be emphasized that laboratory results give only a general indication of the way a vaccine will behave in the field, and that there is no substitute for the large scale testing of new batches, or of new types of vaccine. Patently dangerous methods of immunization have sometimes been advocated through workers not appreciating the wide gulf between the stabled and the free-ranging animal. For example, in the routine tests of the last two years 50 goats have each been inoculated with 10·0 c.c. of undiluted cattle vaccine. Not one has died. If, however, such vaccines were to be used for goats under field conditions, a heavy mortality (20-70 per cent.) would occur with the inoculation of even small amounts of vaccine. Every now and then this point is forcibly brought home to us, when people accidently use cattle or sheep vaccine for goats; the results are usually catastrophic.

Nevertheless laboratory tests, when carefully interpreted, give indispensable information.

Comparison of field results with old and with new type vaccine.—The results of the large scale field inoculations done after 1935 compare favourably with those done earlier. There have been no complaints about lack of immunity, although there has been plenty of positive evidence of the vaccine's efficacy in stopping and preventing anthrax on heavily infected farms. Generally, the immunity has been effective and uniform, in spite of our using more attenuated strains.

A more detailed study of the results of immunization in the field will be given in another paper now in course of preparation.

Although there have been a few complaints of undue virulence, the number has been insignificant. The batches responsible were withdrawn and replaced by milder vaccine. In no case were such batches issued at higher dilutions. In 1935, 1936, and 1937, about 50 deaths from vaccine anthrax were reported out of approximately 16 million cattle inoculated. Forty of these deaths occurred with two batches used on about 300,000 animals and then withdrawn.

Thus the increase in the concentration of spores in the vaccine, together with the reduction in virulence of the strains used, enabled us to produce a vaccine giving a uniform and good immunity, without causing many severe reactions or deaths.

A Note on Types of Post-inoculation Accidents.

(1) Death in 24-72 hours; blood smear showing no anthrax bacilli. Most such prove to be *Cl. chauvoei* infection.

(2) Death in 72-96 hours; anthrax bacilli in blood smear. Natural virulent anthrax is usually blamed for such deaths; but they may be the result of inoculation. Such quick deaths as the result of inoculation are more common in sheep. For example, of one lot of 250 sheep inoculated with a batch of vaccine which was subsequently withdrawn, 8 died in 72 hours of typical apoplectic anthrax. Cultures from the blood showed organisms of the vaccine type.

(3) A swelling, diffuse and oedematous, which may reach the dewlap, abdomen and udder. Such swellings may take a week or ten days to develop. Milk yield and condition go down fast and death may occur in from 1 to 3 weeks. Most animals affected in this way recover. This is the usual type of severe reaction. The oedema is almost invariably of a serous-gelatinous nature, and it is rare to find bacilli in blood smears from animals which have succumbed to this type of reaction.

REFERENCES.

BAIL, O. (1915). Veränderung der Bakterien im Tierkörper. Über die Korrelation Zwischen Kapselbildung, Sporenbildung und Infektiosität des Milzbrandes. *Centr. f. Bakt. I. Orig.*, Vol. 75. pp. 159-173.

KIND, G. (1922). Beiträge zur aktiven Immunisierung gegen Milzbrand. *Thesis.* University of Zurich.

MASON, J. H. (1933). A new culture tube. *Journ. South Afr. Vet. Med. Assoc.*, Vol. 4, No. 2, pp. 89-90.

SCHAEFER, W. (1936). Dissociation de la bactéridie charbonneuse. *Compt. Rend. Soc. Biol.*, Vol. 122, No. 25, pp. 1178-1181.

STAMATIN, N. (1934). Contributions a l'etude de la morphologie et la biologie de la bactéridie charbonneuse. *Arch. Veter.*, Vol. 26, No. 112, pp. 1-28.

STERNE, M. (1937). Variation in *Bacillus anthracis*. *Onderst. Journ. Vet. Science and Anim. Indust.*, Vol. 8, Nos. 1 and 2, pp. 271-350.

VILJOEN, P. R., CURSON, H. H., FOURIE, P. J. J. (1928). Anthrax in South Africa, with special reference to improved methods of protective inoculation. *13th and 14th Reports of Director of Veter. Educ. and Research*, South Africa, pp. 431-531.