

STRAIN 19 BRUCELLA VACCINE: V. MASS PRODUCTION IN  
THE *BRUCELLA* VORTEX AERATED CULTURE APPARATUS.

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INTRODUCTION.

The success of the shake flask technique for *Brucella* vaccine cultures (Van Drimmelen, 1956) suggested that strain 19 would also grow satisfactorily in a mass culture apparatus of the type used by Chain, Paladino, Callow, Ugolini and Van der Sluis (1952).

The live bacteria used in the *Brucella* vaccine hardly multiply in the vaccinated animal. Therefore they have to be inoculated in very large numbers ( $6.0 \times 10^{10}$  viable organisms per dose) if a good immunity is to be expected (Buck, Cotton and Smith, 1938). Besides the bulk supply of viable organisms, contamination and variation are the main problems of culturing slow-growing bacteria. The majority of contaminants thrive extraordinarily well on even a heavy growth of *Brucella abortus*, much more so than in cultures of other vaccine and industrial micro-organisms. Absolute sterility is thus a prerequisite. Selective media and antibiotics tend to slow down growth which may be expected to favour the development of non-immunogenic variants in the population. "Rough" *Brucella* variants, which are worthless for vaccine production, are notoriously often found in the fluid of condensation over agar surface cultures. They must be avoided at all costs in any liquid culture process (Joint FAO/WHO Panel on Bruc., 1951).

The purpose of this report is to describe the apparatus and technique employed for production of strain 19 vaccine in mass culture in a cylindrical culture vessel with a vortex system of aeration.

The current techniques used in large-scale industrial fermentation processes have been developed on a basis more of trial and error than of sound theoretical reasoning from data obtained with small pilot plants (Emery, 1955). The literature on fermenters does not reveal any major works dealing with the production of living organisms. The "Bactogen" (Monod, 1950) and the "Chemostat" (Novick and Szilard, 1950) demonstrate the prototypes of modern, aerobic, biochemical and antibiotic, microbial cultures.

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## STRAIN 19 BRUCELLA VACCINE.

It has been shown that the production of *Brucella* vaccine at Onderstepoort has reached a scale of mass culture (Van Drimmelen, 1955). Consequently fermentation kinetics, which appear to be directly applicable to cultures of this kind, have to be considered (Gaden, 1955). The continuous culture method cannot as yet be applied to the manufacture of live vaccines owing to the likelihood of variants arising (Novick, 1955) and the difficulties involved in testing the immunizing qualities of the product. The batch process is thus favoured.

A heavy inoculum of pure, typically "Smooth" seed material would seem essential for Strain 19 *Brucella* vaccine (FAO/WHO Panel on Bruc., 1951). Although large-scale fermentations with *Penicillium chrysogenum* and *Streptomyces* have generally been successful with relatively much smaller inocula than required for small-scale cultures, the fact that *Brucella* organisms are strict parasites, and normally intracellular parasites, gives an indication of the complexity of the growth factors required during the latent growth phase in order to start a culture.

Steam sterilization of liquid medium in large bulk involves considerable loss in quality due to the time required for heating up to 120° C and again for cooling afterwards. Caramelization of the sugar can be prevented by sterilizing this component in separate containers, but many other growth factors are also involved. High-temperature, short-time (H.T.S.T.) sterilization may offer a suitable alternative (Pfeiffer and Vojnovich, 1952).

It has been shown that Seitz filtering of concentrated medium followed by dilution with sterile water is a method with marked advantages. Some of the usual technical difficulties can be overcome by applying the telescopic, aseptic connections as used at Onderstepoort (Van Drimmelen, 1956).

Temperature control by water jacket or coil cooling is mainly a question of convenience. Jackets are generally too expensive for large industrial fermenters whereas coils are subject to abrasive and corrosive constituents of the culture. Moreover, the wide temperature range between sterilization and growth requires a double set of valves for rough and fine control (Emery, 1955). It has been suggested that the latent heat of water vapour could be employed for rapid reduction of temperature by water evaporation from the surface (Mason, 1956).

Aeration at a rate of a volume equal to that of the medium per minute is usual in small-scale fermenters. In the larger scale plants a third of this appears to be adequate (Emery, 1955). In a vortex system without baffles, with the impeller at half the depth of the medium and the depth of the medium equal to the diameter of the culture vessel, good aeration has been obtained by means of a single orifice sparger or none at all, the air being run into the upper part of the vessel (Chain, Palladino, Callow, Ugolini and Van der Sluis, 1952). The air can be sterilized by electrical heating but larger plants have used sterile charcoal bed filters which are less costly (Emery, 1955).

Agitation with a single vane-disk impeller has provided maximum effect with minimum power consumption if no baffles are used. The impeller diameter should be at least 28 per cent of the culture vessel diameter (Chain *et al.*, 1952).

## MATERIALS AND METHODS.

The apparatus was constructed of a stainless steel culture vessel fitted on a stand next to the laboratory table. The media flasks, seed flasks, air supply and steam supply were connected with rubber pressure tubing (see Figure 1). The lay-out is shown in Figure 2. The culture vessel (CV) had a capacity of 250 litres and a height of 85 cm. A single phase electric motor (M) of  $\frac{1}{2}$  h.p. with a working speed of 2,850 r.p.m. was fitted with a three speed A section belt drive and mounted on a frame bolted on to the reinforced lid. A  $\frac{3}{4}$  inch stainless steel impeller shaft (IS) with ball bearing and tufnal bush was mounted perpendicularly in the lid and hermetically sealed by means of a double Angus oil seal\*. An eight inch impeller (I) was fitted 3 inches from the bottom of the vessel.

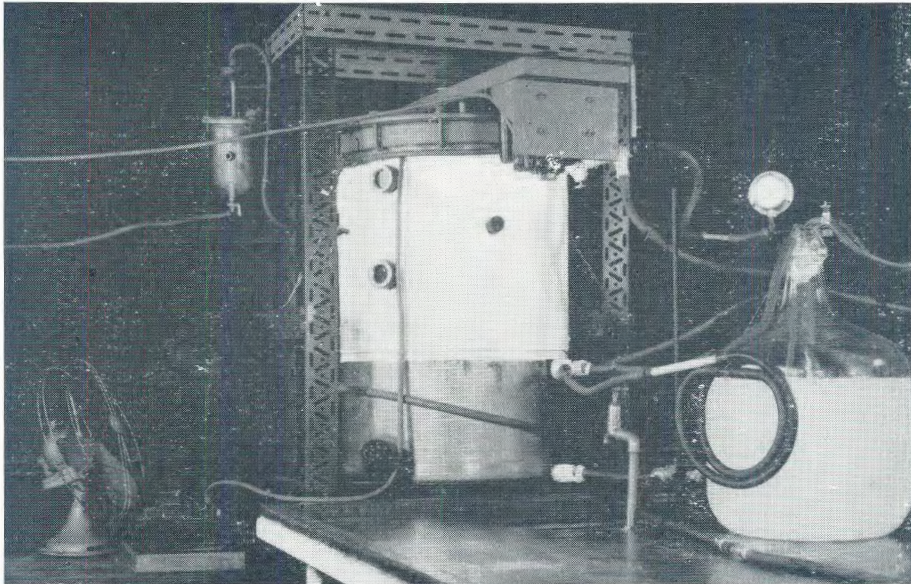


FIG. 1—Photograph of pilot plant for the Brucella Vortex Aerated Culture Apparatus.

Aeration was carried out by an electric pump with air passed through a flow meter (FM), a Berkefeld candle and cotton wool filter (CCWF), and then humidified through a column of water. It was finally sterilized through Seitz filters and entered through a needle valve (Negretti and Zambra, stainless steel) welded into the wall 3 inches from the lid (AI).

Autoclaved flasks were prepared for 1 litre seed (SF), 32 litres concentrated medium (MF) and for 40 litres water with antifoam, which could be connected in series to a  $\frac{5}{8}$  inch diameter inlet (IP) welded into the wall 9 inches above the steam inlet (St) near the bottom of the vessel. Sampling and harvesting outlets were welded to the bottom. The gas outlet (AO) was fitted opposite the air inlet and connected to a pressure gauge (PG) and hot water trap (TP).

\* Manufactured by George Angus Co., Ltd., No. 1 Lower Street, New Doornfontein, P.O. Box 3942, Johannesburg.

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Temperature control was made possible by welding a blind stainless steel tube low into the side. This tube was filled with water in which the thermometer (Tr) and thermostat (Tt) were placed. Heat was removed by a cotton gauze jacket (GJ) moistened through multiple water jets (MWJ) by a thermostatically activated pump (P) and cooled with the draft of a thermostatically controlled electric fan (CF). The return water was collected by a slanting furrow and funnel (WR) into a glass reservoir (R).

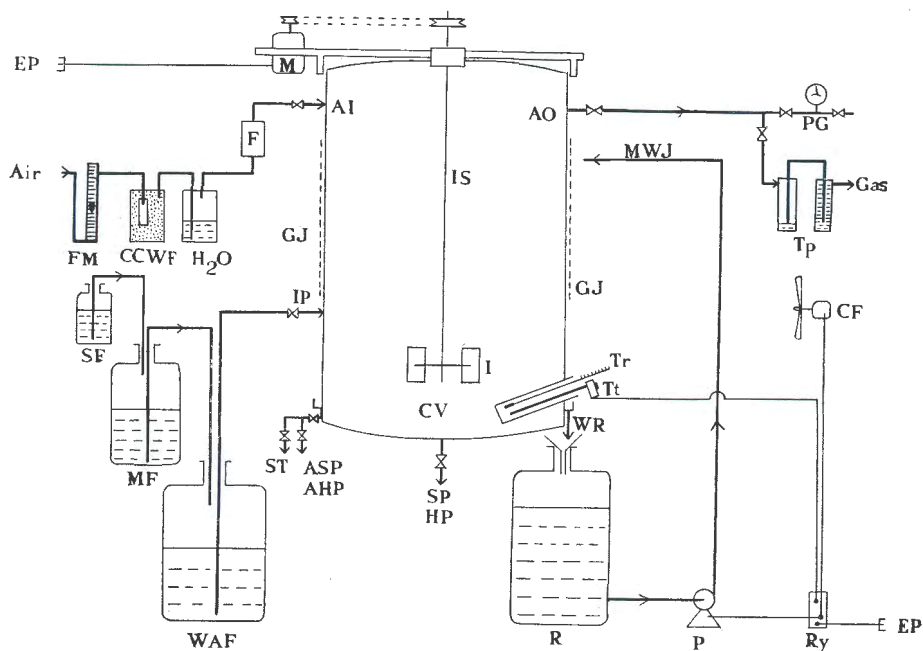


FIG. 2.—Pilot plant for the Brucella Vortex Aerated Culture (Schematic). CV: culture vessel; AI: air inlet; AO: air outlet; I: impeller; IS: impeller shaft; M: motor; EP: electric plug; FM: flow meter; CCWF: Berkefeld candle and cotton wool filter; F: Seitz filter; SF: seed flask; MF: medium flask; WAF: water and antifoam flask; IP: medium inlet point; ST: steam inlet point; SP: sampling point; HP: harvesting point; ASP: alternate sampling point; AHP: alternate harvesting point; PG: pressure gauge; Tp: trap; Tr: thermometer; Tt: thermostat; Ry: Relay; P: pump; CF: cooling fan; GJ: gauze jacket; MWJ: multiple water jets; WR: water return; R: reservoir.

The preparation for each batch of vaccine included the following steps:—

- (1) All fittings for media, sampling, and aeration were autoclaved at 15 lb. for 3 hours.
- (2) Seed production was carried out exactly as for agar culture Strain 19 vaccine according to U.S. Bureau of Animal Industry specifications. (Recently 48 hours shake flask cultures have been used for seeding).
- (3) Forty litres of distilled water with 12 ml. of antifoam\* were autoclaved.

\* A polyoxethylene derivative of ricinoleic acid obtained from Imperial Chemical Industries, Limited.

## (4) Thirty-two litres of concentrated medium consisting of

Peptone.....	2160 gm.
Glucose.....	2160 gm.
Yeast Extract (*).....	720 gm.
Na <sub>2</sub> HPO <sub>4</sub> (anhydrous).....	120 gm.
H <sub>2</sub> O.....	3200 ml.
pH adjusted to.....	pH 6.4.

were Seitz filtered and incubated for four to six days to test for sterility.

## (5) The culture vessel and connections were sterilized by internal steam at 110° C for 3 hours on two successive days. The sterile fittings were connected at the beginning of the second sterilization period.

## (6) The culture was started as follows:—

- (i) With the vessel at 110° C the inlet port was aseptically connected to the water and antifoam flask and this in series to the media and seed flasks.
- (ii) The steam inlet was closed off, the internal pressure released and then all outlets closed.
- (iii) The inlet port was opened and water<sup>4</sup> allowed to enter by suction from the vacuum created by cooling of the water vapour in the vessel.
- (iv) The medium was siphoned into the flask containing water with antifoam, at a rate sufficient to keep this flask more than half full until the medium flask was empty.
- (v) The seed was similarly siphoned into the medium.
- (vi) When the 72 litres of the total liquid charge had entered the vessel the inlet port was closed and air was introduced through the air inlet at the rate of 6,000 cc. per minute.
- (vii) As soon as the vacuum was completely overcome the air outlet was opened and the impeller started at 1,000 r.p.m.
- (viii) The temperature was adjusted by means of direct heating to 37.5° C and the thermostatic control adjusted to remove heat produced by the agitation and growth in the culture.

During the growth period, samples were taken for purity tests and various measurements. It was found best to sample from the steam port in order to be able to sterilize the sampling tube before and after the operation without interfering with the growth process.

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\* A vegetable flavoured yeast extract obtained from S.A. Food Extracts, Ltd., 5 Parson's Street, Industria, Johannesburg.

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Harvesting was carried out at 70 hours by running the culture into eight ten-litre flasks each containing 1,000 ml. of a 0·6 per cent sterilized sodium-carboxymethyl-cellulose solution. These flasks were held at 4° C overnight to allow the cells to sediment. The supernatant, spent medium was then discarded and the sediment mixed with freeze-drying mixture to make a final concentration of:—

Cells.....	40·0%.
Lactose or Sucrose.....	12·5%.
Ascorbic Acid.....	1·9%.
Thiourea.....	2·8%.
Spent medium q.s. to make.....	1,000·0 ml.
pH adjusted to.....	pH 6·4.

This mixture was distributed in 4 ml. amounts into 10 cc. ampoules and freeze-dried.

## RESULTS.

The *Brucella* Vortex Aerated Culture apparatus used in these experiments was a pilot plant, but the results were such that it was immediately applied to routine production of *Brucella* vaccine. A similar apparatus of simpler and more economical construction has been planned.

In Table 1 some pertinent data on fourteen batches are given. It will be seen that the batches received different treatments, some steps having been taken as "feelers". It was found that the apparatus provided a means of producing the equivalent of some 120,000 doses liquid vaccine in a single batch. The harvested material was used for *Brucella* vaccine and applied in the anti-brucellosis campaign in South Africa.

In Table 2 a comparison is drawn between the various production methods for *Brucella* vaccine.

## DISCUSSION.

The *Brucella* Vortex Aerated Culture apparatus which is the subject of this paper provides, for the first time, a device by which the output of *Brucella* vaccine can be determined by the choice of the relevant dimensions of the culture vessel. Labour and glassware requirements have now become secondary considerations.

By means of the "Bru. V.A.C." apparatus a 2,000 to 6,000-fold increase of viable *Brucella* organisms was obtained in 70 hours. The process involved only one series of purity, density, viability, and variation tests thereby reducing bacteriological work to a minimum. The apparatus delivered  $1·44 \times 10^{16}$  viable organisms in one batch i.e. in one week. This is equivalent to more than 120,000 doses of liquid vaccine for which by the Roux flask potato agar culture method, at least 4,000 media flasks would have had to be used.

It is possible that the present favourable results may have been due partly to the shape of the vortex. The depth of the medium was only half the diameter of the vessel. The opposite is usual for industrial plants.

There is a real advantage in the batch process with the "Bru. V.A.C." apparatus in that no moving parts need to be maintained in service over weekends.

Finally, the system of seeding, culturing and harvesting in this set-up has been developed to an almost "closed" system eliminating many of the earlier sources of contamination.

## SUMMARY.

The vaccine organism *Brucella abortus* strain 19 has been cultured in bulk in 72 litres of liquid medium aerated in a vortex system in a stainless steel culture vessel of 250 litre capacity. The lay-out and operation of the apparatus are described and results of a series of batches produced are given. The differences between various production methods are briefly presented.

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TABLE 1.  
Strain 19 Brucella Vaccine: Results of Cultures in the Brucella Vortex Aeration Culture Apparatus.

Culture No.	No. of Viable Organisms (per ml.) ( $\times 10^9$ ) in Culture at				Percentage packed Cell Volume at				pH of Culture at				Age of Culture when Harvested, hr.	Total Cell Volume Harvested, ml.	Total Viable Cells Harvested.	Remarks.			
	0 hr.	22 hr.	46 hr.	70 hr.	94 hr.	0 hr.	22 hr.	46 hr.	70 hr.	90 hr.	0 hr.	22 hr.					46 hr.	70 hr.	90 hr.
1	6.6	16.5	223.0	—	—	—	—	—	—	—	6.4	6.5	6.6	—	—	3,240	$1.6 \times 10^{16}$	48 hr. Potato agar seed culture.	
2	3.3	—	Contaminated at seeding.	—	—	—	—	—	—	—	6.4	—	—	—	—	—	—		
3	6.6	6.7	Contaminated from air inlet.	—	—	—	—	—	—	—	6.4	6.2	—	—	—	—	—		
4	6.6	6.7	100.0	205.0	262.5	0.1	1.2	2.0	2.5	—	6.4	6.4	6.5	6.7	6.7	1,800	$1.9 \times 10^{16}$		
5	3.3	2.9	80.0	215.0	—	—	0.15	2.0	5.25	—	6.4	6.4	6.8	6.8	—	3,780	$1.5 \times 10^{16}$		
6	19.8	5.4	133.0	110.0	—	—	0.2	2.0	3.5	—	6.4	6.4	6.6	6.7	—	2,520	$0.8 \times 10^{16}$		
7	17.2	11.6	120.0	125.0	—	—	0.4	2.25	4.0	—	6.4	6.6	6.8	6.8	—	2,880	$0.9 \times 10^{16}$		
8	6.6	8.0	101.0	103.0	—	—	0.4	2.0	3.0	—	6.4	6.8	7.0	7.0	—	2,160	$0.74 \times 10^{16}$		
9	6.6	10.0	95.0	155.0	—	—	0.1	2.5	6.0	—	6.4	6.5	7.0	7.5	—	4,320	$1.1 \times 10^{16}$		
10	6.6	0.45	40.0	189.0	179.0	—	0.04	1.5	4.5	5.1	6.4	6.5	7.2	6.9	7.0	4,240	$1.2 \times 10^{16}$		Glucose added at intervals during growth.
11	6.6	0.73	69.0	189.0	—	—	0.1	2.5	5.0	—	6.4	6.7	7.2	7.0	—	3,600	$1.3 \times 10^{16}$		
12	100.0	38.0	No ct.	—	—	—	1.0	4.5	—	—	6.4	6.8	6.8	—	—	3,240	No ct.		400 ml. 48 hr. Shake flask seed culture.
13	50.0	35.0	200.0	—	—	—	1.0	6.0	—	—	6.4	6.8	6.8	—	—	4,320	$1.4 \times 10^{16}$		
14	50.0	28.0	200.0	—	—	—	1.0	6.0	—	—	6.4	6.8	6.8	—	—	4,320	$1.4 \times 10^{16}$		



TABLE 2.  
Comparative Data on Production Methods of Brucella Vaccine.

Method of Culture.	Type of Culture Vessel.	Capacity of Culture Vessel.	Amount of Medium per Unit.	No. of Stages of Purity Tested between seeding of Medium and Distribution of Vaccine.	Minimum No. of Purity Tests per Million Doses of Vaccine Suspension.	Minimum No. of Containers cleaned and sterilized per Million Doses of Vaccine in bulk.	Minimum No. of Containers cleaned and sterilized for one Dose of Vaccine.	No. of Doses of Vaccine Suspension per Litre of Medium used.	No. of Doses of Vaccine Suspension per Unit Culture.
Agar surface culture..	Roux flask...	1 Litre...	150 cc...	4	25,500	100,000	8	267	40
Agar surface culture..	Sutton flask..	4 Litre...	900 cc...	4	12,500	50,000	8	150	120
Shake flask aerated liquid culture	Sutton flask..	4 Litre...	400 ml...	4	2,500	4,500	7	1,667	500
Vortex aerated liquid culture	Bru. V.A.C. apparatus	250 Litre	72,000 ml.	3	13	30	6	1,667	120,000