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STRAIN 19 BRUCELLA VACCINE—I. PRODUCTION OF VACCINE BY THE SHAKE FLASK TECHNIQUE.

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The ever-increasing demand for Strain 19 *Brucella* vaccine in South Africa necessitated the investigation of methods designed to speed up production. The available facilities did not allow of the required expansion with agar surface culture methods but the aerated liquid culture technique evolved at Camp Detrick suggested a solution (Gerhardt and Gee, 1946). These results had been obtained with *Br. suis* and the concentrations of living organisms achieved were inadequate to give promise of a sufficiently high bacterial count with Strain 19 to ensure cessation of growth when diluted to vaccine strength. In preliminary work on aerated liquid cultures more desirable results were obtained in Erlenmeyer flasks with small amounts of agitated media containing glycerine, glucose, peptone, glutamic acid, histidine and yeast extract (Sterne and Wentzel, 1950).

Brucella vaccine, which consists of a saline suspension of at least 12×10^9 living bacteria per ml. and contains no preservative of any kind, has to be subjected to the strictest microscopical, cultural, biochemical and biological tests for purity. As no economical entirely closed system has yet been evolved, a proportion of batches must inevitably become contaminated and has to be discarded. Smaller batches are therefore preferable to large ones.

The purpose of this report is to describe the technique adopted to produce the requisite volume of vaccine by means of agitated liquid cultures.

Materials and Methods.

Seeding and harvesting of bulk cultures are carried out by a siphon technique in order to minimize contamination as the room is used for various bacteriological manipulations. Media flasks, seed flasks and vaccine flasks are fitted with hooded pipettes of suitable length and shape (see illustrations). The seed material is prepared as prescribed by the United States Bureau of Animal Industry (1950).

Liquid medium is prepared as follows:—

Glucose	270 Gm,
Peptone	270 Gm,
Yeast Extract ("Marmite")	90 Gm,
Phosphate (Na_2HPO_4– Anhydrous) ...	13·4 Gm.
Warm water	4 Litres,
pH adjusted to	pH 6·3

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This solution is cleared through "Speedex" powder over blotting paper and sintered glass in a funnel and then sterilized by filtering through a Seitz E.K. pad ("ford sterimat"). The Seitz filter is connected over a flame by means of telescoped glass tubes to an autoclaved 12 litre flask containing 5 litres of water plus 3 ml. antifoam agent (a polyoxethylene derivative of ricinoleic acid).

The seed is produced by suspending the growth on a 48 hour potato agar culture in saline and is kept in the refrigerator until purity tests have been performed. An amount of 100 ml. seed suspension containing approximately 3×10^{12} viable *Brucella* organisms is then introduced by siphon into flasks each containing nine litres of culture medium. From these flasks of seeded medium 450 ml. lots are delivered over a flame through a hooded pipette into flat four litre shake flasks. The latter are placed in a shaking machine with the cradle so constructed that each flask lies at a slant of about 1 in 50 with the neck raised (see figure 1). The cradle is subjected to 64 excursions of 12 cm. each per minute. This results in an agitation with to and fro, as well as semicircular forward and backward movements of the liquid. The whole apparatus is kept in a dark incubating room at 37° C. for 66 hours.

Harvesting is carried out by siphoning into vaccine flasks with diluent ready for bottling. A bent hooded pipette is used for this operation. Each batch is tested separately for the following factors:—

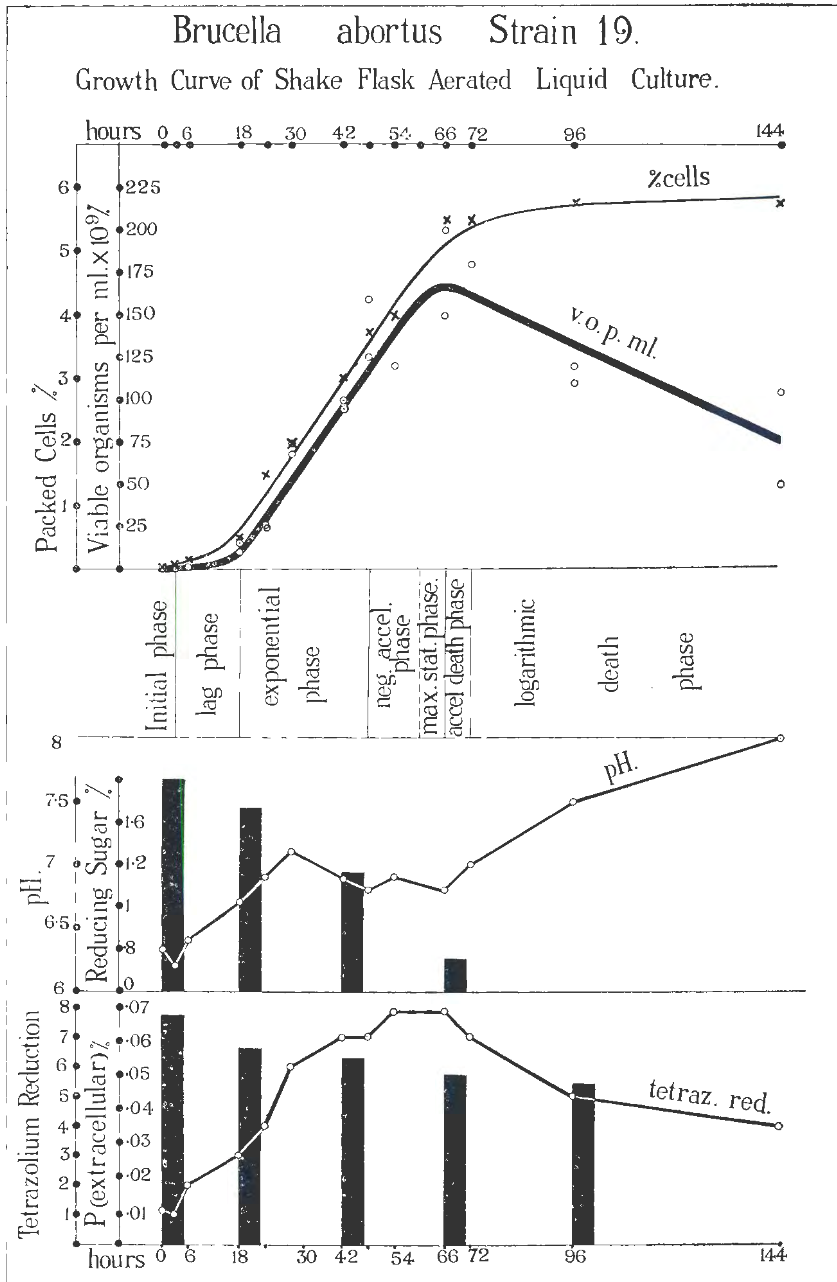
(i) pH—glass electrode potentiometer,
(ii) density—Fitch modified Hopkin's tubes,
(iii) purity—cultural and microscopical,
(iv) viability count—Miles and Misra (1938),
(v) "S" to "R" variations—plate,
(vi) tetrazolium reduction—v. Drimmelen (1953).

Biological tests for safety and immunizing quality are carried out on baulked samples of batches which have passed the above tests.

The pipette is sealed off and the flask stored in the cool room to await bottling when the pipette is opened over a flame and purity tests are repeated on the final product.

RESULTS.

The viability counts (Miles and Misra, 1938), packed cell volume and the pH changes in the growth curve are presented in Graph (*a*). At routine harvesting (66 hours) a final count of 150 to 200×10^9 viable organisms per ml. can be obtained (see Table 1). Exhaustive studies with the technique prescribed by the B.A.I. and others (Huddleston, 1949; Braun, 1947, 1950) fail to show any indication of variants in the culture at the end of the logarithmic phase. The mutation rate for "S" to "R" variants appears to be lower than that encountered in vaccine samples made from conventional potato agar cultures. Purity is checked again after bottling by an independent laboratory using cultural, biochemical and microscopical tests. Antigenicity is tested as a routine in guinea-pigs and occasionally in cattle. Immunity tests in guinea-pigs (Stableforth, 1953) and in cattle (McDiarmid, 1953) are carried out. Results obtained agree entirely with those from surface culture vaccine. The reactions produced also give the usual temperature curves, swellings at the site of injection and swellings of the regional lymph glands in cattle. In guinea-pigs no lesions are found at autopsy and the duration of spleen infection shows nothing unusual.



GRAPH (*a*).—The Growth Curve of *Brucella abortus* Strain 19 in aerated liquid culture in shake flasks of 4 L. capacity; showing packed cell percentage, viable organism count, pH changes, percentage reducing sugar in medium, variations in tetrazolium reducing power and percentage extracellular phosphate.

TABLE I.

Comparative data on the agar surface culture and aerated liquid culture methods of producing *Brucella* vaccine.

Culture Method.		Agar Surface.	Aerated Liquid.
Type of culture flasks used		" Roux " flasks. One litre capacity	Large " Shake " flasks. Four litre capacity.
Number of culture flasks used per Litre of medium		7·7	2·5
Average Number of viable organisms harvested	Per flask	$3 \cdot 6 \times 10^{12}$	$68 \cdot 0 \times 10^{12}$
	Per litre of medium	$2 \cdot 8 \times 10^{13}$	$17 \cdot 0 \times 10^{13}$
Minimum number of containers sterilized	In producing one dose of vaccine.	8	6
	For Production of 8,500 doses of vaccine	457	49
Volume of culture medium consumed per dose of vaccine produced		3·75 c.c.	0·9 c.c.

DISCUSSION.

The stability of Strain 19 has been confirmed repeatedly (Taylor and McDiarmid, 1949). When it was decided to attempt to increase the vaccine output of existing laboratories with a modified production method, due consideration was given to the impossibility of completing immunizing quality tests comparable to those of the originators of the existing Strain 19 vaccine. (Buck, 1930; Cotton, Buck and Smith, 1934). The technique evolved is therefore confined to the use of potato agar surface culture made from selected smooth colonies of the Strain 19 obtained in frozen dried vials from the B.A.I. Only this type of culture has been proved to afford the satisfactory immunity demonstrated by experimental tests (Edward, McDiarmid, de Ropp and McLeod, 1946; Campbell and Rodwell, 1949; Gregory, 1951, 1952, 1953a and b). Consequently the process of vaccine production always follows the recommendations of the B.A.I. The aerated liquid culture method is only introduced at the final stage, when the living organisms are transferred in the logarithmic growth phase from agar to agitated liquid medium, and within 66 hours a 450 fold multiplication is obtained. Variation of Strain 19 in non-aerated liquid is a common source of poor quality vaccine and is frequently found in the condensation water of agar surface culture flasks. The shake flask culture method has no such drawback, since the medium chosen is relatively free from " R " selecting factors (e.g. Mn-ions, Mg-ions, alanine) and the short incubation period does not allow of selective growth of mutants.

Five or more typical " S " colonies are isolated each time a dried culture is used for producing a series of vaccine batches in order to minimize the chances of batches being produced from a single colony possessing imperceptible deficiencies in immunizing qualities.

The shake flask technique permits a greatly increased output of Contagious Abortion vaccine at a reduced price. The potential production capacity of the laboratory has been increased from 250.000 doses per year to about three million. which should meet the requirements of Southern Africa for the near future (van Drimmelen, 1952).

SUMMARY.

A method for the production of *Brucella abortus* Strain 19 Vaccine by a shake flask, aerated liquid culture technique is described.

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Fig. 1.—Apparatus for agitating liquid Strain 19 culture in shake flasks by reciprocating movement.

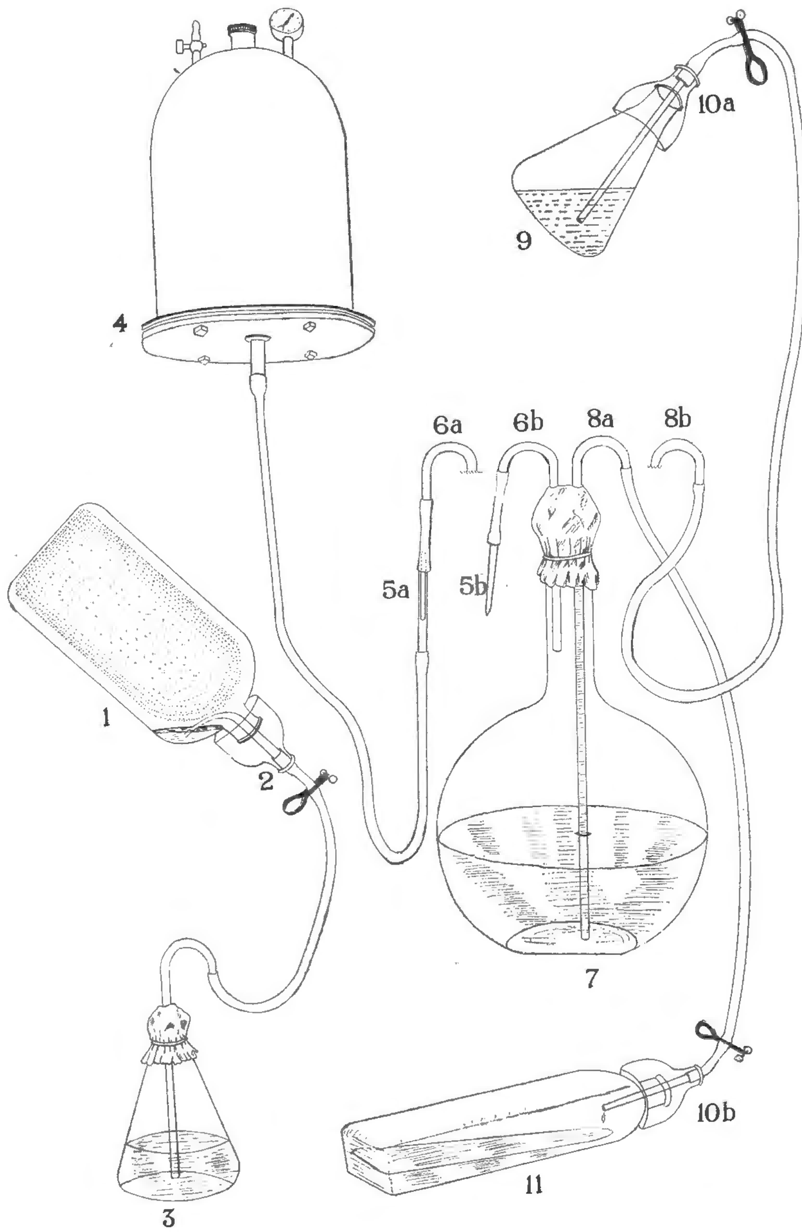

FIG. 2.—Schematic illustration of production methods applied to aerated liquid culture of Strain 19.

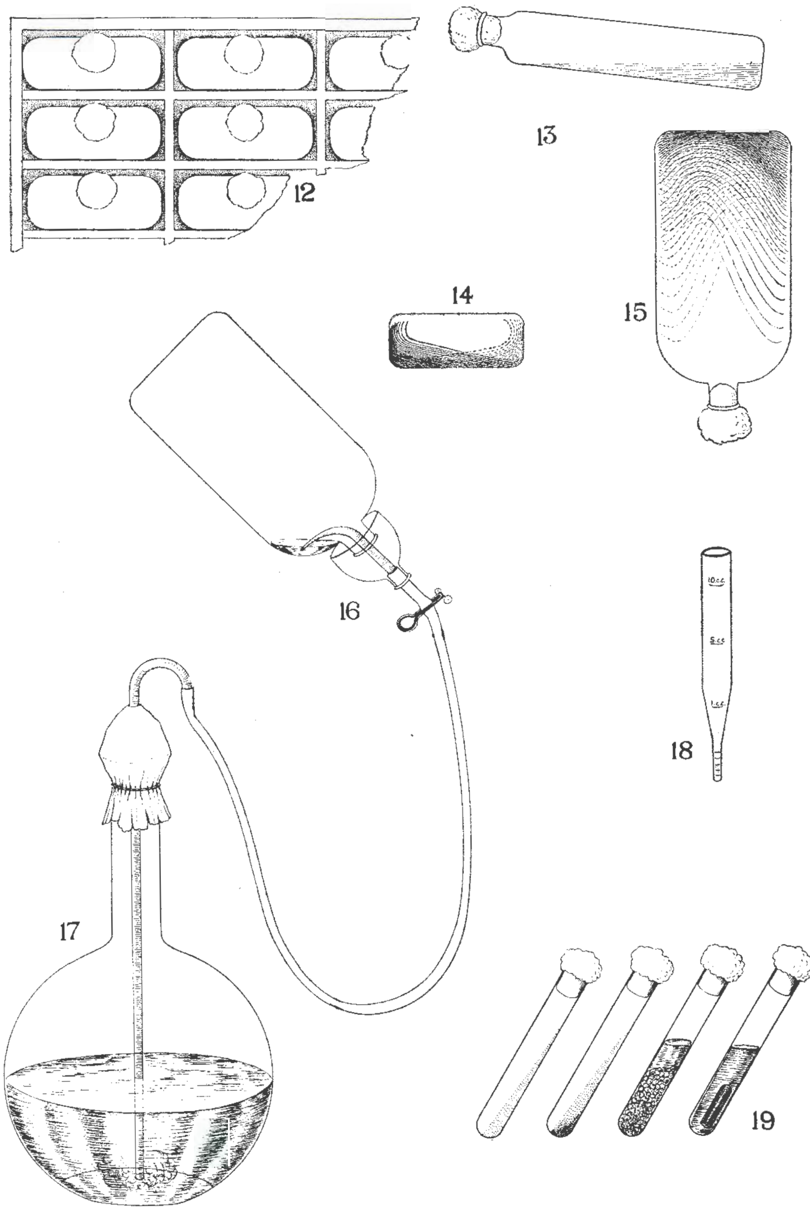

FIG. 3.—Schematic illustration of production methods applied to aerated liquid culture of Strain 19.

LEGEND TO FIGURES 2 AND 3.

1. Seed suspension from agar surface culture in Roux flask.
2. Bent hooded pipette for harvesting seed suspension by siphon into diluent.
3. Seed flask.
4. Pressure filter fitted with Seitz E.K. asbestos pad.

5a. Telescoped glass tube joint connecting filter to medium flask.

5b. Glass tube from disconnected joint sealed off in flame.

6. Bent glass tube for introducing filtered medium.
7. Medium flask.

8a. Bent glass tube filled with seeded medium being siphoned over into shake flasks.

8b. Bent glass tube filled with seed suspension running into medium flask.

9. Seed flask tested for purity.

10a. Hooded pipette for transferring seed suspension by siphon to medium.

11. Shake flasks being filled with seeded medium by means of siphon and hooded pipette.
12. Shake flasks on shaking machine, front view.
13. Shake flask on shaking machine, side view (liquid stationary).
14. Shake flask on shaking machine, back view (liquid agitated).
15. Shake flask on shaking machine, top view (liquid agitated).
16. Harvesting of aerated liquid culture by means of bent hooded pipette and siphon.
17. Vaccine flask with buffered saline diluent.
18. Hopkin's tube for density test.
19. Culture media for purity test.