Onderstepoort Journal of Veterinary Science and Animal Industry, Volume II, Number 1, January, 1934.

A Note on the Cultivation of Anaerobes.

By

J. H. MASON, F.R.C.V.S., F.R.S.E., Empire Marketing Board Research Fellow, Onderstepoort.

THE writer (1930) reported that the addition (5-10 per cent.) of a mixture of equal parts of sheep serum and sheep haemolysed red cells (filtered through a Berkefeld candle) to nutrient agar gave better growth results with anaerobes than the addition of serum alone. The results were nearly, and often as good, as those obtained with blood agar and in addition the medium was clear, a decided advantage in the surface isolation of bacteria. The great disadvantage has been the ensuring of sterility of the serum-cell nutrient. Not uncommonly faulty filter candles allowed bacteria to pass and further the fluid had to be stored and samples taken from it whilst in the unpreserved state. Attempts were therefore made to obtain the serum and haemolysed-cells in as sterile a fashion as possible, to destroy any chance bacteria that might have gained access during manipulations, without altering the consistency, appearance or nutritive properties of the mixture and, if possible, to increase the concentration of laked corpuscles in the haemolysed-cell portion.

OBTAINING THE BLOOD.

Sheep were used as donors of blood and in all instances the wool was closely clipped from the jugular region and the animal bled in a relatively draught free, clean room. The bleeding apparatus consisted of a large bore needle, to which was attached a length of rubber tubing, terminating in a piece of narrow glass tubing provided with a cowl. This and all glassware and media used were autoclaved for half an hour at 120° C. prior to use. The effect, on the sterility of the blood, of disinfecting the skin with Tr. iodi, 5 per cent. phenol and 5 per cent. lysol, of touching one small area with 50 per cent. lysol, of a double sterilization (day interval) with 50 per cent. lysol, and of thoroughly cleansing the skin with soap and water prior to disinfection, was noted, by running about 3·0 c.c. of blood directly into broth and Robertson's meat broth and incubating these for 7-14 days at 37° C. The incidence of infection was rather high although it was noted that growth seldom occurred before the 3rd or 4th day, indicating that only a few germs had been introduced. The method finally decided upon and which gave the highest percentage of sterile blood was as follows: The jugular area was clipped and cleansed with alcohol, followed by ether, the needle was inserted into the vein with one thrust and *about* 30·0 *c.c. of blood allowed to escape;* then the blood was run into sterile tubes or flasks and allowed to clot and a small quantity of blood (to saturation point) laked in distilled water. Whilst this method has

given the best results from a sterility standpoint, the credit for it cannot be entirely allotted to the use of alcohol and ether and the allowing of the first flow of blood to escape. With practice one is able to " hit " the vein at the first thrust, and thus the chances of contaminating the needle point are reduced.

Preparation of the Serum-Haemolysed-Cells Mixture.

The serum is allowed to separate and mixed with an equal volume of haemolysed red cells, sterile precautions being adopted.

Sterilisation and Preservation of the Mixture.

The effect of adding glycerine 20 per cent., acriflavine 1/5,000 to 1/20,000, formalin (40 per cent. formaldehyde) 0·05 per cent. to 0·2 per cent., hydrogen peroxide 2·5 per cent. of 3 vols., petrol (excess), chloroform (excess) and ether (excess) to the mixture, containing a loopful of a dilute suspension of *S. suipestifer*, *Bact. coli*, or a staphyloccus and incubating for 24 hours at 37° C. and then putting up sterility tests, was noted. All except ether were discarded as unsuitable, e.g., acriflavine in non-precipitating concentrations and H_2O_2 did not sterilise, formalin sterilised but was bacteriostatic when the mixture was added to agar and this inoculated with an anaerobe, and petrol and chloroform produced unsightly sediments. Ether answered the purpose satisfactorily. Neither the colour nor the consistency of the mixture was materially altered, excess ether was driven off at 37° C., that remaining in solution being diluted below its effective bacteriostatic range when the mixture was added to agar. To test its bactericidal power a loopful of a dilute suspension of a coliform bacillus, two different staphylococci and a member of the salmonella group was added respectively to tubes (10·0 c.c. amounts) of the mixture and incubated for 24 hours at 37° C. Sterility tests were then put up. The control tubes (mixture plus bacteria without ether) all showed profuse growth on agar plates and one etherised staphylococcus tube showed a few colonies (indicating a definite although incomplete bactericidal effect) whilst the remaining tubes were sterile. As a routine measure, since this test, an occasional tube of mixture has had added to it a small quantity (usually a loopful) of a dilute suspension of *Bact. coli*, a staphylococcus or a streptococcus, plus an excess of ether. After incubation for 24 hours at 37° C. a sterility test is conducted. In no instance has growth of the organism been demonstrated; the mixture is either sterile or the number of germs has been greatly reduced as judged by the paucity of growth on plates compared with that from the control unetherised tubes. Further, from a sterility standpoint the method has proved satisfactory in asmuch as, over a period of 18 months, the incidence of infected agar plates attributable to contaminated mixture has been very low. A few tests, conducted as above, but using anaerobes such as *B. welchii* and *B. sporogenes*, showed that ether was unable to kill these germs, even after incubation for 3 days at 37° C., with a poorly sporulating germ like *B. welchii* there was definite evidence of bactericidal action but with *B. sporogenes*, which sporulates copiously, this could not be demonstrated.

Boiled Alkalised Serum and Serum-Haemolysed-Cells.

It is well known that serum or plasma which has been rendered very alkaline by the addition of KOH may be boiled without coagulation occurring. The same applies to serum-haemolysed-cells. Such boiled material when added to media has proved to be growth stimulating to bacteria [Leusden (1932), and Wahby (1932)].

A few experiments were carried out on the growth-enhancing effect of such boiled material on a number of anaerobes. To a series of tubes containing respectively sheep plasma, serum and serum-haemolysed-cells was added from 0·25 per cent. to 1·5 per cent. of N/1 KOH, and the tubes boiled for from ½ to 1 hour. The material was then neutralized with HCl to pH 7·3-7·4, and added in approximately 10 per cent. quantities to nutrient agar and meat broth. These were inoculated with approximately the same quantities of young broth cultures of different anaerobes (*B. welchii*, *B. cochlearius* 2 strains, *B. chauvoei* 2 strains and *B. multifermentans*), and cultivated, under anaerobic conditions, for periods of from one to three days. In brief, the results were disappointing; in every instance the boiled material was less growth-stimulating than the etherised serum-cells mixture (as above) and frequently as good or better growth was obtained in media containing no addition. Table I records one such result; agar containing approximately 10 per cent. of etherised serum-cells, boiled plasma (1 hour, 1·0 per cent. N/1 KOH) boiled serum-cells (as plasma) and with no addition were poured as plates and after drying overnight on the incubator, were streaked with a young *B. welchii* broth culture. They were then cultured for 24 hours in a McIntosh and Fildes' jar.

TABLE I.

Effect of Boiled Plasma, etc., on Growth of B. welchii.

Addition to agar.	*No. of colonies.*
Boiled plasma	43
Boiled serum-cells	10
Etherised serum-cells	Confluent growth.
Nil	16

In further tests the results varied somewhat, e.g., "boiled serum-cells" was better than "boiled plasma" or "nil" was equal to "boiled plasma" but in all instances the etherised serum-cells gave by far the best results.

Concentration of the Haemolysed-Cell Portion of the Mixture.

The possibility existed that by increasing the concentration of the haemolysed-cell portion of the mixture, better growth results would be obtained. A number of experiments were carried out to determine the haemolysing effect of saponin on oxalated sheep blood. It was found that a dilute solution (1/400) of saponin in distilled water would haemolyse many more red cells than would distilled water alone. A state could be reached where the laked cell fluid was quite mucinous and of a very dark red colour, and further such material mixed with an equal volume of serum and added to agar gave satisfactory growth results. However, comparative tests did not show that such a mixture had any advantage (from a growth-stimulating standpoint) over the routine serum-cell mixture, but on

the other hand had the definite disadvantage of rendering the medium dark in colour and translucent. It was only with difficulty that bacterial colonies could be clearly seen, viewed from the " back " of the medium. Whilst a point could be reached by the use of a suitable concentration of saponin, where more cells could be laked than in distilled water alone, and the final medium was clear, the fact that no better growth-results were obtained than with the usual method rendered further work in this direction superfluous.

The Use of Etherised Serum-Cells in the Cultivation of Anaerobes.

The writer (1930) has already recorded experiments comparing the growth-stimulating effect of serum and serum-cells from the sheep and horse. It may merely be mentioned here that etherised serum-cell mixture has the same effect as filtered serum-cell mixture. With less delicate anaerobes such as *B. welchii*, *Vibrion septique* and *B. sporogenes*, the growth is as good as that obtained upon blood agar; with *B. chauvoei* the result whilst sometimes not quite so good, is nevertheless, perfectly satisfactory. Further, there is the advantage of being able to keep at hand a supply of sterile material and of having a nearly transparent medium.

Conclusions.

1. A method of obtaining and sterilising a serum-haemolysed-cell-mixture is described.

2. Its growth stimulating effect on anaerobes is noted.

3. Boiled alkalised serum and serum-cells have no growth stimulating action on anaerobes.

References.

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