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STRAIN 19 BRUCELLA VACCINE—II. THE PREPARATION OF FREEZE-DRIED LIVE VACCINE.

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Desiccation from the frozen state may be used to preserve the structure and physiological activity of almost all micro-organisms (Elser, Thomas and Steffen, 1935; Flosdorf and Mudd, 1938; Greaves and Adair, 1939; Stamp, 1947) and was first applied to *Brucella* vaccine by Verwey (1944).

In the liquid state Strain 19 *Brucella* vaccine is an extremely labile product. The 5 ml. dose required for subcutaneous inoculation must contain a minimum of 60,000 million viable organisms. No contamination is permissible as most free living bacteria are capable of multiplying in the vaccine at room temperature and may destroy the *Brucella* organisms in a short time. Light, heat and agitation also cause rapid loss of viability. The use of the vaccine is therefore limited to areas with good transport facilities not far removed from the site of preparation. Conveyance over any considerable distance necessitates such refrigerated packing as will keep the suspension cool (4° C.) and yet prevent freezing which would kill the bacteria. Dead *Brucella* organisms have no immunogenic value so that after arrival arrangements for immediate inoculation of the vaccine are essential. In Southern Africa where vaccination is employed on a large scale as the main anti-brucellosis control measure, freeze-dried vaccine meets the requirements of vast areas which could not be served with liquid vaccine.

The technique chiefly employed in the U.S.A. (lyophyle process) consists of "shelf" freezing followed by vacuum drying and vacuum sealing. The "Cryochem" process, based on a method of "shell" freezing is more suited to small units and is used in many laboratories all over the world. In England centrifugal freeze drying *in vacuo* has been developed and this has marked advantages for small units.

The present report deals with the method of production investigated at Onderstepoort. The demands are peculiar to the local circumstances in that unfavourable climatic conditions and limited transport facilities co-exist with long distances and a high precentage of nondescript cattle in the more remote districts. The relative amounts of liquid and freeze-dried *Brucella* vaccine issued from this laboratory for the period January, 1954 to April, 1955, are shown in Graph (*a*).

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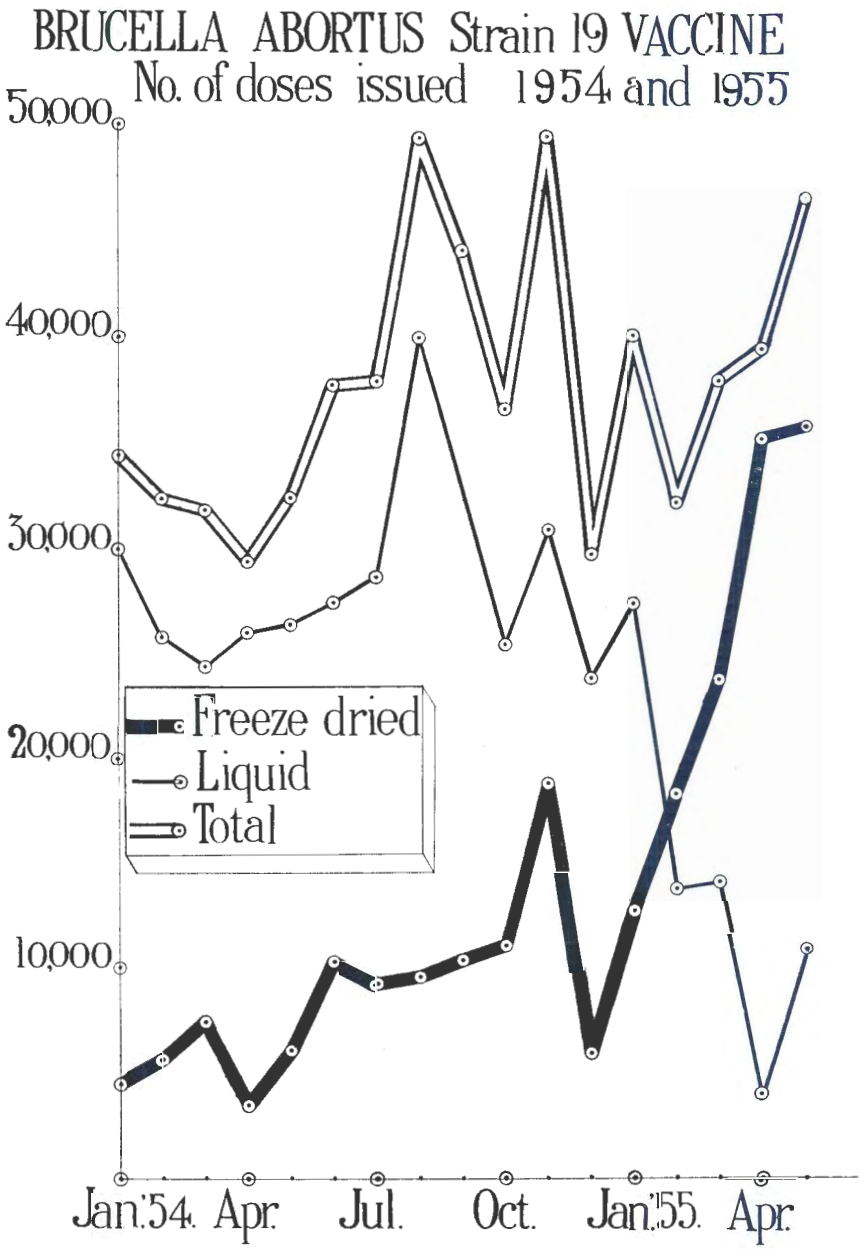


GRAPH (*a*).—Number of Doses Brucella Vaccine issued per month.

Materials and Methods.

Brucella abortus S. 19 was cultivated as prescribed by the United States Bureau of Animal Industry to produce the agar surface culture grown vaccine initially used for freeze-drying. The agar surface grown culture was later used as seed for an aerated liquid culture method of propagation developed at Onderstepoort (v. Drimmelen, 1956). By this method the total number of viable cells could be increased 450 fold in 66 hours and the organisms thus obtained eventually became the source of all freeze dried vaccine.

An Edwards Model 3 PSA centrifugal freeze-drier was used, fitted to receive 10 c.c. ampoules in each of which 3—4 ml. of fluid vaccine could be dried. After drying the ampoules are filled with purified nitrogen on the secondary system and sealed without detachment from the manifold.

Experimental.

The conditions of freeze-drying in the apparatus are more or less fixed. The purpose of the work was to obtain the maximum number of doses of high quality vaccine from every batch dried in one run on the machine. The investigations thus were directed towards two objectives: (1) Concentration of the vaccine before freeze-drying, and (2) Promoting survival of the organisms during drying and storage.

(1) *Concentration of viable* Brucella abortus *S. 19 organisms before freeze-drying.*

(a) Decrease of the amount of suspending saline used for harvesting surface cultures.

Previously, harvesting of *Brucella* culture from agar was carried out without glass beads by agitating 25 ml. buffered saline over the surface growth in one Roux flask to collect a suspension of about 160,000,000,000 viable organisms per ml. Reducing the saline to 10 ml. per flask and adding beads resulted in doubling the concentration but caused increased wastage of cells.

Harvesting the growth in successive flasks by using the same suspension repeatedly provided a method of obtaining suspensions of 1,000,000,000,000 v.o.p. ml. but the losses from increased contamination were excessive.

(b) Increase of final density of viable Brucella *organisms in aerated liquid culture medium.*

Various media and methods had been adapted previously to *Brucella* cultures to achieve a final density of about 90×10^9 v.o.p. ml. The use of a shaker and special flat, 4 litre capacity flasks agitated at an angle, together with simplifying the medium and adding an antifoam agent resulted in a final density of about 175×10^9 v.o.p. ml. (v. Drimmelen, 1956).

Trials with replenishment of growth factors during the exponential growth phase gave indifferent results due to technical limitations in the method of agitation used.

(c) Augmentation of the density of the culture by adding viable organisms harvested from liquid culture by means of centrifuging.

Centrifuge bottles of 550 c.c. capacity were used and the sediment was resuspended in the drying solution together with live *freshly* harvested culture before freeze-drying. Ten litres of culture per day could be concentrated in this way in a 2 litre capacity centrifuge and resuspended in two litres of culture to load

two freeze-drying units. The labour involved and the loss of viability were both considerable although a final concentration of about 10^{12} viable organisms per ml. was attained.

More economical centrifuging was performed with a Sharples' Laboratory Super Centrifuge capable of sedimenting 40 litres of culture per day. Cells in the form of a thick paste were added to fresh culture and stored overnight for purity testing. Drying solution was added before freeze-drying. Pre-drying counts of $1{\cdot}5 \times 10^{12}$ v.o.p. ml. were obtained. Again there was a heavy ballast of dead *Brucella* organisms included in this material. One freeze-drying unit could, nevertheless, deliver 2×10^{14} viable organisms per day in the form of freeze dried vaccine.

(*d*) *Sedimentation of viable cells and removing part of the culture medium.*

This method was investigated with the purpose of reducing losses of viable cells and thus lowering the ballast of dead cell material in the final product.

Promising results with serological agglutinants were discarded on account of the possible antigenic activity of serum and the risk of contaminating virus infections.

Heat sterilizable agglutinants and adsorbents tested were:—

(i) Metallic ions.: e.g. calcium, aluminium, silicon.

(ii) Nitrogenous substances: e.g. casein, gelatin, protamine.

(iii) Gums, carbohydrates: e.g. gum arabic, soluble starch, dextrin, methyl-cellulose, carboxy-methyl-cellulose, sodium-carboxy-methyl-cellulose.

Titration of liquid *Brucella* culture with carboxy-methyl-cellulose showed that a sediment of suitable consistency would be obtained with 0·05 per cent C.M.C. This colloidal solution was clear, uniform and constant unlike solutions of natural gums. As an agglutinant it was superior to solutions of gelatin, agar-agar, gum arabic, gum tragacanth, pectin, starch glue and dextrin glue. C.M.C. was found the most suitable adsorbent for sedimenting live *Brucella* organisms and it was cheap, pure, easily sterilized, inert, harmless and effective in low concentration. (See Table 1).

(2) *Promoting survival of live* Brucella abortus *Strain* 19 *organisms during centrifugal freeze drying and during storage thereafter.*

(*a*) *Addition of an equal quantity of skimmed milk.* (Flosdorf and Mudd, 1938, Verwey and Scheidy, 1946; Fry and Greaves, 1951).

Sterilization of milk by Tyndallizing was a time-consuming factor but the principal objection was its diluting effect. Dried skimmed milk powder was used for a time but this was even more difficult to sterilize on account of the heavy contamination in the commercial product. A survival of 52 per cent was obtained when used with shake flask culture.

(*b*) *Addition of autoclaved drying solution.*

Buffered saline with 25 per cent lactose plus 5 per cent tryptose (pH 6·3) was autoclaved and after cooling, added to 4 times its volume of viable cell suspension. The results of drying were irregular and poor.

TABLE 1.

Variations in Technique of Concentrating Brucella *Organisms for Freeze-drying.*

Method of Culture.	Method of Harvesting.	Method of Increasing Viable Cell Concentration.	Percentage of Estimated Total Cells Cultivated still Viable before Drying.	Viable Organisms per Ampoule before Drying (Average).	Average Survival Rate (Percentage).		Average Number of Doses per Ampoule Issued.	Grams of Medium Used per Dose.
					After Drying.	After One Month.		
Potato Agar Surface Culture	25 ml. saline per Roux flask	—	95	$0{\cdot}2 \times 10^{12}$	46	18	1	15
Potato Agar Surface Culture	10 ml. saline and beads per Roux flask	—	80	$0{\cdot}4 \times 10^{12}$	47	25	1·8	18
Potato Agar Surface Culture	25 ml. saline per Roux flask	Same suspension used in 6–10 flasks successively	60	$3{\cdot}0 \times 10^{12}$	32	20	9·3	30
Aerated Liquid Culture	Direct.....	—	100	$0{\cdot}4 \times 10^{12}$	60	31	2·2	2·5
Aerated Liquid Culture	Direct.....	Adding sedimented cells from centrifuge bottles	30	—	—	—	4·0	5·0
Aerated Liquid Culture	Direct.....	Adding packed cell paste from Sharples centrifuge	25	$1{\cdot}8 \times 10^{12}$	32	23	4·7	5·5
Aerated Liquid Culture	Direct.....	Sedimented in 0·05 per cent C.M.C.	95	$3{\cdot}4 \times 10^{12}$	58	53	18·0	2·5

(*c*) *Addition of autoclaved drying solution containing ascorbic acid to counteract oxidation.* (Stamp, 1947; Naylor and Smith, 1946.)

Buffered saline with lactose 25 per cent, tryptose 5 per cent, ascorbic acid 2·5 per cent and thiourea 1·6 per cent, was sterilized and when cool added to 4 times its volume of resuspended packed cells in buffered saline pH 6·3. A survival rate of only 15 per cent was obtained and the results were erratic. Only a few experimental batches were made.

(*d*) *Addition of Seitz sterilized drying solution to viable cells suspended in spent liquid culture medium.*

It was believed that since the culture was harvested in the maximum, static phase of growth the spent medium might provide the most effective and economical enzyme inhibiter for protection during and after freeze-drying. Seitz filtering a solution of lactose, ascorbic acid and thiourea was adopted to eliminate heat damage to lactose, and ascorbic acid. The filtered sterile drying solution was sealed in glass ampoules which prevented deterioration on storage so that large amounts could be prepared at one time. Spent medium with a final content of lactose 5 per cent, ascorbic acid 0·5 per cent, thiourea 0·25 per cent, and the live cells of the culture gave a survival rate of 48 per cent.

The same mixture augmented by adding packed cells showed a survival rate of 42 per cent for the centrifuge concentrated and resuspended cells.

(*e*) *Addition of Seitz filtered drying solution to Carboxy-methyl-cellulose treated live cells in spent liquid culture medium.*

Methyl-celluloses had shown a favourable effect on the keeping quality of liquid vaccine (van Drimmelen, 1954). Carboxy-methyl-cellulose in low concentration increased the sedimentation rate of cells in the suspension, but the consistency of the sediment was altered from a powdery, dense cell pack to a loose, fluffy deposit.

C.M.C., added in sterile solution to the liquid culture, was thus incorporated in the final drying mixture, i.e. spent medium with a final content of:

Lactose	5 per cent	pH 6·3
Ascorbic acid	0·5 per cent	
Thio-urea	0·25 per cent	
C.M.C.	0·05 per cent	

(the C.M.C. content of the mixture was increased further by adsorption to the sedimented cells, the supernatant being discarded).

Results showed a survival rate of 50 per cent after freeze-drying and there was no significant drop in viable cells after one month storage. (See graph *b*.) This finding is the more advantageous as double the concentration of cells could be incorporated in the freeze-drying mixture. Some batches showed an 80 per cent packed cell volume and in one case a final dried product containing one viable *Brucella* organism per cubic μ was actually obtained.

The present results were obtained with high viscosity (No. "70") C.M.C., a partly purified product priced at less than 10s. per lb. (See Table 2.)

TABLE 2.

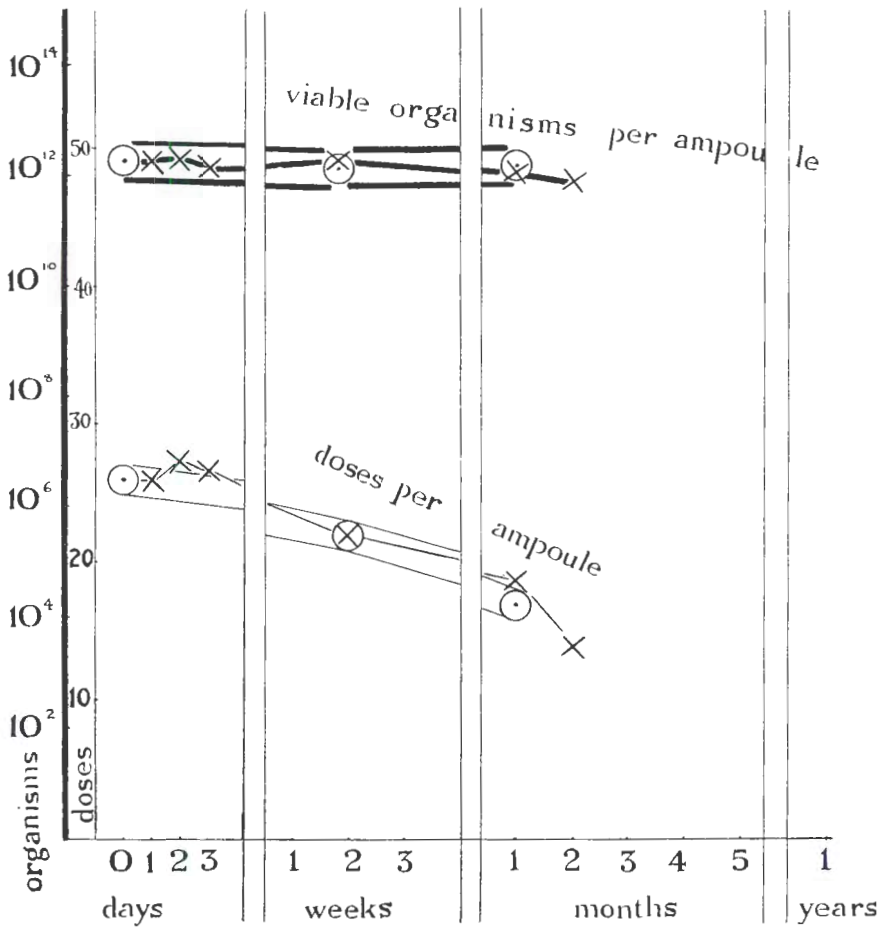
Variation in Composition of Drying Solution Used for Freeze-drying Brucella *Organisms.*

Method of Culture.	Treatment of Cells.	Method of Sterilizing Drying Solution.	Final Mixture Ready for Freeze-drying.	Number of Viable Organisms per ml.	Percentage Survival Rate.	
					After Drying.	After 1 Month.
Agar surface culture.....	Suspended in buffered saline	Tyndallized.....	Skimmed milk... 50% Cells in saline	5×10^{10}	46	21
Agar surface culture.....	Suspended in buffered saline	Autoclaved at 110° C. for 30 minutes	Lactose..... 5% Tryptose..... 5% Cells in saline.	15×10^{10}	25	20
Aerated liquid culture....	Not treated.....	Tyndallized.....	Skimmed milk 10% powder Cells in spent medium.	15×10^{10}	52	52
Aerated liquid culture....	Centrifuged cells resuspended in buffered saline	Autoclaved at 120° C. for 20 minutes	Lactose..... 5% Tryptose..... 1% Ascorbic acid.... 0·5% Thio-urea..... 0·3% Cells in saline	25×10^{10}	15	5
Aerated liquid culture....	Centrifuged cells resuspended in buffered saline	Seitz filtered.....	Lactose..... 5% Ascorbic acid... 0·5% Thio-urea.....0·25% Cells in saline	23×10^{10}	20	11
Aerated liquid culture....	Centrifuged cells resuspended in liquid culture	Seitz filtered.....	Lactose..... 5% Ascorbic acid... 0·5% Thio-urea.....0·25% Cells in spent medium	45×10^{10}	42	31
Aerated liquid culture....	Cells sedimented in 0·05 % C.M.C. in liquid culture	Seitz filtered.....	Lactose..... 5% Ascorbic acid... 0·5% Thio-urea.....0·25% C.M. Cellulose..0·05% Cells in spent medium	105×10^{10}	58	53

Graph (b)

Viability of Concentrated Freeze Dried Brucella Vaccine

○ held at 4°C × held at 37°C



GRAPH (*b*).—Viability of Concentrated Freeze Dried *Brucella* Vaccine.

Bacteriological tests for purity and viability and biological tests for safety and immunizing qualities in guinea-pigs and cattle have given results similar to those with fresh liquid vaccine of the conventional type.

Discussion.

The principal limitation to the application of *Brucella* vaccine in Africa has been the poor keeping quality of the liquid suspension of live bacteria, which constitutes the vaccine.

The aim of the present work was to overcome this defect by freeze-drying. The liquid culture could be dried satisfactorily but this supplied only one to five doses per ampoule. The ultimate object was to produce a vaccine in ampoules containing many viable organisms in a small volume, which could be reconstituted conveniently to supply a large number of doses. Efforts at freeze-drying more viable organisms in the same volume resulted in greater relative loss of viability which in turn created heavier demands on the culture methods. To meet this, the aerated liquid culture technique was perfected in order to supply the required number of live *Brucella* organisms (van Drimmelen, 1956) and survival was promoted.

The breakdown products of bacterial growth were believed to be the best available enzyme inhibiters. Although freezing has a retarding effect on enzyme action, many enzymes have been found to be active at low temperatures (Joslyn, 1949). The shake flask culture medium was therefore, suitably adapted by eliminating glycerine and adjusting the glucose content so that there would be no reducing sugar left in the spent medium. The addition of lactose was necessitated by the demands of freeze drying mainly to regulate the residual moisture necessary for survival. The ascorbic acid performed anti-oxidative functions being protected by thio-urea (thiocarbamide) (Naylor and Smith, 1946).

Centrifugation as a method of concentration is highly detrimental to viability of bacterial cells. This is particularly undesirable in *Brucella* vaccine as dead cells (i) increase the tissue response at the site of inoculation, (ii) increase the agglutinin response in connection with the blood serum test for brucellosis, (iii) have no value as regards immunization.

Spontaneous sedimentation of surface grown vaccine had been observed and investigated (van Drimmelen, unpublished results). The effect was believed to be due to small amounts of agar in solution reacting with capsular constituents of "Smooth" *Brucella* cells. In preventing a tight pack, methyl-celluloses had contributed to the maintenance of viability in liquid vaccine (van Drimmelen, 1954). Originally methyl-celluloses were tested in liquid vaccine for their reputed suspending powers. They are used in foods, cosmetics, soaps, paints and many other products for controlling viscosity, filming, binding properties and suspending qualities of solutions. In the washing industry sodium-carboxy-methyl-cellulose is used to increase dirt-dispersion, by maintaining the particles in suspension to prevent them being redeposited on the material during rinsing. C.M.C. has, however, other properties as well. Even in very low concentration in the aqueous phase it prevents the formation of large crystals on crystallization. C.M.C. consists of long chains of glucose molecules with methylated and carboxylated radicals.

Sodium-carboxy-methyl-cellulose was found the most desirable agglutinant in practice because of its: (i) solubility in water, (ii) purity, (iii) inertness and harmlessness to bacteria, (iv) effectiveness in low concentrations, (v) ease of sterilization, (vi) low cost, and (vii) apparent protective value during freezing. Its incorporation resulted in a technique of vaccine production whereby a single freeze-drying unit could turn out a total number of 3×10^{14} viable *Brucella* cells

in containers suitable for storage and transporation. This amounted to a maximum of 4,400 doses of *Brucella* vaccine per unit per day and enabled distribution of the Strain 19 vaccine at a low cost to remote parts of Africa without loss of effectiveness and without expensive packing or great bulk.

SUMMARY.

A freeze-dried *Brucella* vaccine has been developed which can be issued with all the usual guarantees, e.g. 60×10^9 viable organisms per dose viable for an extended period. This vaccine has been prepared in concentrated form which makes it possible to reconstitute the contents of one ampoule into a number of doses. Such a vaccine is eminently suitable for use in Southern Africa especially in the more remote areas.

Concentration by means of carboxy-methyl-cellulose of the organisms grown in aerated liquid culture increased the dosage per ampoule and the survival rate after freeze-drying and during storage.

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